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ANTISTRESS ACTIVITY OF *Punica granatum* ON RESTRAIN INDUCED OXIDATIVE STRESS IN RATS

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ABSTRACT	Article Info:
<p>Stress and stress-related disorders are a significant cause of disease in modern man, contributing to perhaps 75% of all illnesses. The present study has been aimed to investigate the antistress activity of <i>Punica granatum</i> leaves on restraint stress induced oxidative stress in rats. The following results were observed on treatment with <i>Punica granatum</i> to restraint stress rats. Reduced the oxidative stress markers as MDA and GSH in serum and adrenal gland. Restored the enzymatic antioxidants as SOD, Catalase and Glutathione peroxidase in serum. Restored the vitamin C, E and Ceruloplasmin in serum. The above results demonstrated that <i>Punica granatum</i> leaves extract significantly prevented the restraint stress induced oxidative stress (OS) in rats.</p> <p>Citation: M.Malini,¹ A. Dominic Amalraj^{1*} Velavan S². (2015) Antistress activity of punica granatum on Restrain induced oxidative stress in rats. World Journal of Science and Research. 1(1): 13-18.</p>	<p>Received on 02 April 2015</p> <p>Accepted on 10 May 2015</p> <p>Keywords:</p> <p>oxidative stress, <i>Punica granatum</i>, Stress, Antioxidants</p> <p>*Corresponding author</p> <p>A. Dominic Amalraj, Department of Biochemistry, Arts and Science College Pattukkottai, Tamil Nadu. S. India</p>

INTRODUCTION

In this modern era, stress has become an integral part of human life (Ravindran *et al.*, 2005). It is vital that stress is kept under control and normal functioning is not hampered due to excessive stress. Stress is considered to be any condition which results in perturbation of the body's homeostasis. If the level of stress is extreme, the homeostatic mechanisms of the organism become deficit and the survival of the organism is threatened. Stress has been postulated to be involved in the etiopathogenesis of a variety of disease states, viz; hypertension, peptic ulcer, diabetes, immunosuppression, reproductive dysfunctions and behavioural disorders like anxiety due to involvement of the central nervous system

(CNS), endocrine system, and metabolic system (Lakshmi *et al.*, 2009).

The human body has several mechanisms to counteract oxidative stress by producing antioxidants which are either naturally produced in body, or externally supplied through foods and /or supplements. Endogenous and exogenous antioxidants act as free radical scavengers & therefore can enhance the immune defense and lower the risk of cancer & degenerative diseases. Recently it has been claimed that the imbalances in the levels of free radicals & antioxidants in saliva may play an important role in the onset of periodontal diseases, therefore measurement of oxidative stress in saliva represents major intraoral condition and this would provide a more accurate account of the oral environment (Ceriello, 2008).

In the past, the phytonutrients found in fruits and vegetables were classified as vitamins: Flavonoids were known as vitamin P, cabbage factors (glucosinolates and indoles) were called vitamin U, and ubiquinone was vitamin Q. Tocopherol somehow stayed on the list as vitamin E. Vitamin designation was dropped for the other nutrients because specific deficiency symptoms could not be established. Recent research, however, has enabled scientists to group phytonutrients into classes on the basis of similar protective functions as well as individual physical and chemical characteristics of the molecules. Many plants are known stress relievers and many such more plants need to be investigated for these actions. Therefore, the objective of the present study to investigate the antistress activity of *Punica granatum* leaves and bark through resistant stress induced oxidative stress in rats.

MATERIALS AND METHODS

Experimental animal:

Male albino rats of Wistar strain approximately weighing 180-190g were used in this study. They were healthy animals purchased from the Indian Institute of Science, Bangalore. The animals were housed in spacious polypropylene cages bedded with rice husk. The animal room was well ventilated and maintained under standard experimental conditions (Temperature $27 \pm 2^\circ$ C and 12 hour light/dark cycle) throughout the experimental period. All the animals were fed with standard pellet diet and water were provided *ad libitum*. They were acclimatized to the environment for one week prior to experimental use. The experiment was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India.

Chemicals:

Sodium hydroxide and Trichloro Acetic acid (TCAs) and Thiobarbituric acid (TBA) were purchased for Sigma chemical company, Mumbai All other chemicals and reagents used in this study was of analytical grade with high purity and were obtained from Glaxo laboratories and Sisco Research laboratories, Mumbai, India.

Plant material and preparation of extract:

The leaves of *Punica granatum* were collected from Thanjavur in January 2014. The collected leaves of *Punica granatum* were cut into small pieces and shade dried at room temperature and makes a fine powder using grinder mixture. The powder and material of *Punica granatum* was macerated with 70% ethanol at room temperature for 3 days. After 3 days, the supernatant was transferred into china dish. The supernatant was completely removed by keeping the china dish over a boiling water bath at 45° C. A semi solid extract was obtained after complete elimination of alcohol. The obtained residue was kept in the refrigerator for further use. The extract was made up to a known volume in distilled water just before oral administration.

