Research Article
AMERICAN JOURNAL OF PHARMACY AND HEALTH RESEARCH
www.ajphr.com
2015, Volume 3, Issue 7
ISSN: 2321–3647(online)

Study of Abresham (*Bombyx mori* Cocoon) for Glutathione Mediated Anti-oxidant Effect in Rats Subjected to Immobilization Induced Oxidative Stress

Sauduz Zafar Ali[1]*, Kunwar Mohammad Yusuf Amin[2], Naeem Ahmad Khan[2], Shameem Jahan Rizvi[3]

1. Lecturer Department of Ilmul Advia, Ayurvedic and Unani Tibbia College, Govt. of NCT Delhi, Karol Bagh, New Delhi.05.
2. Professor. Department of Ilmul Advia, Faculty of Unani Medicine, AMU, Aligarh, UP
3. Professor. Department of Forensic Medicine, JN Medical College, AMU, Aligarh, UP.

ABSTRACT

Abresham or silk cocoon (*Bombyx mori*), a well known Unani drug has been previously shown by us to possess striking antilipidperoxidative and ROS generation blocking potential in rats subjected to oxidative stress by immobilization. This effect is likely to be produced by an increase in the concentration of GSH following silk cocoon administration, as it contains a higher concentration of those three amino acids viz. Gglycine, Glutamic acid and Cysteine, which constitute the glutathione and by modulating the activities of its metabolizing enzyme which are responsible for the reduction of oxidized glutathione and break down of GSH. Therefore, in present study silk cocoon was studied for its effects on the level of GSH, GR, GGT and GST in rat subjected to oxidative stress by immobilization. It produced a significant increase in GSH concentration and GR activity and a significant decrease in GGT activity in a dose dependant manner. It can be concluded that silk cocoon exerts antioxidant activity by increasing glutathione and by modulating its metabolizing enzyme.

Keywords: Unani Medicine, Abresham (Silk Cocoon), ROS, Antioxidant.

*Corresponding Author Email: saud_ali7@yahoo.com
Received 16 June 2015, Accepted 24 June 2015

Please cite this article as: Ali SZ *et al*., Study of Abresham (*Bombyx mori* Cocoon) for Glutathione Mediated Anti-oxidant Effect in Rats Subjected to Immobilization Induced Oxidative Stress. American Journal of Pharmacy & Health Research 2015.

INTRODUCTION

Oxidative stress is being incriminated in a growing list of serious and common diseases such as inflammatory conditions, neurodegenerative diseases and malignancies as well as in ageing [1]. Correspondingly, Antioxidant Drugs such as Vit.E, Vit.C etc are being increasingly used as prophylactic and therapeutic agents [2]. Most of the effective and safe antioxidants are of natural origin, so traditional medicines mainly employing natural drugs, are being explored for better antioxidants[3]. Silk Cocoon is used in Unani Medicine since two thousands years back in patients of neuroasthenia, amnesia, cardiac asthenia, ischaemia and several inflammatory conditions[4,5,6]. Our previous report that demonstrated ROS generation blocking effect of silk cocoon mediated through SOD and Catalase, corroborated the Unani usage in various pathological conditions as described above[7]. Though ROS generation blocking potential of antioxidant agents has greater value but their scavenging potential should also be explored as it would be a substantiating factor that completes the task of anti-oxidant agent. Among the several enzymatic and non enzymatic physiological antioxidants, glutathione and its metabolizing enzymes viz, GR, GGT, GST, have a wider role in antioxidant effect as they do not only block the ROS generation but also scavenge and quench the free radicals, terminate the progressing chain reactions and maintain glutathione concentration, the most important physiological antioxidant. The concentration of glutathione, is partly affected by glutathione reductase enzyme that catalyses the reduction of oxidized glutathione and GGT which is responsible for the breakdown of glutathione[8]. So, fluctuation in GR or GGT activity may give a reflection of concentration of physiological GSH and hence may provide an index of oxidative status of bio-molecules as lesser concentration of GSH would be unable to block or scavenge free radicals and terminate the progressing chain reaction.

