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### Beneficial role of Punarnavine against immobilization stress induced gastric ulceration in rats

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#### ABSTRACT

Present study was designed to investigate the effect of Punarnavine in immobilization stress induced gastric ulceration in rats. Immobilization stress was induced by immobilizing the animal for 2 hours daily for 10 days. Punarnavine (20 mg/Kg & 40 mg/Kg p.o) was given to drug treated rat groups 1 hour prior to induction of stress each day. At the end of stress protocol, gastric ulcerations in terms of ulcer index were evaluated. Stress induced changes in body weight, adrenal gland weight, plasma glucose, Serum corticosterone, serum thiobarbituric acid reactive substances (TBARS) and serum reduced glutathione (gSH) levels were evaluated to estimate the effect of Punarnavine on all the above-mentioned biochemical stress markers. In the study, immobilization stress resulted in profound gastric ulcerations as evident by significant rise in ulcer index. Significant increase in the adrenal gland weight and significant decrease in the body weight was also observed. Serum corticosterone levels (marker of stress) were also significantly elevated. Immobilization also increased oxidative stress assessed in terms of increase in serum TBARS and decrease in gSH levels. Treatment with Punarnavine attenuated immobilization stress induced gastric ulcerations, adrenal hypertrophy and increase in corticosterone levels along with normalization of other stress induced biochemical alterations.

**Keywords:** Immobilization Stress; Free radicals, Gastric ulceration; Punarnavine.

#### INTRODUCTION

Gastric ulcer is an illness that affects a considerable number of people worldwide. The etiological factors of this disorder include: stress, smoking, nutritional deficiencies, infections, frequent and indiscriminate use of nonsteroidal anti-inflammatory drugs (NSAIDs) [1]. Out of

the above mentioned factors, psychogenic factors, such as stress, play a major role in the pathogenesis of gastric ulcers in man. Stress has become an inadvertent factor in modern day living. Stressful events are well documented to activate the Hypothalamo-Pituitary-Adrenal (HPA) axis and central monoaminergic systems [2] which ultimately results in the secretion of glucocorticoids from the adrenal cortex [3]. Chronic stress induced excessive release of glucocorticoids has been reported to disturb the functional homeostasis of the body and is also implicated in the etiopathogenesis of a variety of disease states including hypertension, coronary heart disease [4], gastric ulcers [5], diabetes [6], immuno-suppression [7], mental depression, memory loss [8]. The resultant disturbances may vary depending upon type, intensity, and the duration of a particular stressor and the strain/sex differentiation of the subjects [9]. The pathogenesis of gastroduodenal ulcers is influenced by various aggressive and defensive factors, such as mucus secretion, mucosal barrier, acid-pepsin secretion, blood flow, cellular regeneration and endogenous protective agents (prostaglandins and epidermal growth factor) [10]. Although the introduction of proton-pump inhibitors to the classic anti-ulcer therapy had revolutionized treatment of peptic ulcers and other gastrointestinal disorders, but there is still no complete cure for this disease. It has been shown that long term use of these drugs leads to various adverse and side effects. Relapses of the malady due to ineffectiveness of different drug regimens and even resistance to drugs is emerging [11]. Thus, there is an urgent requirement to identify more effective and safe anti-ulcer agents. During the past few decades, a widespread search has been launched to identify new anti-ulcer therapies from natural sources. In the scientific literature, a large number of medicinal plants with gastric anti-ulcer potential have been reported [12-16]

Punarnavine is an alkaloid found in the plant *Boerhaavia diffusa* L. (family Nyctaginaceae) a perennial herb. Medicinal properties of the plant have been utilized since long in the indigenous system of medicine in India. It is widely distributed in the tropics and subtropics [17]. The chemical formula of Punarnavine is  $C_{17}H_{22}N_2O$ , having a melting point of 236-237°C [18]. It is also considered as the active principle in the plant extract [19].

