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Management of stress exerted by hydrogen peroxide in Drosophila melanogaster using Abhrak bhasma

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ABSTRACT

One of the undesirable outcomes of aerobic respiration is development of reactive oxygen species (ROS) and other free radicals. The cellular antioxidant system continuously reduces these into less toxic molecules. A common end product of quenching of ROS is hydrogen peroxide (H_2O_2), though this too is an oxidant and is further converted into water and molecular oxygen by antioxidant enzymes. We monitored selected indicators of oxidative stress after exposing *Drosophila* larvae to sub lethal concentration of H_2O_2. The efficacy of dietary supplementation of abhrak bhasma and ascorbic acid in relieving the oxidative stress, induced by H_2O_2, was also evaluated in this study. The exposure of *Drosophila* larvae to H_2O_2 was found to modulate the activity of SOD and catalase enzymes. The larvae exposed to H_2O_2 but maintained on a medium free of antioxidant supplement (control) exhibited decrease in total GSH content, GSH:GSSG ratio and capacity to scavenge DPPH free radicals. Overall, supplementing the diet with abhrak bhasma seems to enhance the antioxidant machinery of the *Drosophila* larvae. The comparable results were also obtained for the larvae maintained in a medium supplemented with a known antioxidant, ascorbic acid.

INTRODUCTION

A good balance between generation of oxidant moieties and their reduction to harmless products is one of the key processes for defining health of an organism. Tripping of this balance, towards oxidants, results in oxidative stress that culminates into a variety of ailments and finally death. Accumulation of damages caused by oxidants and its age related consequence is among the widely accepted theory of aging (Harman, 1980). Majority of living systems counter free radicals produced via catalytic and/or non-catalytic pathways (Nimse and Pal, 2015). The non-catalytic mechanism is not specific to the type of free radicals while the catalytic pathway targets specific free radicals and is mediated by specific enzymes.

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Hydrogen peroxide is an important intermediate metabolite being synthesized during the catalysis of reactive oxygen species, generated mainly by one or two electron reduction reactions (Gerd *et al.*, 2006). It may be produced in a cell to serve as second messenger or signalling molecule (Hachiya and Akashi, 2005). In granulocytic leucocytes, H_2O_2 is produced for disposing the engulfed pathogens (Segal, 2005). Different types of SOD enzymes (Cu-Zn or Mn based) localized in certain compartments in eukaryotic cell converts superoxide radicals into H_2O_2. A cell can tolerate extracellular H_2O_2 in the concentration range of 0.001-0.1mM with an appropriate physiological response (Jang and Imlay, 2007). However, toxicity of H_2O_2 largely varies according to the cell type and duration of exposure. The increase in extracellular concentration of H_2O_2, beyond these levels, exerts an oxidative stress that may cause an irreversible damage in the cell. Catalase enzyme breaks down H_2O_2 into water and molecular oxygen while peroxidases reduce H_2O_2 to water molecule using a reducing secondary substrate.

The induction of oxidative stress has been linked to various life threatening diseases. Therefore, the development of methods and strategies to reduce oxidative stress has been one of the active areas of research in recent decade (Ichiishi *et al.*, 2016). One of the simplest ways to strengthen the antioxidant capacity of cells and organisms is to supplement the diet with antioxidant compounds. These antioxidant food supplements may have a natural tendency to counter the free radicals either by directly participating into the reduction reaction or indirectly by inducing other parameters that could handle these free radicals. In this regard, various natural compounds like vitamins, flavonoids and polyphenols obtained from fruits and other plant parts are gaining popularity (Menni *et al.*, 2015; Lobo *et al.*, 2010).

Various traditional formulations have been claimed to relieve symptoms of a particular disease by managing oxidative stress (Modak *et al.*, 2007). The scientific validation of these products using modern tools is however necessary. Bhasma is one such traditional preparation that is widely being used for curing various ailments since ages. Abhrak bhasma (AB) is a type of bhasma prepared through repeated incineration of mica with extracts of about 72 medicinal plant species (Subedi *et al.*, 2017). The number of puta (cycles of incineration) defines the quality as well as efficacy of bhasma. Such preparations are generally named as Dashputi (10 putas), Shataputi (100 putas) or Sahastraputi (1000 putas).

The current study explores the effect of H_2O_2 on various physiological as well as molecular parameters of oxidative stress in *Drosophila* larvae. The study also investigates the efficacy of AB and ascorbic acid (ASC), a known natural antioxidant, in relieving the oxidative stress in *Drosophila* larvae exposed to H_2O_2.