The ratio of free and protein bound glutathione concentration also provides an index of protein protection from oxidative damage and its contribution in antioxidant effect of test agent. We have observed in our previous study that anti-lipid peroxidative and ROS generation blocking silk cocoon possesses glycine, glutamic acid and cysteine in higher concentration i.e. 3.3, 20.72 and 7.17 percent, respectively. Since these 3 amino acids make up glutathione, which is a crucial, non-enzymatic physiological antioxidant, therefore, the test drug could be exerting its antioxidant effect by increasing glutathione levels. Our previous study has also demonstrated the presence of methionine and cystine in silk cocoon, which could also be increasing glutathione[7]. So, in this study we have investigated the effect of silk cocoon on total and protein bound

reduced glutathione, glutahione rductase, gamma gutamyl tanspeptidase and glutathione-s-transferase enzymes in rats subjected to immobilization induced oxidative stress.

MATERIALS AND METHOD

Test Drug

Abresham or silk cocoon (Cocoon of *Bombyx mori L)*, was obtained from Dawakhana Tibbiya College, Aligarh Muslim University, Aligarh, India. The identity was initially confirmed in the light of its Unani morphological description and further authenticated by a Pharmacognosist in the Pharmacognosy section of Department of Ilmul Advia. A representative sample (No. B-45/B) has been deposited in the museum of Department of Ilmul Advia. Twenty-five gram of silk cocoon was cut in small pieces and a 50% ethanolic extract was prepared in Soxhlet's apparatus. The extract was filtered and evaporated on water bath. The dried yield of extract was found to be 15% w/w. The extract was dissolved in 0.9% saline water before dosing the animals, while ∝-tocopherol acetate was suspended in 5% gum acacia. The dose was determined by multiplying the Unani clinical dose with conversion factor of 7 and found to be 15 mg/kg for ∝-tocopherol acetate and 175 mg /kg for silk cocoon [9]. Two additional doses (250 mg/kg and 325 mg/kg) were also studied to determine the dose dependant response.

Chemicals

All the chemicals and reagents were of analytical grade and were obtained from various sources. Reduced Glutathione, Oxidized Glutathione, 1,2,dithiobis nitrobenzoic acid (DTNB), reduced nicotinamide adenine dinucleotide phosphate (NADPH), γ-glutamyl p-nitrianilide, Ethylenediamine tetracetic acid disodium salt (EDTA) (SRL, India), 1,chloro-2, 4, dinitrobenzene (CDNB), Bovine serum albumin- Fraction V (Ottokemi, India), Tris (Hydroxymethylaminomethane), Succinic acid, (S.D. Fine, India). Glycyl glycine, ∝-tocopherol acetate (Loba Chemie, India), Trichloroacetic acid (CDH, India), Follin Phenol Ciocalteu Reagent (Merck, India). All other chemical and reagent were of the highest purity commercially available

Animals and Treatment

Forty two male albino rats (Wistar strain), weighing 120-130 gm, divided into 6 groups of 7 animals each, were used. The animals were provided with standard diet (Purina) and tap water *ad libitum* and maintained at temperature ranging from 20-25° C with 12 hour light and dark cycle. They were deprived of food for 12 hours before the treatment (while had free access to water).

They were treated by oral route once a day for 7 days. The different groups were treated as follows:

Group I (Plain Control): Vehicle (0.9% saline) for 7 days (no stress)

Group II (Stress control): Vehicle for 7 days (stress was induced on 7th day)

Group III (Standard Control): ∝-tocopherol acetate (15 mg/g) for 7 days

Group IV (Test group): Silk cocoon extract (175 mg/kg)

Group V (Test group): Silk cocoon extract (250 mg/kg)

Group VI (Test group): Silk cocoon extract (325 mg/kg)

On the 7th day, immediately after giving the treatment, all the animals (except those in Group I), were subjected to immobilization stress in individual cages of their size for 6 hours [10, 11]. The animals were then removed from the cages and post-stress treatment was given as above. Forty-five minute after the post-stress treatment, the animals (including group I) were sacrificed by cervical dislocation.