Various types of extracts of different plant parts (especially roots and leaves) of *B.diffusa* have been documented to possess hepatoprotective [20], antiproliferative and antiestrogenic [21], antibacterial [22], spasmolytic [23], antifungal [24], antidiabetic [25], immunomodulatory [26], antiamebic [27] and anticonvulsant [28] activity. Most of the medicinal properties of the plant have been attributed to the alkaloidal constituent Punarnavine and recently many studies have documented the key role of Punarnavine in immunomodulatory [29] as well as anti-metastatic [30], activities on the plant. Although there has been some recent research regarding the antioxidant potential of leaf extract of *B.diffusa*, yet to the best of our knowledge, no study has been documented till now exploring effect of punarnavine in stress induced ulcers. Therefore, the present study was designed to evaluate the beneficial role of punarnavine, in immobilization stress induced gastric ulceration in rats.

## MATERIALS AND METHODS

### Animals

Male Wistar albino rats (Punjab Agriculture University, Ludhiana, India) of either sex weighing 25±5 g, maintained at standard laboratory diet (Kisan Feeds Ltd., Chandigarh, India) and having

free access to tap water, were employed in the present study. They were housed in the departmental animal house and were exposed to normal cycle of light and dark. The experimental protocol was duly approved by Institutional Animal Ethics Committee and care of the animals was carried out as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India (Reg. No.- 37/ 2007/ CPCSEA).

### **Drugs and Chemicals**

Punarnavine was obtained as a gift sample from Bio-gen Extracts Pvt. Ltd., Bangalore, India. Reduced Glutathione (gSH), 5, 5- dithiobis (2-nitro benzoic acid) (DTNB), and thiobarbituric acid were obtained from Loba Chem., Mumbai, India. 1, 1, 3, 3-tetramethoxy propane was procured from Sigma-Aldrich, USA. Glucose estimation kit was purchased from Transasia Biomedicals Ltd. Daman. India. Other chemicals and reagents used were purchased from SD fine-chemicals Ltd., Mumbai, India. All the reagents and chemicals employed in the study were of analytical grade.

### **Experimental Procedure**

#### ***Induction of immobilizations stress***

Immobilization stress was produced as per the method described by Kvetnansky [31].

#### ***Estimation of stress induced changes in ulcer index***

Ulcer Index (UI) was scored according to the method of Main & Whittle [32].

#### ***Evaluation of stress induced changes in Body Weight Adrenal Gland Weight***

At the end of stress protocol i.e., on 11<sup>th</sup> day, rats from all the groups were first weighed and then sacrificed. Kidneys were dissected out and adrenal gland was located as a small protuberance over the upper medial portion of the kidney. The adrenal gland was carefully dissected out and weighed.

#### ***Estimation of stress induced changes in Plasma Glucose Level***

Blood plasma glucose concentration was estimated by using glucose estimation kit which employed Trinder's method for estimation [33]. All the estimations were done using UV-Visible spectrophotometer (DU 640B Spectrophotometer, Beckman Coulter Inc., CA, USA).

#### ***Estimation of stress induced changes in Serum Corticosterone Level***

The estimation of corticosterone in serum was carried out by the method of Katyare and Pandya [34]

#### ***Estimation of stress induced changes in Serum thiobarbituric acid reactive substances (TBARS) Level***

The quantitative estimation of thiobarbituric acid reactive substances (TBARS), an index of lipid peroxidation in serum was performed according to the method of Satoh [35].

#### ***Estimation of stress induced changes in Serum Glutathione (gSH) Level***

The reduced glutathione (gSH) content in serum was estimated using method of Tietze [36].

**Experimental Protocol**

Seven groups were employed in the present study. Each group comprised of 12 Wistar albino rats.

***Group I (Normal rats)***

Rats were left undisturbed for the whole time period of study i.e. 10 days except for regular cleaning of cages. Rats were weighed on 11<sup>th</sup> day and were then sacrificed to collect blood for determination of various biochemical parameters.

***Group II (Immobilization stress)***

Rats were immobilized for 2 hrs daily, for 10 days, to produce immobilization stress (CS). Rats were weighed on 11<sup>th</sup> day and were then sacrificed to collect blood for determination of various biochemical parameters.

***Group III Punarnavine low dose + Immobilization stress (P1+S)***

Rats were subjected to chronic stress as described in group II. Punarnavine (20 mg/kg *p.o.*) was administered 1 hr prior to stress protocol each day. Rest of the protocol was same as described in group II.

***Group IV Punarnavine high dose + Immobilization stress (P2+S)***

Rats were subjected to chronic stress as described in group II. Punarnavine (50 mg/kg *p.o.*) was administered 1 hr prior to stress protocol each day. Rest of the protocol was same as described in group II.

