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Antistress Potential of Glycyrrhizin in Chronic Immobilization Stress

GOVIND PATIDAR* and ANIS SHAIKH

Institute of pharmacy, Vikram University, Ujjain, India.
*Corresponding author E-mail: anisshaikh63@gmail.com

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ABSTRACT

The word 'stress' is defined as "a state of affair involving demand on physical or mental energy." Stress is a condition which can disturb the normal physiological and psychological functions of an individual. In medical parlance 'stress' is defined as a perturbation of the body's homeostasis. This demand on mind-body occurs when it tries to cope with incessant changes in life. Extreme stress conditions, psychologists say, are detrimental to human health but in moderation stress is normal and, in many cases, proves useful. Stress, nonetheless, is synonymous with negative conditions. During stressful situations the energy requirement of the organism is increased resulting in enhance generation of free radicals that causes oxidation of nucleic acid and proteins. Free radical also damage biomembrane, reflected by increased lipid peroxidation, thereby compromising cell integrity and function. During this process, the ability of the body's defense system to combat the oxidative stress may diminish due to reduced antioxidants. Stress also increases brain serotonin (5-HT) level. The ascending 5-HT neurons from raphe nuclei innervates hypothalamic and limbic sites and have an overall role in regulating secretions of Adrenocorticotropic hormone (ACTH) during stress. The current research concludes that Glycyrrhizin at the doses of 100 and 200 mg/kg, p.o. reversed the behavioral and biochemical changes in Chronic Immobilization Stressed mice. So we can predict that Glycyrrhizin, the active constituent of liquorice shows antistress potential.

Key words: Adrenocorticotropic hormone, Eustress, Hypostress, Antistress activity

INTRODUCTION

The word 'stress' is defined as "a state of affair involving demand on physical or mental energy." Stress is a condition which can disturb the normal physiological and psychological functions of an individual. In medical parlance 'stress' is defined as a perturbation of the body's homeostasis. This demand on mind-body occurs when it tries to cope with incessant changes in life. Extreme stress conditions, psychologists say, are detrimental to human health but in moderation stress is normal and, in many cases, proves useful. Stress, nonetheless, is synonymous with negative conditions. During stressful situations the energy requirement of the organism is increased resulting in enhance generation of free radicals that causes oxidation of nucleic acid and proteins. Free radical also damage biomembrane, reflected by increased lipid peroxidation, thereby compromising cell integrity and function. During this process, the ability of the body's defense system to combat the oxidative stress may diminish due to reduced antioxidants.[2] Stress also increases brain serotonin (5-HT) level. The ascending 5-HT neurons from raphe nuclei innervates hypothalamic and limbic sites and have an overall role in regulating secretions of Adrenocorticotropic hormone (ACTH) during stress.[1,2,3]

MATERIALS AnD METHODS

Procurement and Identification of Crude Drug

The dried roots of glycyrrhiza glabra were procured from the market of Mandsaur (M.P.). The voucher specimen (BRNCP/Z/003/2010) was

submitted in the department of Pharmacognosy, B. R. Nahata College of Pharmacy, Mandsaur (M.P.).

Isolation and Characterization of Glycyrrhizin
Isolation of Glycyrrhizin
Procedure

20g powdered drug was taken Added 20ml of Acetone 2ml of dil. HNO_3 was added Mixed, Corked the flask and Macerated for 2 hours with occasionally shaking Filtered the contents To the marc 20 ml of acetone was added, Warmed it on water bath and filtered Combined both filter and filtrate Added sufficient quantity of dil. Ammonia sol Precipitation of ammonium glycyrrhizin occurred Filtered the precipitate, washed it with 5ml of acetone twice Dried and weighed the product The yield of ammonium glycyrrhizin should be approximately 4.5% w/w.[53]

Determination of percentage yield

The percentage yield of extract was calculated by using following formula: -

$$\text{Percentage yield} = \frac{\text{Weight of Extract}}{\text{Weight of powder drug Taken}} \times 100$$

Characterization of Glycyrrhizin
Physical Parameters

Following parameters were seen-

- Color
- Solubility
- Melting point

Chemical Test
Test for Liquorice

On addition with 80% sulphuric acid powdered drug showed deep yellow color i.e. crude drug found to be liquorice.