MATERIALS AND METHODS

Abhrak Bhasma

Sahastraputi Abhrak bhasma was procured from Dhootapapeshwar Ltd, among the leading manufacturers of ayurvedic medicines (Batch no: P150300110).

Drosophila Husbandry and Test

Drosophila melanogaster (Canton S strain) flies were maintained on Corn-meal agar medium at a temperature of 26^{o}C, with 12hr light-12hr dark cycles. The control flies were allowed to lay eggs on corn-meal agar medium containing 0% AB/ 0.1% AB/ 0.5% AB or 20mM ASC for 6-12 hours. The larvae subsequently hatching from these eggs would feed on the respective media. After 5 days of feeding, the 3rd instar larvae were recovered for analysis. The 3rd instar larvae from each feeding regime were then segregated into control and test sets.

The larvae from control set were kept in petri-plates containing distilled water while larvae from test set were kept in 1% H_2O_2 prepared in distilled water for 1 hour. After 1 hour, the larvae were removed and washed thoroughly with distilled water. The larvae from each set were subsequently segregated into 4 vials containing Trizolin, Protein extraction buffer, 0.154 M KCl and GSH extraction buffer respectively for RNA extraction, enzyme (SOD and catalase) assay, DPPH scavenging capacity/MDA content assay and glutathione content estimation. All the values were estimated in reference to the amount of proteins used per reaction. The protein content of each extract was measured using Bradford's assay (He, 2011). The final results were represented as percent change in comparison to the control larvae not subjected to H_2O_2 stress. Base lines of activity or concentration in each graph (except for PCR results) indicate control larvae not subjected to stress.

Catalase Assay

Catalase assay was performed by monitoring molecular breakdown of H_2O_2 by catalase at 240nm under pH 7 as per the method described by Aebi (1983) and Bai *et al.* (1999). Larvae (20 each) were homogenized in protein extraction buffer containing 20mM tris acetate buffer (pH7.8), 0.1% triton X-100 and 1mM PMSF, using micro-pestle homogenizer (Sigma catalogue no: **Z359955-1EA**).

The tissue extract was then added to 0.1M phosphate buffer containing 17mM H_2O_2 and its degradation was immediately followed at 240nm on Biotek spectrophotometer (Model no: EPOCH –Gen5). One unit of catalase activity was defined as enzyme required for decomposing 1.0 µmole of H2O2 per minute at pH 7.0 at 25°C and expressed as enzyme units.

SOD Assay

SOD assay was performed by pyrogallol auto-oxidation inhibition method as described by Marklund and Marklund (1974) with slight modifications. The method involves blocking of auto oxidation of pyrogallol by SOD. The inhibition of oxidation of pyrogallol was followed at 420nm on a Biotek spectrophotometer (Model no: EPOCH –Gen5). One unit of SOD activity was defined as amount of enzyme that reduces the pyrogallol auto-oxidation by 50% at pH 8.5 at 25°C and expressed as enzyme units.

Glutathione Content (Total Gsh and Gsh:Gssg Ratio)

The amount of GSH and its oxidized form GSSG were measured using enzymatic recycling method described by Rahman *et al.* (2006). The larvae (20 each) were homogenized in Glutathione extraction buffer containing 0.1M Phosphate buffer with 5mM EDTA (pH 7.5), 6mg/ml sulphosalicyclic acid and 0.1% triton X-100. Oxidation of GSH was carried out with DTNB [5, 5'-dithio-bis-(2-nitrobenzoic acid)] to form a yellow derivative, TNB (5-thio-2-nitro-benzoic acid), measurable at 412 nm. The GSSG, thus formed, is recycled using Glutathione reductase enzyme in presence of NADPH. The rate of formation of TNB in comparison to standards was correlated to find the concentration of the GSH and GSSG in samples. The free GSH was derivatized with 2-vinylpyridine for accurate measurement of GSSG. The excess of 2-vinylpyridine was later neutralized with triethanolamine.

Dpph Scavenging Assay

Free radical scavenging capacity of the tissue was measured using DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay (Brand-williams *et al.*, 1995). Larvae (25 each) were homogenized in 0.154M KCl using micropestle homogenizer and the whole body extract was used for the assay. The varying volumes (20µl, 40µl and 100µl) of tissue extract were used for DPPH scavenging; measured at 517nm on BioTek Spectrophotometer. The amount of tissue required (in terms of mg of protein) for scavenging DPPH by 50% was then calculated from the results obtained.

