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ANTISTRESS POTENTIAL OF GLYCYRRHIZIN IN FORCED SWIM STRESS

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ABSTRACT

Stress is an aversive stimulus which perturbs the physiological homeostasis and its impact is reflected on a variety of biological systems. Complex mechanisms contribute to the breakdown in adaptational process resulting in various behavioral and endocrinological changes. Stress involved in the etiopathogenesis of variety of disease such as depression & anxiety, cognitive dysfunction, male impotency, hypertension and ulcerative colitis. The hypothalamic-pituitary-adrenal (HPA) axis and adrenal glands are crucial for the regulation of stress physiology. The activation of the HPA (Hypothalamic-pituitary-adrenal) system due to stress results in secretion of corticotrophin hormone, adrenocorticotropin hormone (ACT), β-endorphin and glucocorticoids into the circulation. Release of ACT in stress stimulates adrenals to increase production of hormones- epinephrine, nor epinephrine and corticosteroids. The current research concludes that Glycyrrhizin at the doses of 100 & 200 mg/kg, *p.o.* significantly reversed the Behavioral and Biochemical alterations in Forced Swim Test induced stress in mice when compared with Fluoxetine (selective serotonin reuptake inhibitor, 10mg/kg, *i.p.*), taken as a standard drug. On the basis of above paradigm we can predict that Glycyrrhizin, the active constituent of Liquorice (*Glycyrrhiza glabra*) shows antistress potential.

Key words: Fluoxetine, Etiopathogenesis, Homeostasis.

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 (Anonymous 1).

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 (Gupta V *et al*., 2004). 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 (Gupta V *et al*., 1999).

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PLANT PROFILE

Botanical Name - Glycyrrhiza glabra Linn.
Family - *Leguminosae*
Synonyms - Glycyrrhiza, Liquroice, Mulethi
Glycyrrhiza; glykas/glukos = sweet, rhiza = root (the one whose root is sweet to taste); glabra = smooth and hairless (Gupta D, 2008).
Glycyrrhizin is a potassium and calcium salt of glycyrrhizinic acid. Glycyrrhizinic acid is a glycoside and on hydrolysis yields glycyrrhetinic acid (glycyrrhetic

acid) which has a triterpenoid structure and three molecules of glucuronic acid (IHP, 2002). Glycyrrhizin is 50 times sweeter than sucrose and used as flavoring and sweetening agent, for taste improvement of vitamin B complex and other nauseous liquid pharmaceuticals. Glycyrrhizin is a triterpene glycoside which have numerous pharmacological effects like anti-inflammatory, anti-viral, anti-tumor, hepatoprotective activities, anxiolytic and in epilepsy (Kazuo O *et al*., 1981).

Marketed products of Glycyrrhizin

(1) Mono-Ammonium glycyrrhizinate (MAG)
(2) Disodium Glycyrrhizinate (Kazuo O *et al*., 1981)

MATERIALS AND METHODS

Pharmacological Study

Drug

Glycyrrhizin was obtained from the Sigma Aldrich Corporation, Mumbai.

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.).

Forced Swim Stress in Mice

Animals in all the groups except control (normal) were forced to swim in groups of 6 in plastic/glass container (38x23x16 cm3) containing water to a height of 7 cm at 25^{o}C ± 3^{o}C for 1 h every day for 7 days. The mice were prevented from clinging to each other or to walls of container (Kaur G *et al*., 2000).

Drugs and Treatment

Glycyrrhizin and Fluoxetine solution were made by dissolving them in distilled water.
Fluoxetine (10 mg/kg, *i.p*.) and Glycyrrhizin (100 and 200 mg/kg, *p.o.*) were administered 30 min and 1 hour before mice subjected to Forced swim stress respectively.

Experimental design

Group-I - Normal (Unstressed)
Group-II - Control (Stressed)
Group-II - Glycyrrhizin (100mg/kg, *p.o.*, Stressed)
Group-IV - Glycyrrhizin (200mg/kg, *p.o.*, Stressed)
Group-V - Fluoxetine (10 mg/kg, *i.p.*, Stressed)

Behavioral Study

All the behavioral parameters were observed on the 7th day of forced swim stress except the measurement of depression which was observed on 8th day.

