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Screening of *Psidium guajava* Leaf Extracts for Antistress Activity in Different Experimental Animal Models

Lakshmi B.V.S.*, Sudhakar M.

Department of Pharmacology, Malla Reddy College of Pharmacy, Dhulapally (via Hakimpet), Maisammaguda, Secunderabad-500014, Andhra Pradesh, India.

*** Author for Correspondence:** *B.V.S. Lakshmi E-Mail: lakshmi_saineha@yahoo.com*
Tel: 09885324334, Fax No.: 040-23792154

ABSTRACT

Ethanolic extract of leaves of *Psidium guajava* was investigated on anoxia stress tolerance test in Swiss mice. The animals were also subjected to acute physical stress (swimming endurance test) and acute heat induced stress to gauge the antistress potential of the extract. Further to evaluate the antistress activity of *Psidium guajava* in chronic stress condition, fresh Wistar rats were subjected to cold restraint stress (4° for 2 h) for 10 days. Stimulation of hypothalamus pituitary adrenal axis in stressful condition alters plasma glucose, triglyceride, cholesterol, BUN and corticosterone levels. There is also alteration in the blood cell counts. Pretreatment with the extract significantly ($P<0.001$) ameliorated the stress-induced variations in these biochemical levels and blood cell counts in both acute and chronic stress models. The extract treated animals showed increase in swimming endurance time and increase in anoxia tolerance time in physical and anoxia stress models respectively. Treatment groups also reverted back increase in liver, adrenal gland weights and atrophy of spleen caused by cold chronic stress and swimming endurance stress models. The results indicate that ethanolic extract of *Psidium guajava* has significant adaptogenic activity against a variety of biochemical and physiological perturbations in different stress models.

Keywords: Acute heat stress, Adaptogenic, Antistress, Cold restraint stress, *Psidium guajava,* Swimming stress.

INTRODUCTION

Stress basically is a reaction of mind and body against change in the homeostasis. The productive stress is called Eustress while harmful stress is called Distress. If the stress is extreme, the homeostatic mechanisms of the organism become deficit and the survival of the organism is threatened. Under these conditions, stress triggers a wide range of body changes called General Adaptation Syndrome (GAS). The stimuli, which produce GAS, are called the Stressors and range from physical to psychological factors including cold, heat, infection, toxins, major personal disappointment etc (1).In the stress-filled environment we live in, successful adaptation to stress is a prerequisite for survival. In the indigenous system of medicine, there are many herbal drugs and formulations recommended to enable one to withstand stress without altering the physiological functions of the body. This, drug induced state of resistance against aversive stimuli is termed as Adaptogenic activity and the drugs, named Adaptogens. Stress alters the equilibrium of various hormones which have a significant impact on the immune response in general. The status of immune system-immunosuppression versus immunopotentiation-will depend upon the net effect of these changes. Stress and depression have been shown to affect immune system functioning, with both immunosuppression and immune activation (2). Correlations between depression and elevated susceptibility for infections or mortality rates have been observed and are associated with immune

suppression (3). The physiological reaction to stress involves alteration in the autonomic nervous system, the endocrine system and the immune system. The secretion of Glucocorticoids is a classic endocrine response to stress (4). Stressful stimulation influences antigen-specific as well as nonspecific reactions (5).

Many herbs reported in ancient literature have potent antistress activity and their utilities in current scenario need to be unveiled. Extensive literature survey revealed that *Psidium guajava*, acclaimed as 'poor man's apple of the tropics', has a long history of traditional use for a wide range of diseases. The fruit as well as its juice is freely consumed for its great taste and nutritional benefits. Much of the traditional uses have been validated by scientific research (6). Toxicity studies in mice and other animal models as well as controlled human studies show both leaf and fruit are safe without any side effects (6). A number of chemicals isolated from plants like quercetin, guaijaverin, flavonoids and galactose-specific lecithins have shown promising activity in many human trials (7). The plant has been extensively studied in terms of pharmacological activity of its major components, and the results indicate potent antidiarrheal, antihypertensive, hepatoprotective, antioxidant, antimicrobial, hypoglycemic and antimutagenic activities (8). *P. guajava* belongs to the family Myrtaceae. *P. guajava* may have been domesticated in India several thousand years ago. In view of the immense medicinal importance of *P. guajava* plant evidenced in the various studies mentioned above and also corroborated in a recent review article by Kamath et al. (2008), there is a strong incentive for further research to evaluate the potential usefulness of leaves of *Psidium guajava* for antistress and adaptogenic activity in experimental animals.