***Group V (Punarnavine low dose per se)***

Rats were administered Punarnavine (20 mg/kg *p.o.*) for 10 days. Rest of the protocol was same as described in group I.

***Group VI (Punarnavine high dose per se)***

Rats were administered Punarnavine (50 mg/kg *p.o.*) for 10 days. Rest of the protocol was same as described in group I.

***Group VII (Vehicle in immobilization stress)***

Rats were subjected to chronic stress as described in group II. 0.5% CMC was administered *p.o.* 1 hr prior to stress protocol each day. Rest of protocol was same as in group II.

**Statistical Analysis**

The results were expressed as mean  $\pm$  standard error of means (S.E.M). The data obtained from various groups were statistically analyzed using one-way ANOVA followed by Tukey's test was used in post hoc analysis for comparison between different groups. The  $p < 0.05$  was considered to be statistically significant.

## RESULTS

### ***Effect of Punarnavine on immobilization stress induced gastric ulceration:***

Immobilization stress produced significant increase in ulcer index. Treatment with punarnavine (20 mg/Kg *i.p* and 40 mg/Kg *i.p*) significantly attenuated stress induced rise in ulcer index in a dose dependent manner.

### ***Effect of Punarnavine on immobilization stress induced changes in body weight and adrenal gland weight.***

Immobilization stress produced significant increase in adrenal gland weight and decrease in the body weight as compared to normal rats group. Treatment with Punarnavine (20 mg/Kg *i.p* and 40 mg/Kg *i.p*) significantly attenuated stress induced rise adrenal gland weight and decrease in body weight in a dose dependent manner.

### ***Effect of Punarnavine on Immobilization stress induced changes in plasma glucose and serum corticosterone level:***

Immobilization stress produced significant increase in plasma glucose and serum corticosterone levels as compared to normal rats group, as noted on 11<sup>th</sup> day after stress protocol. Treatment with Punarnavine (20 mg/kg *i.p* and 40 mg/kg *i.p*) significantly attenuated stress induced rise in plasma glucose (Figure 1) and serum corticosterone (Figure 2) level in a dose dependent manner. However, *per se* treatment with Punarnavine did not modulate the plasma glucose and corticosterone levels of rats.

### ***Effect of Punarnavine on chronic stress and chronic unpredictable stress induced changes in oxidative stress markers:***

Chronic stress and chronic unpredictable stress produced significant increase in serum thiobarbituric acid reactive substances (TBARS) and decrease in reduced glutathione levels as compared to normal rats group. Treatment with Punarnavine (20 mg/kg *p.o* and 40 mg/kg *p.o*) significantly attenuated stress induced rise in TBARS and decrease in glutathione levels in a dose dependent manner. However, *per se* treatment with Punarnavine did not modulate the serum TBARS and glutathione levels of rats (Figure 3) and (Figure 4)

## DISCUSSION

In the present study, exposure to Immobilization stress resulted in significant increase in ulcer index, adrenal gland weight, plasma glucose, serum corticosterone and decrease in the levels of plasma cholesterol and body weight. Increase in the incidences of gastric ulceration is a direct evidence of involvement of hypothalamus pituitary adrenal (HPA) axis, which is highly responsive to stress. The hyper activation of Para Ventricular Nucleus (PVN) in the hypothalamus causes a decrease in the mucosal blood flow and hyper contractility through descending projections which influence the activity of vagal efferent [37,38], and the resulting imbalance between defensive and aggressive factors induces pathogenesis of ulcers [39]. These stress induced biochemical changes may be a consequence of hyper-activation of Hypothalamo-Pituitary-Adrenal (HPA) axis, as stress is well documented to activate HPA axis [40]. Prolonged hyper-activation of HPA axis results in functional hypertrophy of adrenal gland [41] with subsequent increase in the corticosterone release [42]. Stress induced rise in plasma glucose and

decrease in cholesterol may be attributed to corticosterone mediated enhanced metabolism to meet the increased demands of the body organs during stress [43].

The present results demonstrate that administration of Punarnavine (20 mg/Kg and 40 mg/Kg *p.o*) significantly attenuated chronic stress and chronic unpredictable stress induced increase in corticosterone, glucose, cholesterol and creatine kinase levels. These findings clearly indicate the adaptogenic potential of Punarnavine during stressful events.