Measurement of Depression

On the 8th day the depression level (duration of immobility period) was recorded in all groups of mice. The mice were forced to swim individually for 6 min session in a glass jar and the duration of immobility period was recorded (Dhir A *et al*., 2008).

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 (Goyal R *et al*., 2007).

Measurement of Cognitive dysfunction

Cognitive behavior was noted by using elevated plus-maze learning task. Transfer latency (TL) that is the time taken by the animal to move from the open arm to enclosed arm, was considered as an index of learned task (memory process). The elevated plus maze consisted of two open arms (50×10 cm) and two closed arms (50×10×40 cm)with an open roof. The maze was elevated to a height of 25 cm from the floor. The animal was placed individually at the end of either of the open arms and the initial transfer latency was noted on the first day. If the animal did not enter an enclosed arm within 90 s, it was gently pushed in to the enclosed arm and the transfer latency was assigned as 90 s. To become acquainted with the maze, the animals were allowed to explore the plus maze for 20 s after reaching the closed arm and then returned to its home cage. Retention of the learned task was assessed 24 h after the 1st day trial (Kulkarni SK *et al*., 2006).

Measurement of Anxiety

The anxiety level of various groups of mice was measured using mirror chamber and following parameters were recorded.
(i) Latency to enter the chamber
(ii) 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 (Luck H, 1971; Kulkarni MP *et al*., 2009).

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 (Luck H, 1971; Kulkarni MP *et al*., 2009).

Measurement of Hyperalgesia

The hyperalgesic response on the hot-plate is considered to result from a combination of central and peripheral mechanisms. In this test, animals were individually placed on a hot- plate (Eddy's Hot-Plate) with the temperature adjusted to 55± 1^{o}C. The latency to the first sign of paw licking or jump response to avoid the heat was taken as an index of the pain threshold; the cut-off time was 10s in order to avoid damage to the paw (Xia X *et al*., 2007).

BIOCHEMICAL PARAMETERS

On the 8th 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 (Kumar A *et al*., 2008).

Estimation of Corticosterone

Before decapitation, 1 ml of blood was collected directly from the heart in heparinised syringe. The plasma was then separated and processed for plasma corticosterone at once. The reading was taken at 530 nm. The observations were compared with those of stressed, vehicle treated group (Ahmad A *et al*., 2007).

Measurement of Lipid peroxidation

Took 0.5 ml homogenate + 0.5 ml Tris-HCL (P^{H}- 7.4) and incubated at 37^{0}C for 2 hours. Then 1 ml 10% TCA (Trichloro acetic acid) was added. It was centrifuged at 1000 x g for 10 min. To 1 ml supernatant, 1 ml of 0.67% TBA (Thiobarbituric acid) were added and 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 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 (Ishola IO *et al*., 2008).

Estimation of Reduced Glutathione (GSH)

1 ml of homogenate was precipitated with 1 ml of 4% sulfosalicyclic acid by keeping the mixture at 4^{0}C for 1 hour. Immediately Centrifuged 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, P^{H}-8) were taken. The yellow color was measured at 412 nm using UV spectrophotometer. Values were expressed as nanomoles of reduced glutathione per mg of protein.

Catalase Estimation

Catalase estimation was measured on the basis of 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 (Ishola IO *et al*., 2007).

Statistical Analysis

The data were analyzed by Graph Pad Prism software demo version and results are expressed as Mean±S.E.M. The significance of the difference in the responses of treatment groups in comparison to the control was determined by One Way Analysis of Variance (ANOVA) followed by "by Dunnett's test". $P<0.05$ was considered significant, $P<0.01$ considered very significant and $P<0.001$ considered highly significant statistically.