Lipid Peroxidation

Lipid peroxidation was measured by TBARS [Thiobarbeturic acid (TBA) reactive substances] assay method (Zeb and Ullah, 2016; Devasagayam *et al.*, 2003). The whole body extracts of larvae (25 each), homogenized in KCl was made to react with TBA reagent (containing 0.037g TBA, 15% TCA and 0.24N HCl per 10ml). Reaction mixture of samples as well as different concentration of standard MDA was boiled for 15minutes and the optical density of colored product was measured at 532nm on BioTek spectrophotometer. Concentration of MDA in the samples was estimated using the standard MDA plot.

RT PCR and Q-RT PCR

The total cellular RNA was extracted using TRIZOL method as per manufacturer's instruction (Merck) subsequently the cDNA was prepared using SD-prodigy cDNA synthesis kit (SD Fine-chem Ltd.). Primers for actin, cncC, hsp 70 and catalase (Table 1) were designed using NCBI primer design tool.

Table 1: Genes and primers.

Gene	Product size	Forward	Reverse
Actin	469bp	CGGCTCGGACA GTGATAGAC	CCGGTACCAAG TATCCTCGC
cncC	147bp	AGCGCTAGGCT AAAGCAACA	GACAGTTAACG GGACGCTCT
hsp70	188bp	TTGACAACCGG CTAGTCACT	GGTGTAGAAGT CTTGGCCCT
Catalase	366bp	GAACTGCCCGT ACAAGGTGA	GTCAGCATGCG ACCGAAATC

Amplification of these genes was carried out with the help of PCR using the reaction setting as 94^{o}C for 2 minute, repeated cycle of (25 cycles): each of 94^{o}C for 30 second, 62^{o}C for 30 second and 72^{o}C for 30 second with final extension at 72^{o}C for 5 minute. The products thus obtained were then run on 2% agarose gel. The gel was observed under UV trans-illuminator and the images were captured in Uvitech system. The semi-quantitative estimation of changes in gene expression was carried out in the captured gel images using 'ImageJ' software. Following this qRT PCR was carried out using syber based EVAGREEN (Biorad) on CFX96 Real Time System (Biorad). The cT values were analyzed for fold change in expression of genes using actin as an internal control. Data from both the experiments were compiled and any mismatch data were repeated for qRT PCR to obtain accurate results. (Triplicates of real-time PCR were not performed due to paucity of funds.)

Statistical Analysis

Statistical significance between two mean values was analysed using student's t test. For comparing more than two means, ANOVA (Analysis Of Variance) test was performed. ANOVA was followed by Tukey's HSD post hoc analysis. Wherever ANOVA showed significant difference but Tukey's HSD did not reflect any difference between the groups, Bonferroni and Holm multiple comparison method was applied.

RESULTS AND DISCUSSION

Effect of exposure to H_2O_2 on antioxidant enzymes

The *Drosophila* larvae from control as well as antioxidant supplement regime (AB and ASC) at the 3rd instar stage were submerged in 1% H_2O_2 solution. The activity levels of SOD and catalase enzyme were then measured in control as well as test set of each feeding regime. The larvae from different feeding regime exhibited variations in the SOD activity in response to exposure to H_2O_2. In comparison with the control larvae not treated with H_2O_2, those treated with H_2O_2 had only a little change in the activity of SOD. While, the larvae from AB feeding regime showed about 20% decrease in the SOD activity (Figure 1A, Table 2). The larvae fed with ASC exhibited about 50% increase in the activity level of SOD (ANOVA p-value: $3.94*10^{-6}$). The percent increase in the SOD activity of ASC fed larvae was found to be statistically significant in comparison to control larvae as well as larvae fed with 0.1% and 0.5% AB (Tukey's HSD inference: <0.01each between ASC fed larvae and other groups). Exposure to 1% H_2O_2 induced an increase in the activity of catalase in larvae from control and AB feeding regimes (Figure 1B, Table 2). The ASC fed larvae showed no apparent change in catalase activity due to H_2O_2 stress. The percent change in the catalase activity in larvae from AB as well as ASC feeding regime was significantly different in comparison to that in control larvae (ANOVA p-value: 0.000983). The larvae fed on ASC and 0.1% AB showed relatively less alteration in the catalase activity compared to the control larvae (Holm inference: p<0.01 for each). The larvae fed on medium containing 0.5%AB also showed an increase in the catalase activity and this was found to be statistically insgnifiacnt in comparison with control larvae (Holm p-value:0.089).