Preparation of Biological Sample

The liver was removed, washed in cooled 0.9% normal saline, kept in ice, blotted on filter paper, weighed and homogenized at 2500 rpm in cold 0.15M KCl for total and protein bound reduced glutathione estimation, in cold phosphate buffer (0. 2M, pH 6.5) for GST estimation, in chilled 0.5M Tris HCl buffer (pH 9) for glutamyl transpeptidase and in chilled 0.1M Tris-HCl buffer (pH 8.2) for glutathione reductase estimation, separately, using Elvehjen homogeniser. Homogenization procedure was performed as quickly as possible under completely standardized conditions. The homogenates were centrifuged at 10,000 rpm for 20 minutes at 4 ^{0}C and the post-mitochondrial supernatant was kept on ice until assayed.

BIOCHEMICAL INVESTIGATION

Estimation of Total and Protein Bound Reduced Glutathione

Total and protein bound reduced glutathione in post-mitochondrial supernatant was determined by the method of Elman et al. modified by Sedlak and Lindsay[12]. The assay was based on reduction of DTNB by SH group of glutathione in alkaline medium to produce one mole of 2 nitro-5-mercaptobenzoic acid per mole of –SH group. Absorbance of yellow colour, 5 minute after addition of DTNB, was read at 412 nm in spectrophotometer against a blank using reduced glutathione as standard. Results were expressed as μM /gm of tissue.

Estimation of Glutathione Reductase (GR) activity

GR activity in PMS of liver was estimated by the method of Hazelton et al. [13]. Assay was based on the ability of GR to induce reduction of oxidized glutathione by NADPH to reduced

glutathione observed by decrease in absorbance at 340 nm spectrophotometrically. Specific activity of enzyme was expressed as ηM of NADPH oxidized/min/mg protein.

Estimation of γ-Glutamyl Transpeptidase (GGT) activity

The GGT activity in post-mitochondrial supernatant of liver was estimated by the method of Tate et al.[14]. Assay was based on the ability of GGT to induce transfer of γ glutamyl group from glutathione to amino acids and hydrolyse the γ-glutamyl bond. Change in absorbance was recorded at 410 nm in spectrophotometer. Specific activity of enzyme was expressed as μM of P-nitroanilide formed /min/mg protein.

Estimation of Glutathione-S-Transferase (GST) activity

The GST activity in PMS of liver was estimated by the method of Habig et al.[15]. Assay was based on the ability of GST to induce catalysis between glutathione and 1-chloro-2, 4 dinitrobenzene. The reaction was started by the addition of 0.2ml of 1mM CDNB and increase in absorbance due to GSH-CDNB conjugate formation was recorded at 340 nm in spectrophotometer. Results were expressed as μM of GSH-CDNB conjugate formed /min/mg protein.

Protein estimation

Protein estimation was made since the concentration of all the test parameters, namely, GR, GGT and GST, was expressed per mg of Protein. The estimation was carried out by the method of Lowry et al [16].

Statistical Analysis

The concentration of each parameter in various animal groups (I - VI) were statistically compared for determining the significance of difference by one-way ANOVA Test followed by pair-wise comparison of various groups by LSD. The analysis was carried out by using the software of the website, www.analyseit.com

RESULTS AND DISCUSSION

Immobilization/Restraint stress is a well known method for induction of physical and psychological stress that results in restricted mobility and aggression and produces a considerable pituitary-adreno-cortical activation[17,18,19]. It increases free radical generation, brings about changes in activities of various ROS generation blocking enzyme in plasma of rat, increases the lipid peroxidation in plasma and brain and decreases the concentration of glutathione and Vit C [20,21]. Demonstration of increased lipid peroxidation and decreased SOD and Catalase activities in our previous study also support the oxidative effect of restraint stress[7].

Table 1: Effect of 50% ethanolic extract of Silk Cocoon and α-tocopherol acetate on the concentration of Total and Protein bound reduced Glutathione and the activity of GR in PMS of rat liver subjected to restraint stress

Group	Total Reduced Glutathione*(µ mole of GSH/ gm of tissue)	Protein Bound Reduced Glutathione**(µ mole of GSH/ gm of tissue)	Glutathione Reductase***(n mole of NADPH oxidized /min/mg of protein)
Plain Control (Non Stressed)	4.2±0.13	3.56±0.12	0.71±0.01
Stressed (Control)	2.25±0.14 b3	1.44±0.15 b3	0.33±0.006 b3
Standard	4.4±0.15 a^3	3.58±0.16 a^3	0.85±0.13 a^3b3
Silk Cocoon (175mg)	3.9±0.12 a^3 (57)	3.12±0.13 a^3 (51)	0.64±0.011 a^3 (67.5)
Silk Cocoon (250mg)	5.8±0.17 a^3b3c^3 (87)	5.01±0.19 $a^3b^3c^3$ (85)	0.82±0.011 a^3b^2 (90)
Silk Cocoon (325mg)	6.6±0.19 a^3b3c^3	5.86±0.19 a^3b3c^3	0.90±0.012 a^3b3