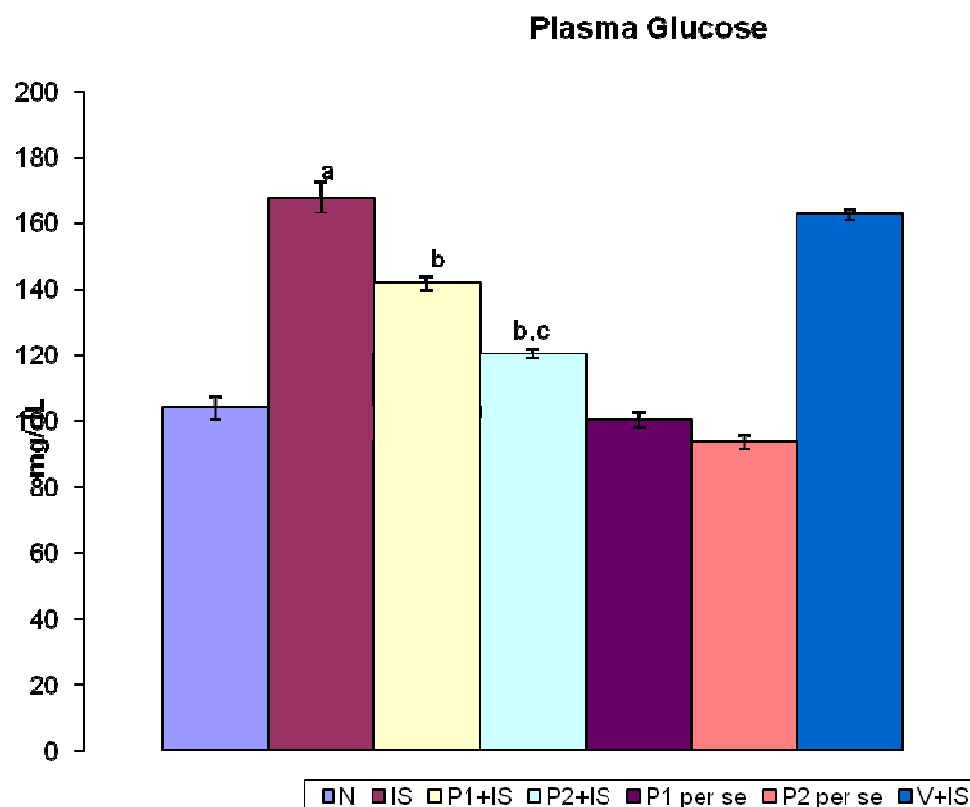
The beneficial effects of Punarnavine on immobilization induced gastric ulcerations may possibly be linked to decrease in corticosterone levels, which in turn may be the consequence of normalization of the activated HPA axis. Punarnavine may have acted directly on the hyper-activated HPA axis or may have had an indirect effect for its normalization. However, the present study data is not sufficient to delineate the molecular mechanism of Punarnavine mediated decrease in corticosterone secretion.

**Table1. Changes in average body weight, adrenal gland weight and ulcer index under chronic stress and chronic unpredictable stress of the normal, stress and curcumin-treated groups.**

Groups	Avg. body weight (gm) day 11	Adrenal gland weight (mg)	Ulcer index
N	25.3 ± 0.5	5.0 ± 0.4	0
IS	15.8 ± 0.6 <sup>a</sup>	7.5 ± 0.4 <sup>a</sup>	1.9 ± 0.1 <sup>a</sup>
P1+IS	20.1 ± 0.3 <sup>b</sup>	6.2 ± 0.3 <sup>b</sup>	0.18 ± 0.05 <sup>b</sup>
P2+IS	23.5 ± 0.4 <sup>b,c</sup>	5.1 ± 0.3 <sup>b,c</sup>	0.23 ± 0.04 <sup>b,c</sup>
Punarnavine 20 mg/kg <i>per se</i>	24.6 ± 0.4	4.8 ± 0.2	0
Punarnavine 40 mg/kg <i>per se</i>	25.6 ± 0.4	4.7 ± 0.3	0
Vehicle + IS	16.1 ± 0.4	7.8 ± 0.3	2.1 ± 0.01