RESULTS

Table 1. Physical Parameters

S.No.	Parameters	Standard
1.	Color	White to Brownish yellow powder
2.	Solubility	Freely soluble in hot water and alcohol
3.	Melting point	292^{0}C

Table 2. Effect of Glycyrrhizin and Fluoxetine treatment on Immobility time on 8th day of forced swim stress in mice

S. No.	Groups	Immobility Time (sec) Mean ±SEM
1	Normal	204.2±2.574
2	Control (stressed)	262.2±3.497
3	Glycyrrhizin (100mg/kg)	229.2±1.990***
4	Glycyrrhizin (200mg/kg)	215.7±1.430***
5	Fluoxetine (10mg/kg)	206.3±1.563***

Table 3. Effect of Glycyrrhizin and Fluoxetine treatment on Muscle coordination on 7th day of forced swim stress in mice

S. No.	Groups	Fall off Time (sec.)Mean ±SEM
1	Normal	114.5±2.930
2	Control (stressed)	36.33±2.404
3	Glycyrrhizin (100mg/kg)	98.83±2.774***
4	Glycyrrhizin (200mg/kg)	82.50±3.096***
5	Fluoxetine (10mg/kg)	78.00±2.082***

Table 4. Effect of Glycyrrhizin and Fluoxetine treatment on Memory on 7th day of forced swim stress in mice

S. No.	Groups	Latency to Enter (Sec) Mean ±SEM
1	Normal	10.33±0.146
2	Control (stressed)	27.67±0.7149
3	Glycyrrhizin (100mg/kg)	14.17±0.4773***
4	Glycyrrhizin (200mg/kg)	12.00±0.5774***
5	Fluoxetine (10mg/kg)	10.67±0.7149***

Table 5. Effect of Glycyrrhizin and Fluoxetine treatment on Locomotor activity on 7th day of forced swim stress in mice

S. No.	Groups	No. of counts/5 min Mean ±SEM
1	Normal	394.2±7.120
2	Control (stressed)	150.0±38.50
3	Glycyrrhizin (100mg/kg)	385.0±41.69**
4	Glycyrrhizin (200mg/kg)	299.2±9.867***
5	Fluoxetine (10mg/kg)	211.7±9.718*

Table 6. Effect of Glycyrrhizin and Fluoxetine treatment on Hyperalgesia on 7th day of forced swim stress in mice

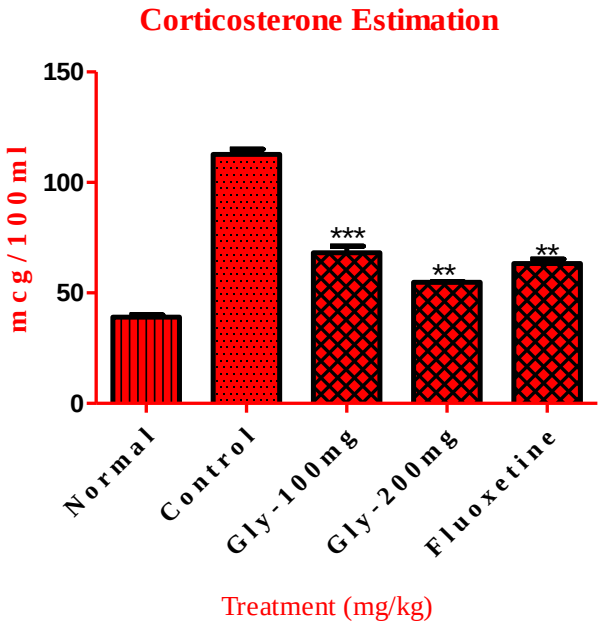
S. No.	Groups	Paw licking/Jump response (Sec) Mean ±SEM
1	Normal	5.000±0.3651
2	Control (stressed)	4.500±0.2236
3	Glycyrrhizin (100mg/kg)	6.333±0.4944
4	Glycyrrhizin (200mg/kg)	8.167±0.7923***
5	Fluoxetine (10mg/kg)	7.500±0.5627**

Table 7. Effect of Glycyrrhizin and Fluoxetine treatment on Anxiety level on 7th day

S.No.	Groups	Latency to Enter (sec) (Mean ±SEM)	No. of Entries (Mean ± SEM)	Time spent (Sec) (Mean ± SEM)
1	Normal	53.00±2.251	4.353±0.4944	22.83±3.038
2	Control (stressed)	96.67±2.108	1.833±0.3073	4.833±0.7923
3	Glycyrrhizin (100mg/kg)	52.83±1.376***	4.667±0.4216***	22.33±1.476***
4	Glycyrrhizin (200mg/kg)	55.33±1.820***	6.667±0.3333***	26.17±2.522***
5	Fluoxetine (10mg/kg)	29.17±1.424***	11.33±0.6667***	36.67±2.231***