Test for Saponin Glycoside

On addition and shaking with water glycyrrhizin produced froth i.e. presence of triterpenoid saponin glycosides.54

Characterization Techniques

Following Techniques were used for the characterization of the glycyrrhizin.

- TLC (Thin layer chromatography)
- UV-Spectroscopy
- IR Spectroscopy
- TLC (Thin Layer Chromatography) -

TLC for Glycyrrhizin
Procedure

Applied small amount of each of test solution and standard solution in two different tracks on a precoated silica gel plate. Developed the plate in the solvent systemto a distance of 12 cm.

Solvent system- Toluene: Ethyl acetate: Glacial acetic acid

(12.5: 7.5: 0.5)

Detection-

- UV visible
- Anisaldehyde sulphuric acid reagent14

The Rf value of the spot was calculated using the formula-

$$\text{Rf} = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

UV-Spectrophotometer

UV-Spectrophotometer is the best method for the identification of functional groups of unknown substances.[55]

IR Spectroscopy

IR is one of the most powerful analytical technique which offers the possibility of chemical identification and provides useful information about the structure of molecules.[56]

Pharmacological study
Drug

Glycyrrhizin was isolated from Liquorice (unpeeled root) purchased from the local market of Mandsaur.

Animals

Male Albino mice weighing between 22-30 g of weight were obtained from B.R.N.C.P. Mandsaur Animal House. The animals were stabilized for 1 week; they were maintained in standard condition at room temp; 60 ± 5% relative humidity and 12 h light dark cycle. They had free access to food and water. Animals were acclimatized to laboratory conditions before the

experiment. All the experiments were carried out between 09:00 and 15:00 h. The experimental protocols were approved by Institutional Animal Ethics Committee of B. R. Nahata College of Pharmacy, Mandsaur, (M.P.).

Chronic Immobilization Stress

The animals in all the groups except control (normal) were subjected to immobilization stress daily in a prone position for 150 min for 5 consecutive days using simple adhesive tape (chronic stress). Animals were released by removing the tape after moistening with acetone.[57, 58]

Drugs and Treatment

Glycyrrhizin suspension was made by suspending glycyrrhizin in 1% CMC (Carboxy methyl cellulose) in distilled water. Fluoxetine solution was made by dissolving it in distilled water. Fluoxetine (10 mg/kg, i.p.) 59 was administered 30 min, Glycyrrhizin (100 and 200 mg/kg, p.o.) 36 and vehicle (1% CMC solution, p.o.) were administered 1 hour before subjected to chronic immobilized stress.

Groups
Group-I - Normal (Unstressed)
Group-II - Control (Stressed)q
Group-II - Glycyrrhizin (100mg/kg, p.o.)
Group-IV - Glycyrrhizin (200mg/kg, p.o.)
Group-V - Fluoxetine (10 mg/kg, i.p.) [36, 59]

Behavioral study

All the behavioral parameters were observed at the 6th day of chronic immobilization stress.

Measurement of Hyperalgesia

The hyperalgesia of animals were determined by Tail-flick method. In this method, the tip (last 1-2 cm) of the tail of animals were placed on the radiant heat source Analgesiometer). The tail withdrawal from the heat (flicking response) was taken as the end point (normal withdrawal time is 3-5 sec). A cut off period of 10-12 sec observed to prevent any damage to tail.[58]

Measurement of Anxiety

The anxiety level of various groups of mice was measured using mirror chamber and following parameters were recorded

- Latency to enter the chamber
- Number of entries and time spent in mirror chamber

The mirror chamber consisted of a wooden chamber having a mirror enclosed within it.