Preliminary phytochemicals screening: Chemical tests were carried out on the alcoholic extract using standard procedures to identify the preliminary phytochemical

screening following the methodology of Sofowara (1993), Trease and Evans (1989) and Harborne (1973).

Experimental induction:

Rats were randomly divided into 3 groups of 6 animals each. Groups I and II received distilled water (1ml/100g). Group III animals received 500 mg/kg, oral administration doses of *Punica granatum*. All the treatments were given continuously for 12 days. On day 12, one hour after the last treatment, the forelimbs and hind limbs of the mice in groups II, and III were tied with adhesive tape thereby immobilizing them for 2 h. after the induction of stress for 2h, the adhesive tapes were removed and blood was collected from retroorbital plexus of the stressed and non-stressed rats (Joshi *et al.*, 2012). The rats were then sacrificed and the adrenal glands were weighed. The EDTA blood obtained from retroorbital plexus was centrifuged and the plasma obtained was used for the estimation of MDA, GSH, glucose, triglyceride and cholesterol levels. Adrenal glands were homogenized with phosphate buffer saline and the supernatant used to analyze the MDA, GSH, SOD and Catalase.

Collection of blood and preparation of serum sample:

At the end of the experimental period, the animals were killed cervical dislocation after an overnight fasting. The blood sample was collected. The blood was allowed to clot by standing at room temperature for 30 minutes and then refrigerated for another 30 minute. The resultant clear part was centrifuged at 3000 rpm for 10minutes and then the serum (supernatant) was isolated and stored at refrigerated until required for biochemical analysis.

Tissue homogenate:

Immediately after blood collecting, the animals were sacrificed by cervical dislocation and the adrenal gland was dissected out, washed with ice-cold physiological saline. The required amount was weighed and homogenized using a Teflon homogenizer. Tissue homogenate was prepared in 0.1 M Tris Hcl buffer (pH 7.4) and used for the estimation of various biochemical parameters.

BIOCHEMICAL ESTIMATIONS

Malondialdehyde was estimated by the thiobarbituric acid assay method of Beuge and Aust (1978). Reduced glutathione was estimated by method of Moron *et al* (1979). Superoxide dismutase activity was determined by the procedure of Kakkar *et al.* (1984). The activity of catalase was assayed by the method of Beers and Sizer (1952). The level of ascorbic acid was estimated by the method of Omaye *et al* (1979). α -tocopherol was estimated by the method of Baker *et al* (1980). Ceruloplasmin is an oxidase and so has been termed copper oxidase. It can catalyse the oxidation of some polyamines and its action of para-phenylenediamine was used by Ravin (1961) as a measure of the amount present in serum.

Statistical Analysis:

Values were expressed as mean \pm SD for six rats in the each group and statistical significant differences between mean values were determined by one way analysis of variance (ANOVA) followed by the Tukey's test for multiple comparisons. The results were

statistically analyzed by Graphpad Instat Software (Graphpad Software, San Diego, CA, USA) version 3 was used and $p < 0.05$ were considered to be significant.

RESULTS

The present study was carried out to evaluate the Anti-stress activity of *Punica granatum* leaves on restrain induced oxidative stress in rats. The observations made on different groups of experimental and control animals were compared as follows.

The qualitative phytochemical analysis of methanolic extract of *Punica granatum* leaves extract contains flavonoids, saponin, terpenoids, steroids, polyphenols and tannin.

Table 1 shows the Qualitative Analysis Punica granatum leaves

Phytochemicals	Observation
Tannin	+
Phlobatannins	+
Saponin	+
Flavonoids	+
Steroids	+
Terpenoids	+
Triterpenoids	+
Alkaloids	-
Carbohydrate	-
Amino acid	-
Anthroquinone	-
Polyphenol	+
Glycoside	+

(+) Present (-) Absence

Table II represents the levels of MDA in serum and adrenal gland of normal and experimental rats. Group II Stress induced rats showed a significant increased in the level of MDA when compared to Group I rats. Group III Stress induced rats treated with *Punica granatum* significantly decreased in the level of MDA when compared to group II.

Group II Stress induced rats showed a significant decreased in the level of GSH in serum and adrenal gland of when compared to Group I rats. Group III Stress induced rats treated with *Punica granatum* significantly increased in the level of GSH as compared to group II.

Table III represents the levels of SOD and Catalase in serum of normal and experimental rats. Group II Stress induced rats showed a significant decreased in the activity of SOD when compared to Group I rats. Group III Stress induced rats treated with *Punica granatum* significantly increased in the activity of SOD when compared to group II.

Group II Stress induced rats showed a significant decreased in the activity of Catalase when compared to Group I rats. Group III Stress induced rats treated with *Punica granatum* significantly increased in the activity of Catalase as compared to group II.