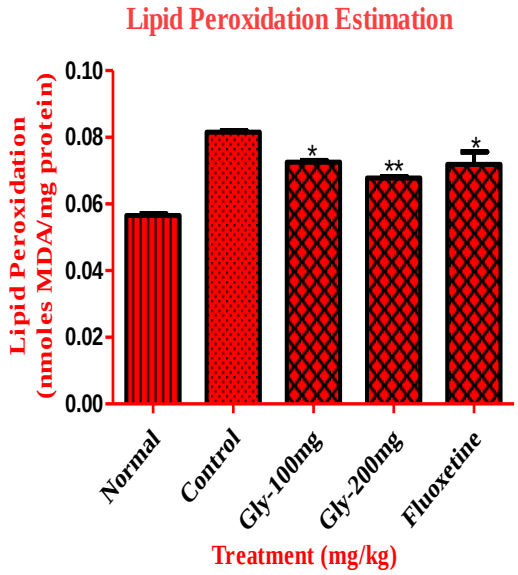
Values are express in Mean ±SEM., P <0.01**Very significant, P <0.001 ***Highly Significant as compare to Control group. (ANOVA followed by Dunnett's test), n=6.

Fig 1. Effect of Glycyrrhizin (100 & 200 mg/kg, p.o.) on corticosterone level in forced swim stress induced biochemical alteration in the whole brain of mice.



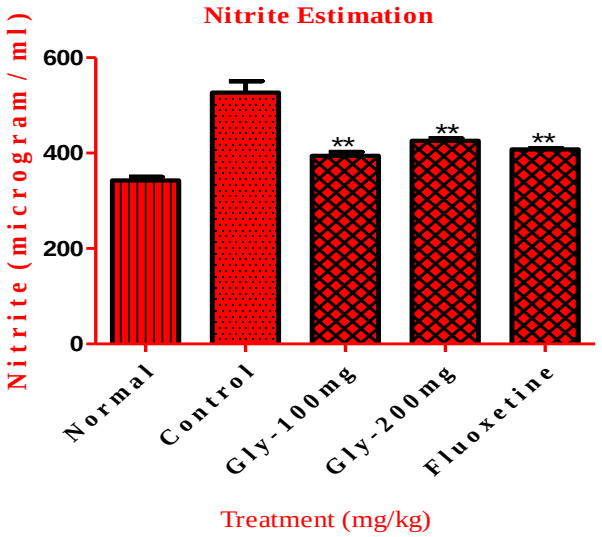
Values are express in Mean±SEM. $P<0.01$ **Very Significant, $P<0.001$ ***Highly Significant as compare to Control group. (ANOVA followed by Dunnett's test), n=6.

Fig 2. Effect of Glycyrrhizin (100 & 200 mg/kg, p.o.) on lipid peroxidation level in forced swim stress induced biochemical alteration in the whole brain of mice.



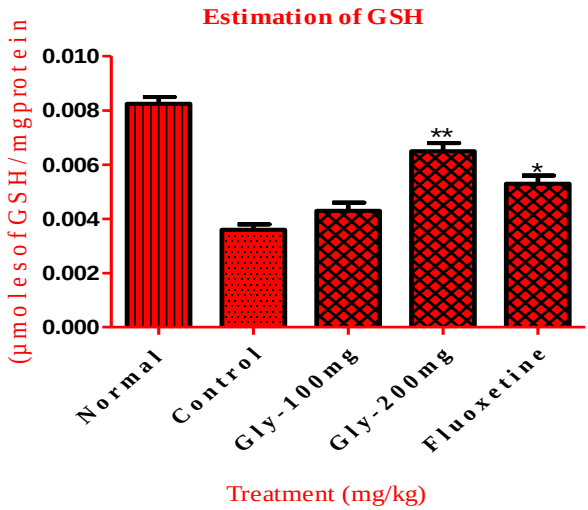
Values are express in Mean±SEM. $P<0.05$, *Significant, $P<0.01$**Very Significant as compare to Control group. (ANOVA followed by Dunnett's test), n=6.