Animal were placed individually at the distal corner of the mirror chamber at the beginning of the test.58, 6

Measurement of Locomotor activity

The locomotor activity was assessed using digital activity meter (Actophotometer). The activity meter consisted of an arena (29x22x22 cm) and operated on photoelectric cells that were connected in circuit with a counter. When the animal cuts off the beam of light falling on photoelectric cell, a circuit was recorded. After subjecting mice to the stress and 30 minute after drug administration mice were placed gently in this arena and number of counts (locomotor activity scores) recorded for 10 minutes[58, 60].

Measurement of Muscle co-ordination

Mice were subjected to motor function evaluation by placing them individually on Rota rod, which was adjusted to the speed of 25 rpm. The fall-off time was recorded for each mouse and the longest period any animal was kept on the rod was 300s.[60] 2.4.5.

Measurement of Cognitive dysfunction

The Elevated plus maze served as the exteroceptive behavioral model to evaluate learning and memory in mice. The apparatus consisted of two open arms (16 cm X 5 cm) and two covered arms (16 cm X 5 cm X 12 cm) extending from a central platform (5 cm X 5 cm), which was elevated to a height of 25 cm from the floor. On the first day, each mouse was placed at the end of an open arm, facing away from the central platform. Transfer latency (TL) was taken as the time taken by the mouse to move into any one of the covered arms with all its four legs. TL was recorded on the first day. If the mouse did not enter into one of the covered arms within 90 sec, it was gently pushed into one of the two covered arms and the TL was assigned as 90 sec. The mouse was allowed to explore the maze

for 10 sec and then was returned to its home cage. Memory retention was examined 24 h after the first day trial on the second day.61

Biochemical parameters

On the 6th day of study, the animals were sacrificed by decapitation. The brains were removed, rinsed in isotonic saline and weighed. A 10% (w/v) tissue homogenate was prepared with 0.1 M phosphate buffer (pH 7.4). The post nuclear fraction for enzyme assay was obtained by centrifugation of the homogenate at 12,000 ×g for 20 min, at 4 °C.[60]

Measurement of Lipid peroxidation

Took 0.5 ml homogenate + 0.5 ml Tris HCL (PH- 7.4) and incubated at 370c for 2 hours Then 1 ml 10% TCA (Trichloro acetic acid) was added Centrifuged at 1000 x g for 10 minTo 1 ml supernatant, 1 ml of 0.67% TBA (Thiobarbituric acid) were added Kept the tubes in boiling water bath for 10 min Cooled the solution and added 1 ml of distilled water Absorbance measured at 532 nm using UV spectrophotometer values were expressed as nmol of malondialdehyde per mg protein

Estimation of Reduced Glutathione (GSH)

1 ml of homogenate was precipitated with 1 ml of 4% sulfosalicyclic acid by keeping the mixture at 40c for 1 hour Immediately Centriguged at 1200 xg for 15 min Then 1 ml of supernatant, 0.2 ml of DTNB (Dithiobisnitrobenzoic acid) and 2.7 ml of phosphate buffer (0.1 M, PH-8) were taken The yellow color was measured at 412 nm using UV spectrophotometer value were expressed as nanomoles of reduced glutathione per mg of protein

Estimation of Nitrite

The accumulation of nitrite in the supernatant, an indicator of the production of nitric oxide (NO). Equal volumes of supernatant and Greiss reagent were mixed, the mixture was incubated for 10 min at room temperature in the dark and the absorbance at 540 nm was determined with UV spectrophotometer. The concentration of nitrite in the supernatant was determined from a sodium nitrite standard curve and expressed as micromoles nitrite per millimeter of homogenate[63].