MATERIALS AND METHODS

Chemicals

All the chemicals used were of the analytical grade. Ashwagandha was purchased from Himalaya Company Ltd. Kits for the examination of serum cholesterol, triglycerides, BUN, corticosterone and glucose were purchased from SPAN Diagnostics Kits Ltd.

Preparation of extract

The leaves of *Psidium guajava* Linn. were collected in Ranga Reddy district of Hyderabad and was positively identified and confirmed by botanist in Osmania University, Hyderabad. The voucher specimen is deposited in our college herbarium with specimen no. MRCP-04. The collected leaves were dried under shade. The shade dried leaves were coarsely powdered and extracted with 99% ethanol using Soxhlet apparatus at a temperature below 60°C for 24 hours. The solvent was evaporated under vacuum, which gave semisolid mass (yield: 25% w/w) with respect to the dried powder. Oral suspensions containing 100 mg/ml, 200mg/ml and 400mg/ml of the ethanolic extract of *Psidium guajava* were prepared in 1% w/v gum acacia.

Animals

Swiss albino mice weighing 20–25 g and Albino Wistar rats weighing 180–250 g of either sex, 4 months of age were used for this study. The experimental animals were housed in polypropylene cages and maintained under standard conditions (12 h light and dark cycles, at 25±3°C and 35–60% humidity). Standard pelletized feed and tap water were provided *ad libitum*. The Institutional Animal Ethical Committee (IAEC) of Malla Reddy College of Pharmacy, Hyderabad, with Reg. No. 1217/a/08/CPCSEA, approved the study.

Anoxia stress tolerance test in mice (9)

Swiss mice of either sex were divided randomly into 5 groups, each group containing 6 mice. Group I mice received 0.1% gum acacia in saline; (vehicle control). Group II mice were treated with water soluble powder of Aswagandha (100 mg/kg, p.o.) and stress; Group III, IV and V mice were treated with ethanolic extract at doses of 100, 200 and 400 mg/kg, p.o. and stress. The drug treatment was carried out daily for a period of 21 days. At the end of each week i.e. 1st, 2nd and 3rd weeks of drug treatment, the animals were exposed to the anoxia stress and anoxia tolerance time was noted. Hermetic vessel of one litre air capacity was used to induce anoxia stress. Each animal was kept in the hermetic vessel and the time to show the first sign of convulsion was noted, and were immediately removed from the vessel and resuscitated if needed.

Forced swimming endurance test (physical stress) (10)

Rats of either sex (200–250g) were used for forced swim endurance stress. Group I rats received 0.1% gum acacia in saline; (vehicle control). Group II mice were treated with 0.1% gum acacia in saline and stress; (negative control). Group III rats were treated with water soluble powder of Aswagandha (100 mg/kg, p.o.) and stress; (positive control). Group IV, V and VI mice were treated with ethanolic extract at 100, 200 and 400 mg/kg, p.o. and stress. The rats were subjected to swimming stress by keeping them in propylene tank of dimension (37×37×30 cm), filled with water to a height of 25cm.

Extracts were given to rats, once daily for period of 7 days. On 8th day the rats were allowed to swim till complete exhaustion and the endpoint was taken when the animal started drowning. The mean swimming time for each group was calculated. Then animals were killed and blood was collected by cardiac puncture to estimate biochemical parameters like serum glucose, triglycerides, cholesterol, BUN, corticosterone and blood cell count (RBC, WBC and DLC). The weights of organs such as liver, adrenals, spleen were recorded after washing with alcohol.

Chronic cold restraint stress (11)

Treatment groups were similar to forced swimming endurance stress. Rats were subjected to cold stress by exposing them to 4 ± 1°C, daily for 2hrs for a period of 10 days. Animals were sacrificed at the end of the study period and blood was collected for estimation of various biochemical parameters such as Serum cortisol, glucose levels, RBC count, total leukocyte count, differential count as well as lipid profile. Similarly the weights of organs i.e. liver, spleen and adrenal glands were also recorded.

Acute heat induced stress (12)

Treatment groups were similar to forced swimming endurance stress. Albino rats (180–250 g) of either sex were subjected to stress. All the animals were subjected to heat stress by exposing them to a controlled temperature of 40 ± 2°C daily for a period of 8 days. After which the animals were sacrificed. The blood collected was centrifuged at 5000 rpm for 10 mins for separation of plasma for estimation of Biochemical parameters like glucose, cholesterol, triglycerides and BUN.