Table 2: Percent change with reference to control, not treated with H_2O_2, in various oxidative parameter in Drososophila after exposure to H_2O_2.

Parameters Studied	% Change			
	Control	ASC	0.1%AB	0.5%AB
SOD activity	-8.8±9.4	50.7±8.3	-21.8±5.7	-23.0±11.2
Catalase activity	18.5±4.8	-2.7±3.5	3.4±2.6	9.7±3.0
DPPH scavenging activity	3.3±4.9	8.6±4.3	-10.9±3.8	22.1±3.5
MDA content	4.2±18.4	43.2±26.1	-10.4±16.0	-21.1±8.8
Total GSH	-24.3±9.1	-1.9±12.9	0.7±5.8	-6.6±13.2
GSH:GSSG ratio	-37.1±4.6	53.0±16.9	68.3±33.9	20.5±24.9

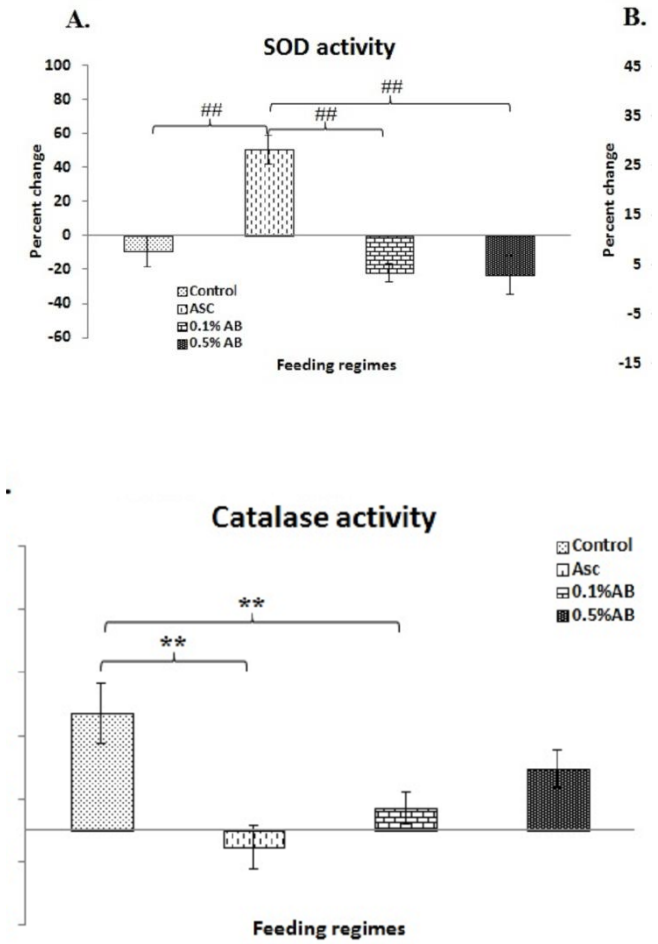

Fig. 1: Change in enzyme activity after exposure to H_2O_2 as percent of the activity in control larvae not treated with H_2O_2 A. Superoxide dismutase (SOD) B. Catalase enzyme

Changes in free radical scavenging capacity

DPPH is a synthetic free radical and the extent of it's scavenging in vitro by tissues gives an indirect estimation of free radical scavenging capacity of an organism *in vitro*. This capacity was measured in terms of mg of protein required (equivalent to amount of tissue required) for scavenging 50% of the DPPH free radicals. The higher the amount of tissue required for this, lower will be the general free radical scavenging capacity of the organism. It was noticed that the free radical scavenging capacity decreased due to exposure to H_2O_2, in all groups, except in the larvae from 0.1% AB feeding regime that exhibited an increase in the free radical scavenging capacity (ANOVA p-value: $4.83*10^{-5}$). The feeding of AB at 0.1% and 0.5% concentrations resulted in contrasting alterations in the free radical scavenging capacity (Figure 2A, Table 2). The feeding of 0.1% AB increased the free radical scavenging capacity under H_2O_2 stress while a decrease in free radical scavenging capacity was observed in the larvae from 0.5% AB feeding regime in comparison to the response in control larvae (Holm inference: $p<0.05$ and $p<0.01$ respectively). Whereas, the percent change in DPPH free radical scavenging capacity of larvae from ASC feeding regime under stress condition was statistically insignificant in comparison to that of control larvae (Holm's p-value:0.374). The percent increase in the free radical scavenging capacity in 0.1% AB fed larvae was also significantly different in comparison to the response registered in ASC and 0.5% AB fed larvae (Tukey hsd inference: $p<0.05$ and $p<0.01$ respectively).