Protein Estimation

The protein content was measured according to the method of Lowry using bovine serum albumin as standard. In test tube 1ml of 1N NaOH solution was transferred and heated up to 100°C. Then suspended 1 ml of homogenate into the above solution for 5 minutes. Add 5 ml of alkaline copper reagent mix properly and leave the mixture at room temperature for10 min. Add 0.5 ml of Folin-ciocalteau reagent rapidly with immediate mixing. Leave it for 30 min., measure the absorbance of solution at 750 nm.[64]

Catalase Estimation

In this, we measured breakdown of hydrogen peroxide (H_2O_2) at 240 nm. Assay mixture consisted of 3ml of H_2O_2, phosphate buffer and 0.05 ml of supernatant of tissue homogenate (10%) and change in absorbance recorded at 240 nm. The result were expressed as micromole H_2O_2 decompose/mg of protein/min.[65]

Adrenal Ascorbic acid Estimation

The adrenal glands removed, rinsed in isotonic saline and weighed. A 1% (w/v) tissue homogenate was prepared with 0.1 M phosphate buffer (PH-7.4) and centrifuged at 12000 xg for 10 min, at 40c. The adrenal ascorbic acid levels were determined by 2, 4- dinitrophenyl hydrazine method. The value were expressed as microgram of ascorbic acid per mg of adrenal tissue[66].

Statistical analysis

The data were analyzed by Graph Pad Prism software demo version by one way analysis of variance (ANOVA) followed by "Dunnett's test" and p value less than 0.05 were considered as statistically significant.

RESULTS

Isolation And Characterization

Percentage yield

The percentage yield of Glycyrrhizin was found to be 4.2%.

Characterization of Glycyrrhizin

Following parameters showing the characterization of glycyrrhizin.

Physical parameters

Parameters Standard Test

1. Color White to Brownish yellow powder Brownish yellow powder
2. Solubility Freely soluble in hot water and alcohol, Sparingly soluble in hot water and alcohol
3. Melting point 292°C, 285°C

Table 1: Showing interpretation of standard and test glycyrrhizin sample

S. No .	Peaks Standard	Sample
1.	-OH Stretching (Acid group)	2964 cm^{-1} 2923 cm^{-1}
2.	C=O (Acid group)	1712 cm^{-1} 1699 cm^{-1}
3.	C-O (Acid group)	1217 cm^{-1} 1213 cm^{-1}
4.	C-H Stretching	2873 cm^{-1} 2854 cm^{-1}
5.	C=C group	1643 cm^{-1} 1610 cm^{-1}
6.	C=0 Ketone group	1712 cm^{-1} 1725 cm^{-1}
7.	OH Stretching	3400-2400 cm^{-1} 3398 cm^{-1}

Table 2: Effect of Glycyrrhizin and Fluoxetine treatment on Hyperalgesia at 6th day of chronic immobilization stress in mice

S. No.	Groups Tail	Withdrawal Time (Sec) Mean ±SEM
1	Normal	7.167±0.3651
2	Control (stressed)	5.000±0.3651
3	Glycyrrhizin	(100mg/kg) 10.67±0.4944***
4	Glycyrrhizin	(200mg/kg)11.50±0.4282***
5	Fluoxetine	(10mg/kg) 9.500±0.4282

Values are express in Mean±SEM. P<0.05, **Very Significant, ***Highly Significant as compare to Control group. (ANOVA followed by Dunnett's test), n=5.

Table 3: Effect on Anxiety Daily treatment with Glycyrrhizin and Fluoxetine significantly decreased latency to enter, increased the no of entries and time spent in mirror chamber as compared to control (stressed) group

S. No.	Groups	Latency to Enter(Sec) (Mean ±SEM)	no.of Entries (Mean±SEM)	Time spent (Sec) (Mean±SEM
1	Normal	58.83±7.786	4.667±0.4216	31.33±3.528
2	Control (stressed)	110.0±6.952	2.000±0.3651	16.83±2.272
3	Glycyrrhizin (100mg/kg)	72.33±4.256***	5.500±0.5627**	25.17±1.537*
4	Glycyrrhizin (200mg/kg)	78.00±5.000**	6.500±0.1000**	37.50±2.500**
5	Fluoxetine (10mg/kg)	80.00±5.323**	4.833±0.3073**	26.67±1.978*

Values are express in Mean ±SEM. P < 0.05 *Significant, ** Very Significant ***Highly Significant as compare to Control group. (ANOVA followed by Dunnett's test), n=5.