Table II Effect of Punica granatum on MDA and GSH in serum and adrenal gland of experimental rats

Parameters	Group I	Group II	Group III
MDA (Serum) (nmole/dl)	6.56±1.67	13.5±1.71*	8.48±1.17**
MDA(Homogeni) (nmole/gm)	1.96±0.98	5.66±1.54*	1.88±1.05**
GSH (Serum) (mg/dl)	2.72±0.47	1.47±0.37*	2.8±0.32**
GSH (Homogeni) (µg/gm)	2.02±0.51	0.67±0.43*	1.87±0.27**

Values were expressed as mean ± SD for six rats in each group.

* Significantly different from Group I (P<0.05)

** Significantly different from Group II (P<0.05)

Group II Stress induced rats showed a significant decreased in the activity of GPx when compared to Group I rats. Group III Stress induced rats treated with *Punica granatum* significantly increased in the activity of GPx as compared to group II.

Table II Effect of Punica granatum on SOD, Catalase and GPX in experimental rats

Parameters	Group I	Group II	Group III
SOD (U/ml)	4.75±0.74	2.005±0.68*	4.44±0.89**
Catalase (U/ml)	5.81±2.59	2.2±1.05*	6.81±2.14**
GPx (U/ml)	3.13±0.40	1.97±0.38*	3.04±0.30**

Values were expressed as mean ± SD for six rats in each group.

* Significantly different from Group I (P<0.05)

** Significantly different from Group II (P<0.05)

Table IV represents the levels of Vitamin-E, Vitamin-C and Ceruloplasmin in serum of normal and experimental rats. Group II Stress induced rats showed a significant decreased in the level of c Vitamin-E, Vitamin-C and Ceruloplasmin when compared to Group I rats. Group III Stress induced rats treated with *Punica granatum* significantly increased in the level of Vitamin-E, Vitamin-C and Ceruloplasmin when compared to group II.

Table IV Effect of *Punica granatum* on Vitamin-E, Vitamin-C and Ceruloplasmin in experimental rats.

Parameters	Group I	Group II	Group III
Vitamin-E (mg/dl)	12.07±1.96	10 ± 1.44*	16.2±1.24**
Vitamin-C (mg/dl)	6.16±0.12	4.90±0.76*	6.19±0.17**
Ceruloplasmin (µg/dl)	10.6±0.93	3.74±0.66*	8.68±1.07**

DISCUSSION

The stress-induced effects are an outcome of altered activity of different systems such as central neurotransmitters, neurohormonal factors particularly those linked with the pituitary-adrenal axis, and free radical generation (Zafir and Banu, 2009; Jankord and Herman, 2008). These responses are believed to be developed during the first exposure of stress and/ or novel environment. Various types of stressors have been used to study stress-induced alterations, among them; immobilization has been used extensively for the study of stress-related biological, biochemical and physiological responses in research. Among the various stress models, immobilization has been used extensively and accepted widely for studying stress-induced alterations (Pacak and Palkovits, 2001). The distinct advantage of using immobilization as a stressor lies in the fact that it produces both physical as well as an in-escapable psychological stress (Marty *et al.*, 1997). Therefore in our studies, we have used an immobilization stressor in acute stress (AS) as well as chronic stress (CS) models. Furthermore, besides AS, CS alters important neurological, behavioral, and biochemical parameters possibly in a different manner than AS (Ferreti *et al.*, 1995). Therefore, the objectives of the present study to investigate the antistress activity of *Punica granatum* leaves and bark through restrain stress induced oxidative stress in rats.

Lipid peroxidation (LPO), a consequence of oxidative stress, is associated with progressive loss in membrane potential, increase in membrane permeability, and finally cell death. Malondialdehyde (MDA) is the major aldehyde resulting from the peroxidation of biological membrane polyunsaturated fatty acid. MDA, a secondary product of lipid peroxidation is used as an indicator of tissue damage by series of chain reactions. MDA is one of the indicators of oxidative stress. Acute ethanol intake induces an increment in MDA levels in brain, an effect that is abolished by antioxidants. Glutathione is a ubiquitous thiol containing tripeptide, which plays a central role in cell biology. It is implicated in the cellular defence against xenobiotics and naturally occurring deleterious compounds, such as free radicals and hydro peroxides. Glutathione status is a highly sensitive indicator of cell functionality and viability. GSH depletion is linked to a number of diseases states including cancer, neurodegenerative diseases, kidney and cardiovascular diseases (Pastore *et al.*, 2003).