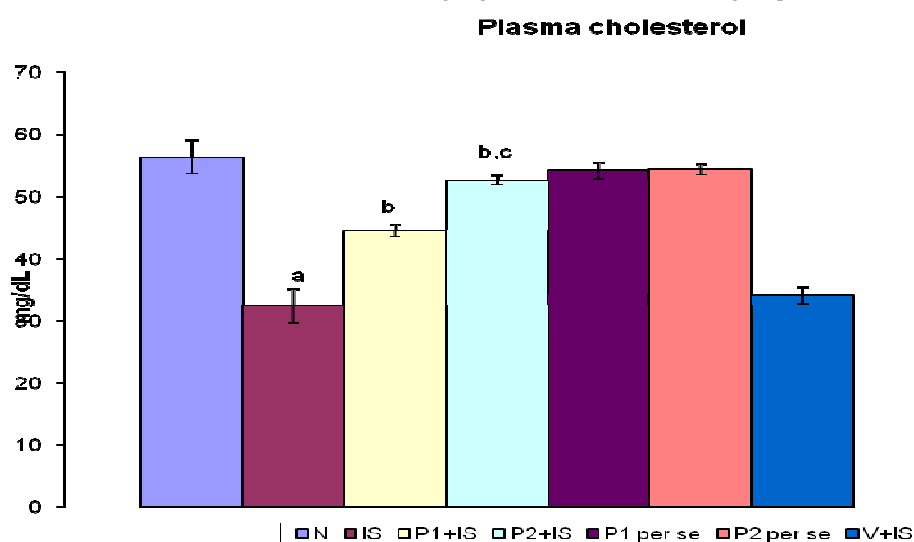
(N= Normal; IS= Immobilization Stress; P1+IS= Punarnavine 20mg/Kg *i.p* plus Immobilization Stress; P2+IS= Punarnavine 40mg/Kg *i.p* plus Immobilization Stress; V= Vehicle) Values are represented as the mean ± S.E.M, n = 12 in each group. <sup>a</sup> *p*<0.05, Vs N group, <sup>b</sup> *p*<0.05, Vs IS group, <sup>c</sup> *p*<0.05, Vs P1+IS group

Further, in the present study, immobilization stress resulted in significant elevation of TBARS (a marker of lipid peroxidation) and reduction in the levels of glutathione (an endogenous anti-oxidant). These findings imply the key role of oxidative stress in mediating immobilization stress induced gastric ulceration. Stress has been well documented to increase the production of free radicals [44], which has also been linked to hyper-activation of HPA axis with consequent increase in corticosterone secretion [45]. Conversely free radicals may also participate in HPA overactivation and increase in corticosterone secretion by producing damage to hippocampal neurons, which maintain the homeostasis of HPA axis by negative feedback mechanisms [46]. Recently, Yasukawa *et al.* reported that reactive oxygen species (ROS) are associated with gastric ulcer [47]. Uteshev *et al.* indicated that a stronger intensity of lipid peroxidation and less activity of the antioxidant system of the blood are correlated with gastroduodenal ulcerous hemorrhages [48]. Previous studies in experimental gastric ulcers show that the elimination of free radicals by an anti-ulcer agent that has antioxidant and free-radical scavenging activities may contribute to reduce the severity of ulcer recurrence [49, 50].



**Figure 1. Changes in plasma glucose under immobilization stress (IS) of the normal, immobilization stress and Punarnavine treated groups.**