Statistical analysis

All the values are expressed as mean ±SEM and data was analyzed by one-way ANOVA, using Graph pad INSTAT. The post-hock analysis was carried out by Dünnet's multiple comparison test to estimate the significance of difference between individual groups.

RESULTS

Preliminary phytochemical analysis of the extract revealed the presence of flavonoids-quercetin, saponins, alkaloids, fixed oils, triterpenes, proteins and steroids.

Effect of ethanolic extract in anoxia stress tolerance test

In the anoxia tolerance test (Table 1), the extract at 100, 200and 400 mg kg^{-1}doses statistically produced a dose dependant significant ($P<0.001$) increase in mean time to convulsion in mice subjected to anoxia stress.

Effect of ethanolic extract in forced swimming endurance stress

The results of the study revealed that the extract possess antistress property as it significantly increased swimming time (Figure 1). Swimming endurance stress resulted in significant increase in adrenal gland weight and liver weight with concomitant decrease in spleen weight in stress control group, which was significantly reverted by *Psidium guajava* pretreatment at 100mg/kg, 200 mg/kg and 400 mg/kg. Stress induced elevated blood cell counts of RBC and DLC i.e. lymphocytes, neutrophils, eosinophils and monocytes have been significantly reduced by the ethanolic extract in a dose dependant manner (Table 2). Pretreatment of animals with *Psidium guajava* at three doses also significantly restored back forced swimming stress induced alterations in plasma corticosterone, glucose, triglyceride, BUN and cholesterol (Table 3). The results were comparable to that of reference standard Ashwagandha.

Effect of ethanolic extract in cold restraint stress

In cold restraint stress, ethanolic extract at 100, 200 and 400mg/kg offered significant ($P<0.001$) protection against the change in the weights of liver, spleen and adrenal

Table 1. Effect of ethanolic extract of Psidium guajava in Anoxia Stress Tolerance in Mice

	Mean duration of tolerance time (min)		
Groups	**1st week**	**2nd week**	**3rd week**
Control	90.36±0.56	91.45±1.94	93.86±1.56
Ashwagandha 100 mg/kg p.o.	149.62±2.05**	153.49±1.21***	157.06±2.35**
P.guajava ext 100 mg/kg p.o.	111.49±2.59	114.62±2.96*	116.56±3.01*
P.guajava ext 200 mg/kg p.o.	123.35±1.73*	127.46±3.84**	129.43±4.01**
P.guajava ext 400 mg/kg p.o.	135.29±3.84***	139.39±2.49**	146.84±3.12**

The values are expressed as mean±SEM, n=6. Significance at
**P<0.05,*
***P<0.01,*
****P<0.001 when compared to control as determined by ANOVA followed by Dunnet's t test.*