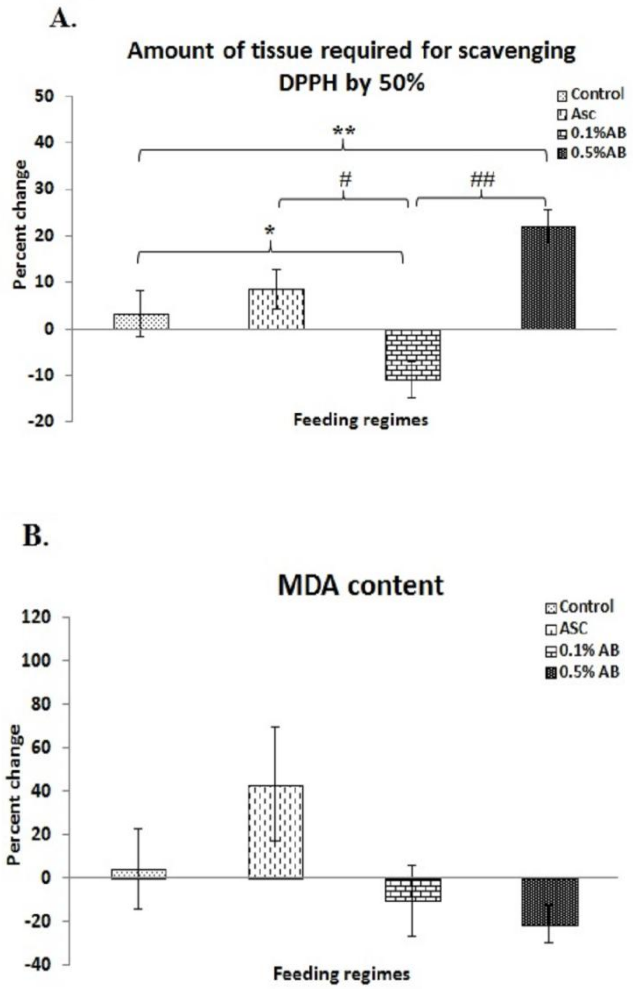

Fig. 2: Percent change as compared to the control, not subjected to H2O2 treatment, in A. DPPH free radical scavenging capacity B. extent of lipid peroxidation (measured as MDA content in tissue)

Another crucial parameter of general antioxidant capacity of the cells and organism is the total GSH content and GSH:GSSG ratio. The percent change, in GSH contents and GSH:GSSG ratio, in larvae from various feeding regimes, due to H_2O_2 stress was statistically insignificant when compared with the larvae of any other feeding regime (ANOVA p-value: 0.3928). On the contrary, a distinct change in response to the induced stress, in the form of GSH:GSSG ratio, was observed between the control larvae and larvae from AB as well as ASC feeding regimes (ANOVA p-value: 0.015). The percent change in GSG:GSSG ratio of control larvae was statistically different from 0.1%AB as well as ASC fed larvae (Holm inference: $p<0.05$ for each). No such differences were observed between other feeding groups (Figure 3, Table 2).