Table 4: Effect of Glycyrrhizin and Fluoxetine treatment on Locomotor activity at 6th day of chronic immobilization stress in mice

S. No.	Groups	No. of counts/10min Mean ±SEM
1	Normal	386.8±37.54
2	Control (stressed)	560.5±59.39
3	Glycyrrhizin (100mg/kg)	394.5±47.61*
4	Glycyrrhizin (200mg/kg)	270.5±25.50**
5	Fluoxetine (10mg/kg)	233.0±11.13***

Values are express in Mean±SEM. P<0.05 *Significant, **Very Significant ***Highly
Significant as compare to Control group. (ANOVA followed by Dunnett's test), n=5.

Table 5: Effect of Glycyrrhizin and Fluoxetine treatment on Muscle coordination at 6th day of chronic immobilization stress in mice

S. No.	Groups	Fall off Time(sec.)Mean ±SEM
1	Normal	115.5±9.025
2	Control (stressed)	40.50±7.018
3	Glycyrrhizin (100mg/kg)	165.5±36.21**
4	Glycyrrhizin (200mg/kg)	270.5±25.50***
5	Fluoxetine (10mg/kg)	114.2±4.167*

Values are express in Mean±SEM. P<0.05 *Significant, **VerySignificant ***Highly
Significant as compare to Control group. (ANOVA followed by Dunnett's test), n=5.

Table 6: Effect of Glycyrrhizin and Fluoxetine treatment on Memory at 6th day of chronic immobilization stress in mice

S. No.	Groups	Latency to Enter (Sec)Mean ±SEM
1	Normal	5.525±1.404
2	Control (stressed)	10.72±0.6536
3	Glycyrrhizin (100mg/kg)	4.543±0.1299***
4	Glycyrrhizin (200mg/kg)	5.827±0.9467*
5	Fluoxetine (10mg/kg)	4.627±0.3495**

Values are express in Mean±SEM. P<0.05 *Significant, ** Very Significant ***Highly Significant as compare to Control group. (ANOVA followed by Dunnett's test), n=5.

FIG. 1: Chronic Immobilization Stress in Mice

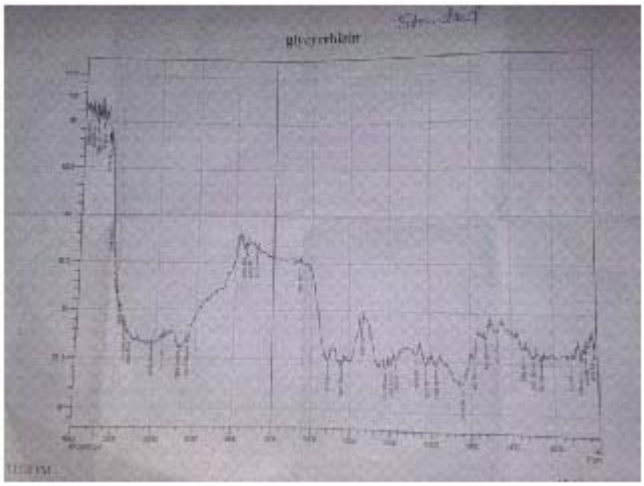

Fig. 2(a): Showing IR Spectra of Standard sample

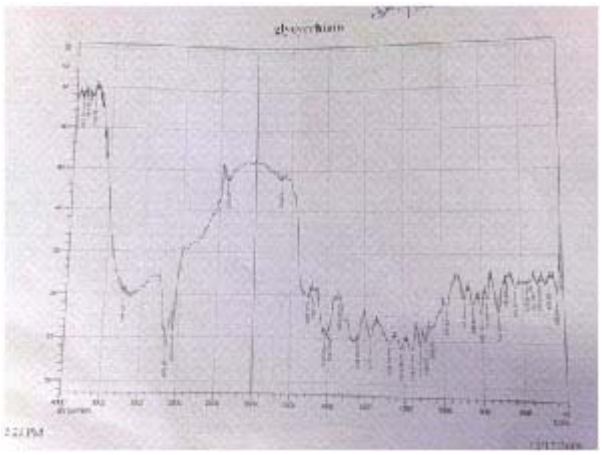

Fig. 2(b): Showing IR Spectra of Test sample

Chemical Test

- Test for Glycosides - Positive
- Test for Liquorice - Positive
- Test for Saponin glycoside - Positive