* (µ mole of GSH/ gm of tissue)

** (µ mole of GSH/ gm of tissue)

*** (n mole of NADPH oxidized /min/mg of protein)

a = Against Stress, b = Against Plain Control, c = Against Standard

3 = P < 0.001, 2= p < 0.01, 1= p< 0.05

Table 2: Effect of 50% ethanolic extract of Silk Cocoon and α-tocopherol acetate on the activity of GGT and GST in PMS of rat liver subjected to restraint stress

Group	γ-Glutamyl Transpeptidase (µ mole of p-nitroanilide formed/min/ mg of protein)*	Glutathione S Transferase (µ mole of GSH-CDNB conjugate formed/min/mg protein)**
Plain Control (Non-Stressed)	0.513±0.016	69.71±2.8
Stressed (Control)	0.576±0.016 b3	48.93±1.8 b3
Standard	0.490±0.12 a^3	88.75±2.66 a^3b3
Silk Cocoon (175mg)	0.442±0.10 a^3b^2 (84)	50.18±1.1NS
Silk Cocoon (250mg)	0.429±0.10 a^3b^3 c^1 (87)	51.37±1.3NS
Silk Cocoon (325mg)	0.386±0.12 a^3b^3 c^3	53.42±1.1NS

*(µ mole of p-nitroanilide formed/min/ mg of protein)

**(µ mole of GSH-CDNB conjugate formed/min/mg protein)

a = Against Stress, b = Against Plain Control, c = Against Standard

3 = P < 0.001, NS- Non significant, 2= p<0.01, 1= p <0.05

In present study, restraint stress, applied on 7th day for six hours, induced marked decrease in total and protein bound GSH concentration and its metabolizing enzyme, GR and GST activity and marked increase in GGT activity in stressed group. Data was compared with the plain control group (Non-stressed group) and it was found to be statistically significant (P<0.001). Similarly, in the standard control, treated with α tocopherol acetate, the concentration of total

and protein bound reduced GSH and the activities of GR and GST were found to be increased as compared to the Stressed group (P<0.001), whereas, the activity of GGT and was found to be lesser than that in the stressed group (P<0.01).These data validate the genuineness of restraint stress model for oxidative stress and antioxidant study. The ability of antioxidant drugs to reduce or block the free radical generation and scavenge them would be an index of protection against oxidative stress. Similarly, increase in physiological antioxidant enzymes and non-enzymes by the test agents will determine their anti-oxidant effect and possible mechanisms. In present study, the effects of multiple doses of Silk Cocoon extract on restraint stress mediated depletion of liver glutathione contents and in the activities of its metabolizing enzymes, are shown in table 1 and 2. All the three test doses produced a significant increase in total and protein bound GSH concentration and GR activity and significant decrease in GGT activity as compared to the stressed group (P<0.001). However, no significant effect was observed on GST activity. The effect of test drug was found in a dose dependant manner. Higher dose (325 mg/kg bw), produced a significant increase in total and protein bound glutathione concentration and significant decrease in GGT activity as compared to the stressed (P<0.001), plain control (P<0.001) and the standard groups (P<0.001). The increase in GR activity was only significant in comparison to the stressed group (P<0.001) and plain control group (P<0.001). The magnitude of effect of test drug was found more significant in group VI followed by group V and IV (Table-1 and 2). These findings are suggestive of significant antioxidant effect in test drug, as the reduced glutathione is one of the most important non-enzymatic physiological antioxidants, which mainly scavenges and blocks the hydroxyl radical and singlet oxygen [23]. Highly reactive hydroxyl radicals that don't have any enzymatic defense have greater affinity for lipid molecules and are known to be one of the most important factors for initiation of chain reaction of lipid. So, OH blockage by an increase in the concentration of GSH indicates antioxidant activity possessed by the test drug. Glutathione also opposes the ROS by acting as a co-factor with glutathione peroxidase enzyme to catalyze the catalase-like conversion of H2O2 to water and molecular oxygen that additionally catalyzes the conversion of Lipid hydroperoxide to lipid alcohols [24]. Thus, it blocks the generation of reactive oxygen and terminates the progressing chain reaction of lipid peroxidation. Secondly, by re-reducing the oxidized enzyme, it also causes their reactivation[23]. Therefore, in the light of above mentioned protective role of glutathione, the augmentation of total reduced glutathione by the test drug shows it to be very effective antioxidant agent. Glutathione protects not only lipid from oxidative damage but also the proteins to which it is bound [25, 26]. So, the extent of protein-binding shows the degree of protein