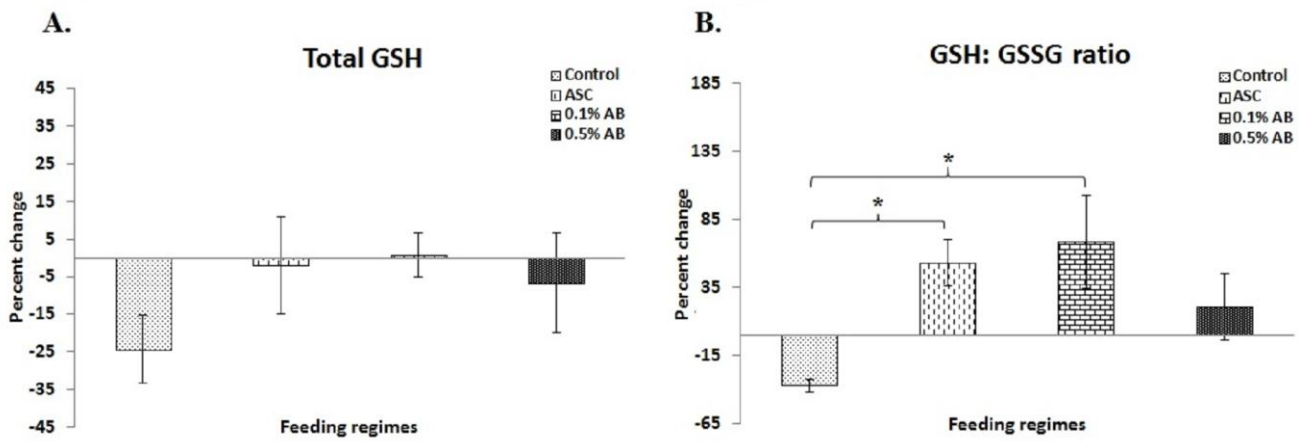

Fig. 3: Effect of exposure to H2O2 on A. Total GSH content B. GSH:GSSG ratio (as compared to the control larvae not treated with H2O2)

No effect on the extent of lipid peroxidation due to stress

Any stress condition ultimately brings about the peroxidation of lipid molecules, which can be measured in terms of MDA contents in the tissues. The increased lipid peroxidation levels can terminally induce apoptotic response. Exposure of larvae from different feeding regimes to 1% H_2O_2 for 1 hour did not cause significant changes in the MDA contents (Figure 2B, Table 2). The percent change in the MDA contents in the larvae fed with AB as well as ASC, and subjected to the stress, was also statistially insignificant in comparison to that in control larvae (**ANOVA p-value: 0.116**).

Transcriptional changes in response to H_2O_2 stress

Apart from the physiological responses, organisms under stressful condition also exhibit changes in the expression of genes such as cap-n-collar isoform C (cncC) and heat shock protein 70 (hsp 70). Being an important transcription factor, cncC, regulates synthesis of other proteins that may have a role in anti-oxidative processes. The larvae from Control, ASC and 0.1% AB feeding regimes exposed to H_2O_2 showed about 1.5 fold increase in cncC expression in comparison with their respective controls (Figure 4). The larvae from 0.5% AB feeding regime exhibited 3.5 fold increase in cncC transcription under H_2O_2 stress. This increase was also comparatively higher than the response of larvae from other feeding regimes. Similarly, in response to H_2O_2 stress, hsp70 gene expression in the larvae from 0.5% AB feeding regime was higher (1.5 fold) than that in the larvae from control as well as 0.1% AB and ASC feeding regimes. The dietary supplement of AB and ASC also increased (1.5 fold) the catalase gene expression under H_2O_2 stress in comparison to respective controls as well as to the larvae fed with control media. Hydrogen peroxide is one of the key metabolite and regulator under the condition of oxidative stress. It is mainly produced by the superoxide dismutase enzyme in most of the organisms. Management of H_2O_2 is crucial due to its capacity of modulating various other parameters in stressful situations (Hachiya and Akashi, 2005; Jang and Imlay, 2007). Therefore, it becomes inevitable to comprehend the effect of exposure to H_2O_2 on the organisms. In the current study we used *Drosophila melanogaster* as a model system to understand the effect of exposure to H_2O_2 on various parameters indicating oxidative stress. We evaluated the efficacy of dietary supplement of AB and a standard antioxidant, ASC, in ameliorating the effects of stress induced by H_2O_2.

In response to the H_2O_2 stress, the larvae fed on control media show a decrease in the SOD activity whereas the catalase activity increases notably. This is an expected observation since with the increase in H_2O_2 load, the first response of the organism would be increase in the catalase activity resulting in breaking down of H_2O_2 into water and oxygen molecule (Izawa *et al.*, 1996). However, this increase in the catalase activity is without any increase in the expression of catalase gene. As there is already an extra burden of H_2O_2 experienced by the cells, the SOD enzyme, which is responsible for H_2O_2 formation, shows decrease in its activity. Feeding of AB to *Drosophila* larvae also result in similar response but with much lesser intensity. This is likely to be due to the relatively larger proportion of iron based constituents in AB. Iron in various forms is known to catalyse breakdown of H_2O_2 *in vitro* (Haber and Weiss, 1934). At the transcriptional level also, in response to exposure to H_2O_2, larvae fed on AB show an increase in the expression of catalase gene, which might boost the capacity of the organism to degrade H_2O_2. Feeding the larvae on ASC, however, has shown contrasting results in the present investigation. In response to the exposure to H_2O_2, the larvae fed on ASC have shown an increase in SOD activity. In these larvae there is, however, no change in the catalase activity though an increase in the transcription of catalase gene has been observed. Since ASC itself is a reducing molecule (Deutsch, 1998), it can reduce H_2O_2 to water molecules; catalase activity is thus compensated for at least partially. The total GSH content and GSH:GSSG ratio are important indicators particularly related to antioxidant capacity of an organism. The decrease in the total GSH content as well as GSH:GSSG ratio has been linked to greater capacity to mitigate stress being experienced by the organism (Noctor and Foyer, 1998; Townsend *et al.*, 2003).