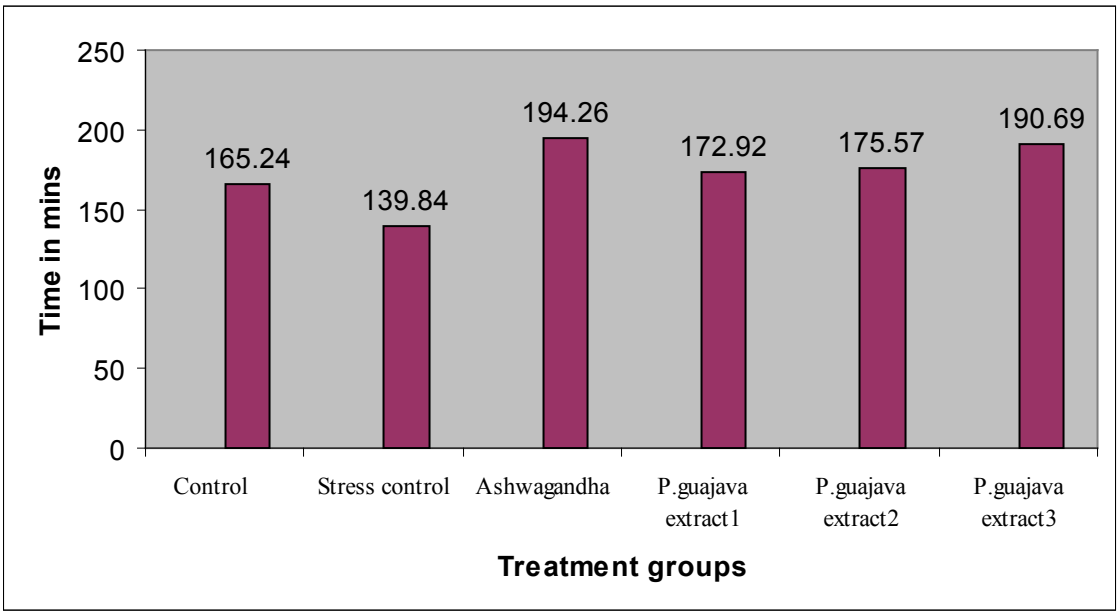

Figure 1: Effect of ethanolic extract of Psidium guajava in forced swimming endurance test in rats (Swimming time). The forced swimming endurance time was measured in rats.

Table 2. Effect of ethanolic extract of Psidium guajava on Organ weights and Blood cell counts in Forced swimming endurance stress in rats

	Organs weight			Blood cell counts					
Treatment	Spleen (mg /100g)	Liver (g/100g)	Adrenal glands (g/100g)	RBC in Millions	WBC (no of cells/mm^3)	DLC No. of cells/Cumm L	N	E	M
Control	185.01±5.21	3.71±0.23	13.76±0.26	7.59±0.45	5960±23.98	4537±24.19	1354±11.24	61.12±1.36	9.84 ±0.34
Stress Control	129.22*±4.69	5.95*±1.84	39.93*±0.65	10.62*±1.52	8974*±21.23	6761*±24.26	2106*±14.56	95.83*±3.96	10.72*±1.01
Ashwagandha 100 mg/kg p.o.	190.45**±5.01	3.99**±0.56	18.87**±0.91	7.84**±1.65	6133**±24.23	4657**±13.34	1396**±21.89	69.54**±2.32	9.89**±0.23
P.guajava ext 100 mg/kg p.o.	143.68*±3.89	5.10±1.22	29.96*±0.59	9.91*±4.15	7519*±26.25	5464*±15.45	1955*±11.74	89.63*±4.23	10.54*±0.99
P.guajava ext 200 mg/kg p.o.	181.35**±2.45	4.47*±2.19	28.84*±0.36	8.82*±2.24	6985*±14.25	5232*±17.98	1662**±12.36	81.59**±2.54	10.23*±0.67
P.guajava ext 400 mg/kg p.o	188.46**±4.81	3.42**±1.81	20.21**±0.57**	8.13**±3.72	6496**±18.64	4985**±28.24	1429**±13.23	72.12**±6.12	10.01*±1.28

The values are expressed as mean±SEM, n=6. Significance at

**P<0.01 when compared to control,*

***P<0.001 when compared to stress control as determined by ANOVA followed by Dunnet's t test.*

Table 3.Effect of ethanolic extract of Psidium guajava on Biochemical parameters in Swimming Endurance Stress in rats

Groups	Corticosterone µg/dl	Glucose mg/dl	Cholesterol mg/dl	Triglycerides mg/dl	BUN mg/ml
Control	91.60±1.56	118.62±1.58	35.42±3.63	6122±2.85	29.32±2.69
Swimming Stress Control	156.13±3.95	195.84±1.96	61.29±1.89	95.87±5.02	57.84±1.24
Ashwagandha 100mg/kg p.o.	99.62±2.57**	121.56±0.42**	32.63±3.59*	67.52±0.89*	32.96±0.78*
P.guajava ext 100mg/kg p.o.	126.39±1.85*	162.35±2.98*	53.25±4.05*	82.13±2.64*	51.36±2.58*
P.guajava ext 200 mg/kg p.o.	109.48±3.87**	135.41±3.02**	46.29±3.82**	77.62±3.98*	46.89±3.58*
P.guajava ext 400mg/kg p.o.	101.64±3.35**	127.98±1.85**	37.84±1.56**	68.93±2.63**	35.74±2.34*

The values are expressed as mean±SEM, n=6 in each group.