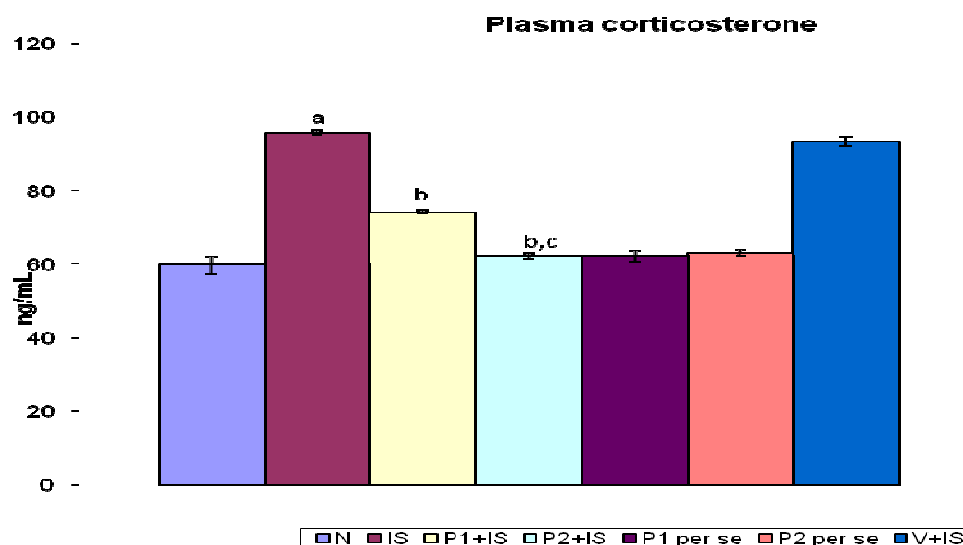
Results are represented as the mean  $\pm$  S.E.M. with  $n = 12$  in each group. <sup>a</sup> $p < 0.05$ , as compared to the Normal (N) group; <sup>b</sup> $p < 0.05$ , as compared to immobilization Stress (IS) group; <sup>c</sup> $p < 0.05$ , as compared to Immobilization stress + 20 mg/Kg Punarnavine (P1+IS) group.



**Figure 2. Changes in plasma cholesterol under immobilization stress (IS) of the normal, immobilization stress and Punarnavine treated groups.**

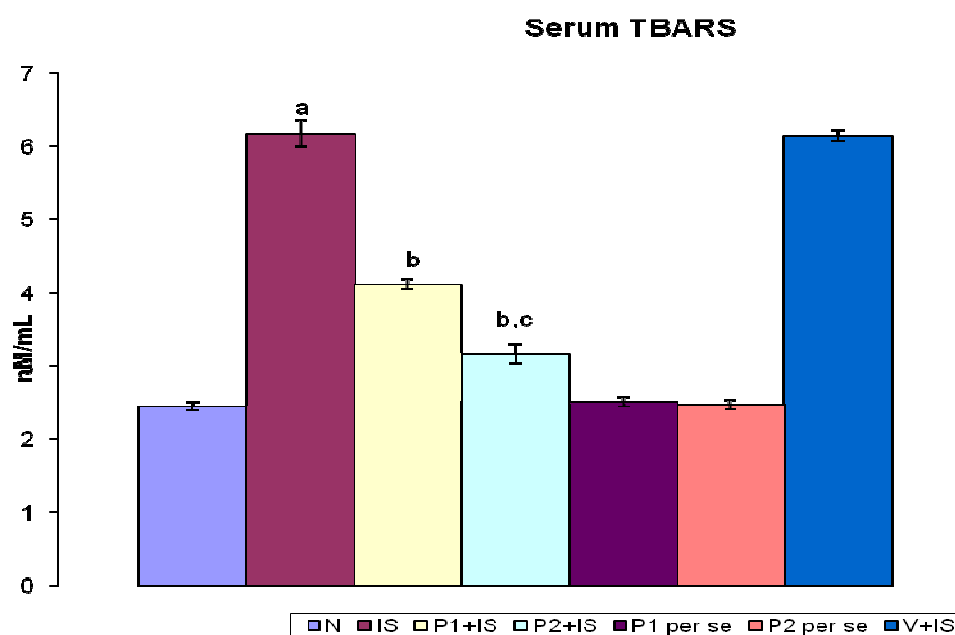
Results are represented as the mean  $\pm$  S.E.M. with  $n = 12$  in each group. <sup>a</sup> $p < 0.05$ , as compared to the Normal (N) group; <sup>b</sup> $p < 0.05$ , as compared to immobilization Stress (IS) group; <sup>c</sup> $p < 0.05$ , as compared to Immobilization stress + 20 mg/Kg Punarnavine (P1+IS) group.





**Figure 4. Changes in serum corticosterone under immobilization stress (IS) of the normal, immobilization stress and Punarnavine treated groups.**

Results are represented as the mean  $\pm$  S.E.M. with  $n = 12$  in each group. <sup>a</sup>  $p < 0.05$ , as compared to the Normal (N) group; <sup>b</sup>  $p < 0.05$ , as compared to immobilization Stress (IS) group; <sup>c</sup>  $p < 0.05$ , as compared to Immobilization stress + 20 mg/Kg Punarnavine (P1+IS) group.