Fig 3. Effect of Glycyrrhizin (100 & 200 mg/kg, p.o.) nitrite level in forced swim stress induced biochemical alteration in the whole brain of mice.



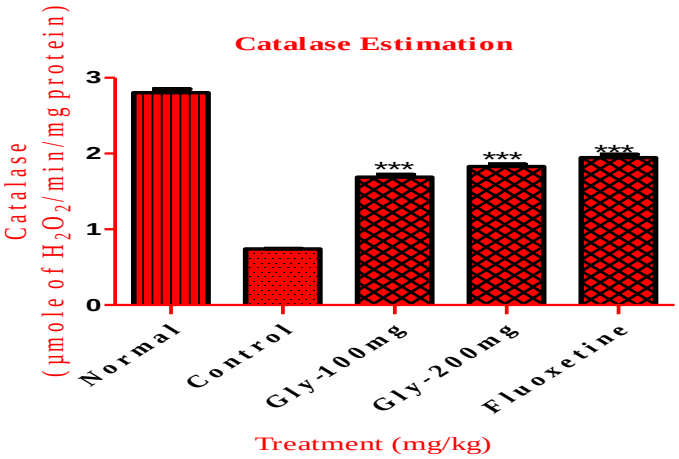
Values are express in Mean±SEM. $P<0.05$, *Significant, $P<0.01$**Very Significant as compare to Control group. (ANOVA followed by Dunnett's test), n=6.

Fig 4. Effect of Glycyrrhizin (100 & 200 mg/kg, p.o.) on reduced glutathione (GSH) level in forced swim stress induced biochemical alteration in the whole brain of mice.



Values are express in Mean±SEM, $P<0.05$, *Significant, $P<0.01$**Very Significant as compare to Control group. (ANOVA followed by Dunnett's test), n=6.

Fig 5. Effect of Glycyrrhizin (100 & 200 mg/kg, p.o.) on catalase level in forced swim stress induced biochemical alteration in the whole brain of mice.



Values are express in Mean±SEM, $P<0.05$, *Significant, $P<0.01$**Very Significant as compare to Control group. (ANOVA followed by Dunnett's test), n=6.

DISCUSSION AND CONCLUSION

Stress is an aversive stimulus which perturbs the physiological homeostasis and its impact is reflected on a variety of biological systems. Complex mechanisms contribute to the breakdown in adaptational process resulting in various behavioral and endocrinological changes. Stress involved in the etiopathogenesis of variety of disease such as depression & anxiety, cognitive dysfunction, male impotency, hypertension and ulcerative colitis.The hypothalamic-pituitary-adrenal (HPA) axis and adrenal glands are crucial for the regulation of stress physiology. The activation of the HPA (Hypothalamic-pituitary-adrenal) system due to stress result in secretion of corticotrophin hormone, adrenocorticotropin hormone (ACT), β-endorphin and glucocorticoids into the circulation. Release of ACT in stress stimulates adrenals to increase production of hormones- epinephrine, nor epinephrine and corticosteroids. CRF (corticotrophin releasing factor) as a neurotransmitter or neuromodulator in brain, is known to act within the central nervous system to modulate a number of behavioral, neuroendocrine and autonomic responses to environmental stimulation through its action on the HPA-axis, resulting in increased level of serum cortisol. Increased cortisol level has been linked with anxiety like behavior and depressed motor response in humans. During stressful conditions, changes in monamines (NA, DA & 5-HT) are well associated with transient behavioral aberrations in memory, learning and other mood disorders. Deregulated function of monoamines is one of the principle reasons for memory dysfunction during stressful conditions.

The current research concludes that Glycyrrhizin at the doses of 100 & 200 mg/kg, *p.o.* significantly reversed the Behavioral and Biochemical alterations in Forced Swim Test induced stress in mice when compared with Fluoxetine (selective serotonin reuptake inhibitor, 10mg/kg, *i.p.*), taken as a standard drug. On the basis of above paradigm we can predict that Glycyrrhizin, the active constituent of Liquorice (*Glycyrrhiza glabra*) shows antistress potential.

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