TLC (Thin layer chromatography)

- RF value of Standard was found to be- 0.45
- RF value of Test was found to be- 0.41

UV-Spectrophotometer

- UV maximum of Standard- 248 nm
- UV maximum of Test - 230 nm

IR-Spectroscopy

Behavioral study

Effect on Hyperalgesia

The daily treatment with Glycyrrhizin and Fluoxetine increases the tail withdrawal time of stressed animals significantly.

Effect on Locomotion

Daily treatment with Glycyrrhizin and Fluoxetine decreases locomotor activity when compared with control (stressed) group.

Effect on Muscle Co-ordination

The daily treatment with Glycyrrhizin and Fluoxetine increases the fall off time significantly when compared to control (stressed) group.

Effect of Memory

Daily treatment with Glycyrrhizin and Fluoxetine prevented the cognitive dysfunction significantly as compared to control (stressed) group.

DISCUSSION

Stress is known to induce alterations in various physiological and psychological responses even leading to pathological diseases. The stress induced effects are supposed to be an outcome of altered activity of different mechanisms such as Central neurotransmitter, Neurohormonal factors, particularly those linked with the pituitaryadrenal axis and free radical generation. Exposure to stress caused significant behavior and biochemical changes. Chronic immobilization stress is the most widely used method for assessing the antistress property of a novel compound. In the present study, chronic immobilization stress caused impairment of muscle coordination, locomotion, anxiety, cognitive functions and hyperalgesia. Immobilization stress increases 2-3 fold of plasma corticosterone level due to activation of Hypothalamic-Pituitary-Adrenal axis (H-P-A axis) resulting in increased production of corticosterone. In humans and animals, adrenal cortex contains a higher concentration of ascorbic acid than other tissues and the acute administration of adrenocorticotropic hormone (ACTH) caused decrease in ascorbic acid levels. Increased cortisol level has been linked with anxiety like behavior and painful responses in humans. Stress may also cause oxidative stress and the formation of free radicals. Oxidative stress can cause cellular damage and neurodegeneration by inducing the reactive oxygen species (ROS) that oxidizes vital cellular components such as lipids, proteins and DNA. Stressed animals showed an early fall-off from the Rota-rod, increased anxiety response in mirror chamber, increased locomotor activity in actophotometer, hyperalgesic response and cognitive dysfunction with altered concentration and memory. Chronic immobilization stress also caused significant oxidative damage in animals brains indicated by increased lipid peroxidation, protein, nitrite activity and depleted reduced glutathione and catalase level in stressed brain. Daily treatment with Glycyrrhizin (100 & 200 mg/kg, p.o.) and Fluoxetine (10 mg/kg, i.p.) causes significantly increased the fall-off time, decreased latency to enter in mirror chamber, decreased locomotor activity, decreased the hyperalgesic responses and prevented the memory dysfunction. Glycyrrhizin also significantly decreased the level of lipid peroxidation, protein, nitrite and increased the activity of endogenous antioxidants such as reduced glutathione and catalase in the brain. Glycyrrhizin also reversed the decrease level of adrenal ascorbic acid in stressed animals. Antistress activity of Glycyrrhizin may be due to attenuating the H-P-A axis activation and free radical scavenging activity (Antioxidant activity). In summary, the present study revealed that daily treatment with Glycyrrhizin (100 & 200 mg/kg, p.o.) was effective in reversing chronic immobilization stress induced various behavioral and biochemical alteration in mice.

CONCLUSION

The current research concludes that Glycyrrhizin at the doses of 100 and 200 mg/kg, p.o. reversed the behavioral and biochemical changes in Chronic Immobilization Stressed mice. So we can predict that Glycyrrhizin, the active constituent of liquorice shows antistress potential.

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