**P<0.01 significant as compared to control,*

***P<0.001, significant as compared to stress control, statistical test employed is ANOVA followed by dunnet's t test.*

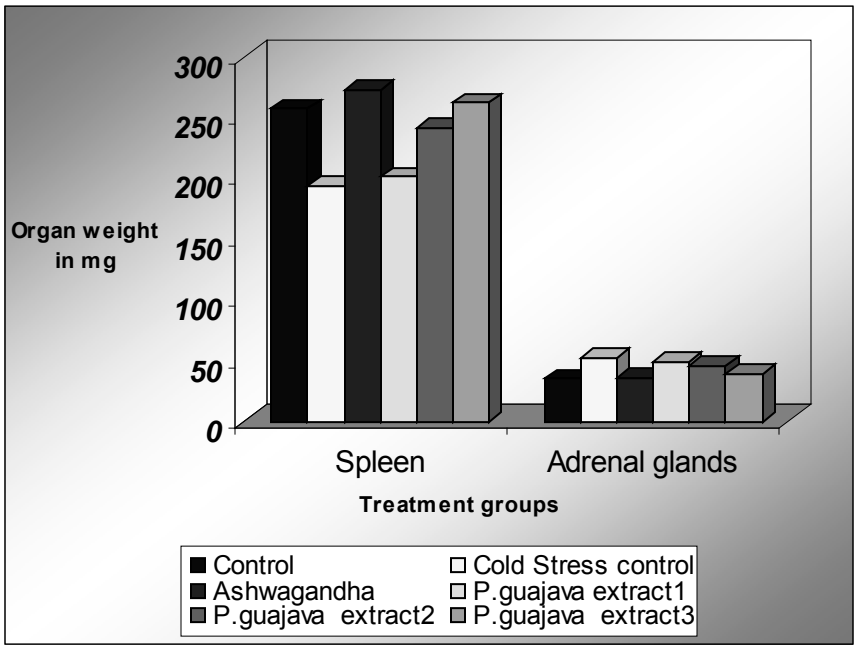

Figure 2: *Effect of ethanolic extract of Psidium guajava on Organ weights in cold restraint stress in rats. The values are expressed as mean±SEM, n=6 in each group.*

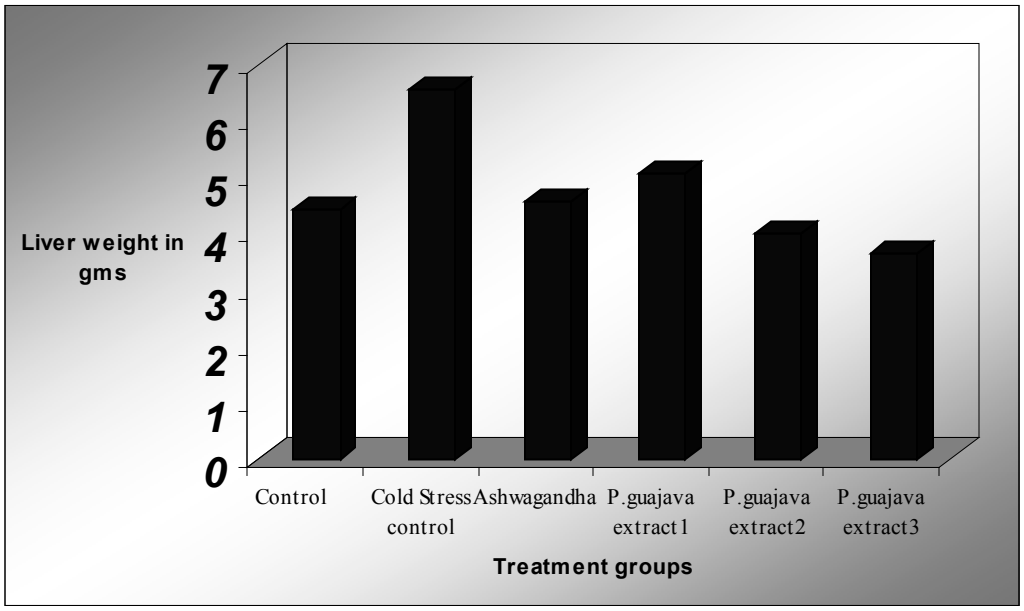

Figure 3: *Effect of ethanolic extract of Psidium guajava on Organ weights (liver) in cold restraint stress in rats. The values are expressed as mean±SEM, n=6 in each group.*

gland when compared to stress control (Figures 2, 3). The extract dose dependently reduced the elevated levels of biochemical parameters when compared to stress control (Table 4). The extract at 400mg/kg ($P<0.01$) significantly reduced all the blood cell counts (Table 5).