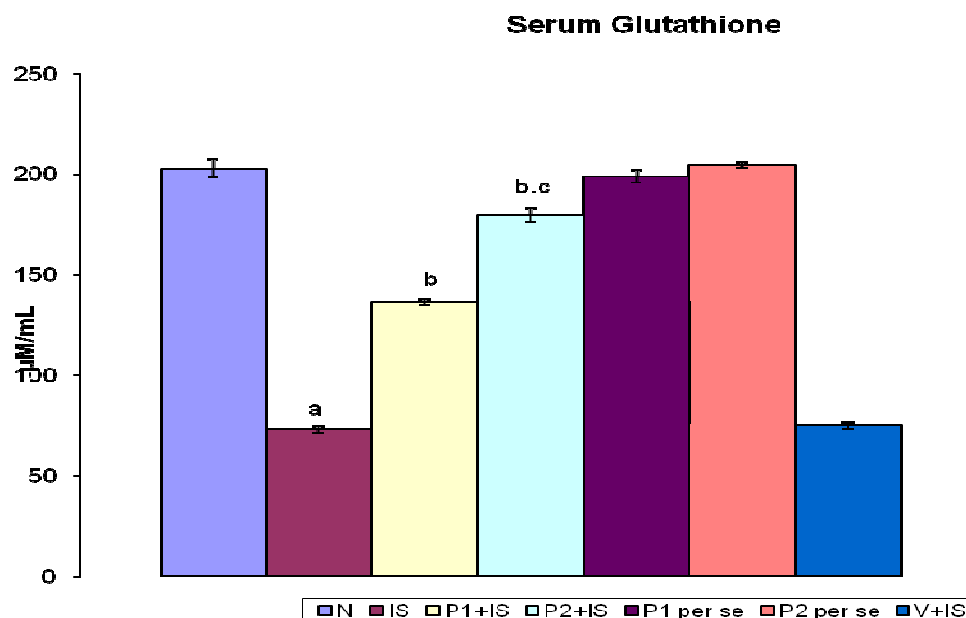
**Figure 5. Changes in serum TBARS under immobilization stress (IS) of the normal, immobilization stress and Punarnavine treated groups.**

Results are represented as the mean  $\pm$  S.E.M. with  $n = 12$  in each group. <sup>a</sup>  $p < 0.05$ , as compared to the Normal (N) group; <sup>b</sup>  $p < 0.05$ , as compared to immobilization Stress (IS) group; <sup>c</sup>  $p < 0.05$ , as compared to Immobilization stress + 20 mg/Kg Punarnavine (P1+IS) group.

Further, administration of Punarnavine attenuated immobilization stress associated increase in oxidative stress in terms of reduction in TBARS and elevation of glutathione levels, suggesting that free radical scavenging property of Punarnavine may also be playing a key role in its anti-



ulcer activity. *Boerhaavia diffusa* has been documented as potent antioxidant [19]. This hypothesis is supported by other reports demonstrating the anti-stress effects of antioxidants such as vitamin C [51], Lipoic acid [52], Vitamin E [53] and *Triphala* [54].



**Figure 6. Changes in serum Glutathione under immobilization stress (IS) of the normal, immobilization stress and Punarnavine treated groups.**

Results are represented as the mean  $\pm$  S.E.M. with  $n = 12$  in each group. <sup>a</sup>  $p < 0.05$ , as compared to the Normal (N) group; <sup>b</sup>  $p < 0.05$ , as compared to immobilization Stress (IS) group; <sup>c</sup>  $p < 0.05$ , as compared to Immobilization stress + 20 mg/Kg Punarnavine (P1+IS) group.

Therefore, it may be proposed that Punarnavine mediated antioxidant actions and normalization of hyper-activated HPA axis with subsequent decrease in corticosterone secretion (may also be due to antioxidant action) is responsible for its preventive effects in immobilization stress induced gastric ulcerations. Nevertheless, further studies are needed to explore the protective role of Punarnavine in other models of gastric ulcers and to delineate the molecular mechanism of Punarnavine mediated normalization of stress induced hyper-activation of HPA axis which possibly resulted in protection against ulceration in the present study.

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