The mechanism of RS stress-induced depletion of GSH involves conjugation of GSH with acetaldehyde, the reactive intermediate of ethanol oxidation, or enhanced utilisation of GSH for the detoxification of free radicals and oxidants produced as a result of ethanol exposure. Our study is in agreement with earlier observations on ethanol induced oxidative stress in the rat adrenal gland (Joshi *et al.*, 2012). In this study, the ethanolic extract of the leaves of *Punica granatum* prevented LPO induced by ethanol in the rat brain. Further, *Punica granatum* extract treatment restored the GSH level and as such boosted the basal GSH level.

It was observed that RS significantly enhance the activities of SOD, Gpx and CAT activity was increased significance. The increased activity of SOD during RS is an indicator of a relative increase in the superoxide radical production, which could stimulate the second line of defence including CAT (Lahiri *et al.*, 2009). The increased CAT activity may also indicate the increase in cellular peroxide levels, whereas, the decreased GSH concentrations could be due to its increased rate of utilization during oxidative stress. Pre-treatment with *Punica granatum* leaves prevented the RS-induced perturbations in the antioxidant enzyme activities, GSH content and the extent of lipid peroxidation. These differential alterations in the antioxidant systems and variable effect of OS compounds in adrenal regions may arise from the differences in antioxidant buffering capacities or differential susceptibilities to oxidative stress. The prevention of altered redox state in RS group by MEL is in agreement with reports demonstrating its potent antioxidant capacity (Fridovich, 1995).

Vitamin C and Vitamin E:

Living organisms have developed complex antioxidant systems to counteract reactive oxygen species. These antioxidant systems include enzymes such as superoxide dismutase, catalase and glutathione peroxidase; macromolecules such as albumin, ceruloplasmin and ferritin and a variety of small molecules, including ascorbic acid, alpha-tocopherol, reduced glutathione, methionine, uric acid and bilirubin (Goraca and Skibska, 2005).

An antioxidant has been defined as “any substance that when present at low concentrations compared with those of an oxidizable substrate significantly delays or prevent oxidation of that substrate. When ROS /RNS are generated in view, their actions are opposed by intricate and coordinated antioxidant lines of defense systems. This includes enzymatic and non – enzymatic antioxidant that keeps in check ROS / RNS level and repair oxidative cellular damage (Halliwell and Gutteridge, 1993). Circulatory non-enzymatic antioxidant such as vitamin E and vitamin C are free radical scavengers. Their synergistic action in scavenging oxygen-derived free radicals is well documented (Wojacki *et al.*, 1995). Vitamin E reacts with lipid peroxy radicals acting as a chain terminator of lipid peroxidation while vitamin C helps to maintain the level of vitamin E at optimum concentrations. Serum levels of vitamin E and vitamin C in the present study were significantly reduced in stress induced rats as compared with control. This reduced content of vitamin C and vitamin E indicates that

utilization for scavenging of free radicals which are produced in stress induced rats Administration of *Punica granatum* significantly increased in the levels of vitamin C and vitamin E in stress induced rats.

Ceruloplasmin:

A major contributor to the antioxidant defence system of plasma is reported to be ceruloplasmin (Halliwell and Gutteridge, 1989). Ceruloplasmin (Cp) is a copper-containing alpha globulin and synthesized in liver. It performing a wide variety of biological functions: it increases stability of cellular membranes, participates in immunological reactions, iron exchanges and stimulates haemopoiesis. Ceruloplasmin is also considered to be a multicopper enzyme with ferroxidase and amine oxidase activities (Inoue, 1999). Ceruloplasmin acts as an antioxidant by several mechanisms: 1) Inhibiting iron dependent lipid peroxidation and OH[•] formation from H₂O₂ via ferroxidase activity, 2) Reacting with and scavenging H₂O₂ and superoxide anion, 3) Inhibiting copper-induced lipid peroxidation by binding copper ions (Halliwell and Gutteridge, 1989). . Serum levels of ceruloplasmin in the present study were significantly reduced in stress induced rats as compared with control rats. The decreased level of ceruloplasmin in *Punica granatum* due to impairment of antioxidant defense. Administration of *Punica granatum* significantly increased in the levels of ceruloplasmin in stress induced rats.

The present study has been aimed to investigate the antistress activity of *Punica granatum* leaves on restraint stress induced oxidative stress in rats. The following results were observed on treatment with *Punica granatum* to restraint stress rats. Reduced the oxidative stress markers as MDA and GSH in serum and adrenal gland. Restored the enzymatic antioxidants as SOD, Catalase and Glutathione peroxidase in serum. Restored the vitamin C, E and Ceruloplasmin in serum. The above results demonstrated that *Punica granatum* leaves extract significantly prevented the restraint stress induced oxidative stress (OS) in rats. In conclusion, this study identified the anti-stress potential of *Punica granatum* leaf relation to their simultaneous modulatory effects on the central monoaminergic and antioxidant systems during acute stressful condition. Further studies need to be done to understand the mechanism of such pharmacological interventions with OS constituents in the prevention of stress-induced neurological and related disorders.

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