protection. Since, the test drug is shown to increase PB-GSH more than that by the standard drug Vit. E therefore, it can be concluded that it produced a striking increase in the protection of proteins against oxidative damage. It has been seen that the test drug also increased both total as well as free glutathione. The F-GSH:PB-GSH ratio is also useful in understanding the mechanism of antioxidant action of an agent [27]. A small ratio indicates that a large fraction of the total glutathione present in a biological sample is bound with proteins which in turn imply that glutathione is playing a prominent role in the overall anti-oxidative effect of the agent [27]. Since, the F-GSH: PB-GSH ratio in plain control, stress control, standard group and silk cocoon treated group was found to be 0.197, 0.651, 0.342, 0.302, 0.291, 0.274, respectively, that is smaller in Silk Cocoon Gps., than that of Vit. E treated group, so, the study indicates that glutathione plays the greater role in the antioxidant activity of silk cocoon. Different doses of test drug are seen to increase glutathione concentration. One of the very important means of increasing the concentration of reduced glutathione is an increase in the activity of glutathione reductase that catalyzes the reduction of oxidized glutathione with NADPH as a co-factor [8]. Similarly, a decrease in the activity of γ- Glutamyl Transpeptisdase (GGT), which is responsible for the breakdown of Glutathione, could also result in increased concentration of this[14]. Therefore, a striking increase in GR activity and significant decrease in GGT activity, which are mostly greater than the activity produced by the standard drug, Vit E, and the plain control not exposed to oxidative stress, indicates that the test drug may be increasing glutathione concentration by increasing the activity of GR and decreasing the activity of GGT.

CONCLUSION

The study demonstrated that silk cocoon possessed striking antioxidant activity. It produced this effect mainly by increasing glutathione concentration and glutathione reductase activity, and decreasing GGT (γ-Glutamyl Transpeptidase) activity in the liver.

REFERENCES

1. Stadman R, Berlett B S. Reactive Oxygen-Mediated Protein oxidation in aging and Disease, in, Reactive Oxygen Species in Biological System: An Interdisciplinary Approach. Edited by Gilbert GD and Colton CA, Kluwer Academic Plenum Publishers, New York. 1999:657-671.
2. Rice-Evans, Arif S. Dietary Antioxidant and Nutrition, in, Reactive Oxygen Species in Biological System: An Interdisciplinary Approach. Edited by Gilbert GD and Colton CA, Kluwer Academic / Plenum Publishers, New York. 1999: 384.