Effect of ethanolic extract in heat induced stress

The extract at different doses offered significant protection against the heat stress induced changes in biochemical parameters (Figure 4).

Table 4. Effect of ethanolic extract of Psidium guajava on Biochemical Parameters in Cold Restraint Stress

Groups	Corticosterone µg/dl	Glucose mg/dl	Cholesterol mg/dl	Triglycerides mg/dl	BUN mg/dl
Control	95.84±1.85	74.82±5.91	41.29±2.35	63.98±2.85	31.22±1.63
Cold stress Control	163.91±2.95*	182.05±6.84*	83.33±1.91*	146.80±3.65*	71.25±2.83*
Ashwagandha 100 mg/kg p.o.	85.82±3.04**	92.82±5.46**	42.86±1.84**	89.37±1.32**	45.01±1.59**
P.guajava ext 100 mg/kg p.o.	125.21±3.33	122.28±4.91*	61.90±1.25*	136.80±3.58*	68.38±0.85*
P.guajava ext 200 mg/kg p.o.	113.48±2.85*	109.54±3.89*	56.67±3.49*	115.53±4.05*	63.12±1.93*
P.guajava ext 400 mg/kg p.o.	91.46±1.75**	82.56±4.63**	46.90±1.23**	79.79±2.25**	48.13±0.68**

The values are expressed as mean±SEM, n=6 in each group.

**P<0.01 significant as compared to control,*

***P<0.001, significant as compared to stress control, statistical test employed is ANOVA followed by dunnet's t test.*

Table 5. Effect of ethanolic extract of Psidium guajava on Blood cell counts in Cold Restraint stress in rats

Treatment	Blood cell counts					
	RBC in millions	WBC (no of cells/mm³)	DLC No. of cells/Cumm			
			L	N	E	M
Control	9.56±3.78	6180±13.98	4981±19.89	1129±10.35	59.84±1.25	10.01±0.23
Cold stress Control	13.84a±2.56	10085a±21.56	7972a±15.89	2019a±21.21	82.96a±2.36	11.56a±0.58
Ashwagandha 100mg/kg p.o.	8.62b±1.19	7562ab±25.96	6106b±14.56	1384b±13.11	62.22ab±3.35	10.12b±0.48
P.guajava ext 100 mg/kg p.o.	11.56±2.45	9854b±23.12	7801±14.23	1962b±12.24	79.18±5.23	11.42±1.02
P.guajava ext 200 mg/kg p.o.	10.67a±3.09±11.87	8261ab±9.84	6329ab±11.58	1853a	67.91b±5.36	10.98b±0.57
P.guajava ext 400 mg/kg p.o.	9.51b±1.79*	7691ab±17.24	5933ab±28.25	1691ab±21.23	55.23ab±4.65	10.76b±0.47

The results are expressed as mean±SEM, n=6 in each group. Significantly different at P<0.01 as determined by ANOVA followed by unpaired t test.