3. Farnsworth NR, Soejarto DD. Potential consequence of plant extinction in the United States on the current and future availability of prescription drugs. Econ. Bot, 1985; 39:231-246.
4. Attar HZ. Ikhteyaaraate Badaee, Matbaa Munshi Nawal Kishore, Lucknow,1888(Pub):17-18.
5. Antaki SDA., Tazkira-ulil-Albab le Jamae al Ajab al Ajaib. 2nd edition.Vol.1. Matbaa Al-Amirah–Sharqeeyah, Egypt, 1924(Pub.): 29.
6. Ghani MN, Khazainah-al- Advia.Vol.2. Matbaa Munshi Nawal Kishore,Lucknow,1920: 652.
7. Ali SZ, Khan NA, Rizvi SJ, Zaidi SMKR, Banu B, Amin KMY. Antioxidant activity of a standardized sample of the Unani drug, Abresham (Bombyx mori Coccon) with Vit E as standard. Hamdard Medicus. 2008; 51(1): 44-51.
8. Halliwell MC. Gutteridge. Free Radical in Biology and Medicine. Oxford Press, London, 1985: 62
9. Dhawan BN. Organization of biological screening of medicinal plants with special reference to CDRI programme. Appendix-1, lectures UNESCO-CDRI Workshop on the Use of Pharmaco Techniques for evaluation of Natural Product. Lucknow: CDRI. 1982: 61.
10. Hasan M, Ali SF. Organophosphate pesticide dichrovos induced increase in the rate of Lipid Peroxidation in the different region of rat brain, supporting ultra structural findings. Neurotoxicity. 1980; 2: 43-52.
11. Zaidi SMKR, Al-Qirim TM, Hoda N, Bano N. Modulation of restraint stress induced oxidative changes in rats by antioxidant vitamins. The Journal of Nutritional Biochemistry. 2003; 4: 633-636.
12. Sedlak J, Lindsay R.H. Estimation of total, bound and non-bound protein sulfhydryl group and tissue with Ellman's reagent. Analytical biochem. 1968; 25: 192-205.
13. Hazelton GA, Land CA. Glutathione Peroxidase and Glutarthione Reductase in aging mouse. Mech Age Develop. 1985; 29: 71-82.
14. Tate SS, Meister A. γ-Glutamyl Transpeptidase, in, Methods in Enzymology. Vol.113. Edited by Meister A. Academic Press Inc, New York, 1985: 400-419.
15. Habig WH, Pabst MJ, Jakoby WB. Glutathione –S-Transferase in the Mercapturic acid formation. J Boil Chem. 1974; 47: 469-74.
16. Lowry OH, Roserbrough H, Farro AI, Randal RJA. Protein measurement with follin phenol reagent. J. Biol. Chem. 1951; 193: 265-268.
17. Kvetnansky R, Mikulaj L. Adrenal and urinary catecholamines in rats during adaptation to repeated immobilization stress. Endocrinol. 1970; 87: 738-43.

18. Singh LK, Ray X, Alexacos N, Netaumen R., Theoharides. Acute immobilization stress triggers skin mast cell degranulation via corticotropin releasing harmone neurotension and substance link to neurogenic skin disorders. Brain Behav. Immunol. 1993; 3: 225-239.
19. Culman J, Kvetnansky A, Kiss E, Mczey K, Murgas K.. Interaction of serotonin and catecholamines in individual brain nuclei in adrenocortical activity regeneration during stress, in, Catecholamine and Stress. Edited by E. Usdin, R. Kventnansky, I. Kopin, (Elsevier/ North Holland. Biochemical Press.) Netherlands. 1980: 113.
20. Liu J, Wang X, Mori V. Immobilization stress induced antioxidant defense changes in rat plasma: effect of treatement with reduced glutathione. Int J Biochemistry. 1994; 26: 511-517
21. Hoidal JR, Van Asbek BS, Mann J, Jacob HS, Kenedy TP, Therapy with red blood cell decreases hyperoxic pulmonary injury. Exp. Lung Res. 1988; 14 (Suppl): 977-85.
22. Manolli L, Gamaro GD, Silveira PP, Dalmaz C. Effect of chronic variate stress on thiobarbituric acid reactive species and on total radical trapping potential in distinct regims of brain. Neurochemical Research. 2000; 25: 915-921.
23. Meister A. Methods for selective modification of glutathione metabolism and study of glutathione transport, in, Methods in Enzymology; Vol. 113, Edited by A. Meister, Academic Press. New York. , 1985: 571-585.
24. Larson RA. Naturally Occurring Antioxidants. Lewis Publishers, New York, 1997: 6-9.
25. Klatt P, Lamas S. Regulation of protein function by S-glutathiolation in response to oxidative stress. Eur J Biochem. 2000; 267: 4928-44.
26. Thomas JA, Mallis RJ. Ageing and oxidation of reactive protein sulfhydryls. Exp Geront. 2000; 36: 1519-26.
27. Iciek M, Chwatko G, Lorenc-Koci E, Bald E, Wlodek L. Plasma levels of total, free and protein bound thiols as well as sulfur in different age groups of rats. Acta Biochimica Polonica. 2004; 3: 815-824.