[a]*significantly different from control*

[b]*significantly different from stress control*

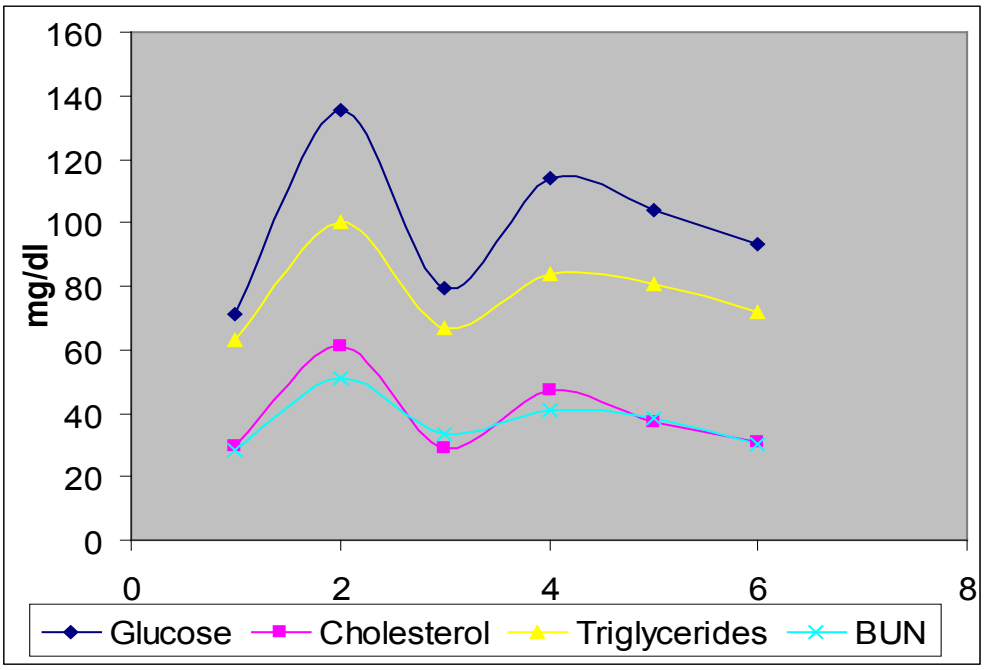

Figure 4: Effect of ethanolic extract of Psidium guajava on Biochemical Parameters in Heat Induced Stress. The values are expressed as mean±SEM, n=6 in each group.

DISCUSSION

Adaptogens are the substances meant to put the organisms into a state of non-specific heightened resistance in order to better resist stressor and adapt to extraordinary challengers. They normalize body functions, strengthen systems and functions that are compromised by stress and have a protective effect against a wide variety of environmental and emotional stress.

The forced swimming is the most widely used method for assessing the anti-stress property of a novel compound (13, 14). This paradigm is based on the observation that animals forced to swim in water eventually assumed a characteristic immobile posture, devoid of any activity (15). The appearance of immobility therefore, reflects a state of tiredness, fatigue, reduced stamina with the end point being the moment when the rat could not swim further and started drowning. However, increased swimming time has been reported in rat pre-treated with antistress and adaptogenic agents (16). *Psidium guajava* at the doses of 200mg/kg and 400mg/kg significantly prolonged the swimming time. This ability of the ethanolic extract of *Psidium guajava* to prolong the swimming time in rat, therefore, suggests an antistress property.

The two main systems involved in stress response are the HPA axis and the sympathetic nervous system. Triggered primarily by an area in the brain stem (lowest part of brain) called the locus ceruleus, the sympathetic nervous system secretes catecholamines. The hypothalamus is a major integrating center for receiving messages from divergent centers and converting them to hormonal signals, via the control of the pituitary gland and by neural pathways (17). The activation of this HPA system 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, norepinephrine and corticosteroids (18). These hormones have profound effect on metabolic functions. Increased plasma cortisol influences the mobilization of stored fat and carbohydrate reserves (19), which in turn increase blood glucose, total protein, BUN, cholesterol and triglyceride levels. In the present study, pretreatment with *Psidium guajava* as well as the reference standard drug Ashwagandha significantly ($P<0.001$) reduced the elevated levels of glucose, cholesterol, triglycerides, BUN and corticosterone indicating their suppressant effect on hyper activity of adrenal cortex and sympathetic nervous system. Thus *Psidium guajava* maintained the homeostatic mechanism in all three models i.e. swimming endurance stress, acute heat induced stress and cold restraint stress models.

The increased requirement of adrenal cortical hormones during stress may also be one of the reasons for increased liver and adrenal gland weights in stress control group. Pretreatment with ethanolic extract of *Psidium guajava* resulted in reversion of increase in liver and adrenal gland weight caused due to the stress besides preventing spleen atrophy thus inhibiting the basic signs of stress response. This observation is evident from swimming and cold restraint stress models. During stress, heart rate, blood pressure and blood flow rate increases. To meet these extra demands RBC and WBC counts will be increased. In the present study *Psidium guajava* extract has decreased the elevated levels of RBC and WBC in both Forced swimming and cold restraint stress models. In case of anoxia stress tolerance test, it was observed that a significant adaptogenic activity was observed with extract at 400mg/kg of *Psidium guajava*.

A variety of biological activities including adaptogenic activity were reported with flavonoids, tannins and phenolic glycosides (9). *Psidium guajava* contains biologically active chemicals that include flavonoids-quercetin, saponins, alkaloids, fixed oils, triterpenes, proteins and steroids (7). The adaptogenic activity may be due to these constituents where as standard drug Ashwagandha an established adaptogenic drug too contains glycosides, steroids and flavoniods (13).

CONCLUSION

The Anti-stress and Adaptogenic activity exhibited by ethanol extract of *Psidium guajava* in the present study suggests the extract may be useful in the treatment of several disorders caused by stress by its immunostimulating, immunomodulating properties and also by enhancing the homeostatic mechanisms.

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