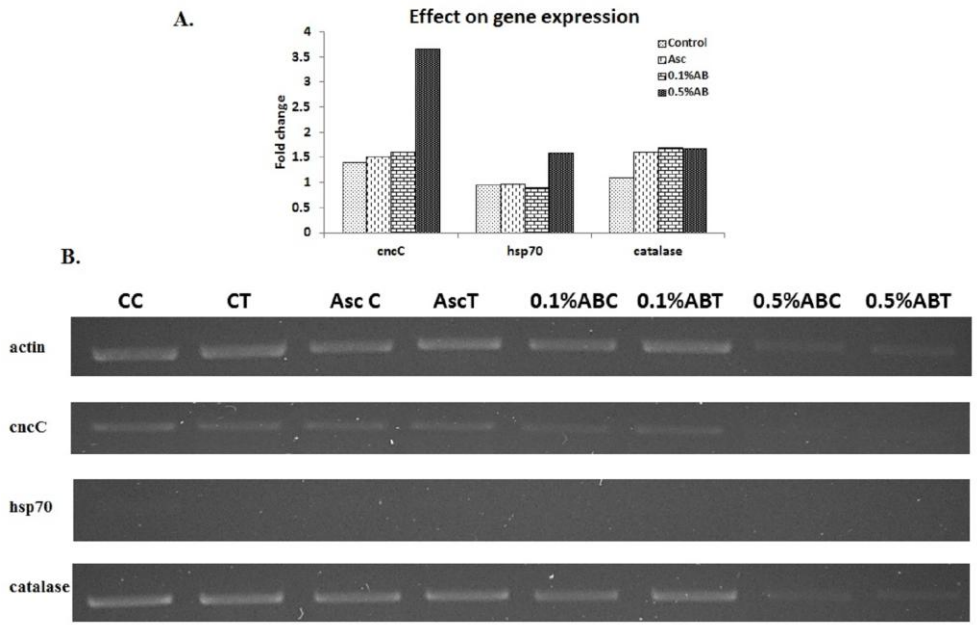

Fig. 4: Alterations in the Gene expression due to H_2O_2 exposure A.
Real time PCR results B. RT PCR products run on 2% agarose gel (Results were later analysed using Image J software).

The exposure of larvae to H_2O_2 leads to decrease in the total GSH content as well as GSH:GSSG ratio in control group. Whereas, in response to H_2O_2 stress, the larvae fed with diet supplemented with AB and ASC do not show any drastic change in the total GSH content as well as in the GSH:GSSG ratio. It indicates the efficacy of AB as well as ASC in ameliorating the effects of excessive amount of H_2O_2 in the cells. The feeding of larvae with 0.1% AB produces relatively more robust response to the induced stress in comparison with the larvae from 0.5% AB as well as ASC feeding regimes. It is supported by the observation that after exposure to H_2O_2 there is an increase in the general free radical scavenging capacity of larvae fed with 0.1% AB. On the contrary, larvae fed with 0.5% AB show drastic decrease in the general free radical scavenging capacity. This can possibly be attributed to increase in hydroxyl and other free radicals in reactions similar to Fenton reaction. This can occur due to presence of relatively greater content of Fe related components in 0.5% AB that can react with H_2O_2 to produce these radicals (Winterbourn, 1995).

The exposure of larvae to H_2O_2 does not seem to produce significant changes in the extent of lipid peroxidation. This indicates that the larvae from all feeding regimes are capable of tolerating exposure to 1% H_2O_2 without altering the extent of lipid peroxidation and thus less likely to undergo cell destruction. Diet supplementation with AB and ASC boosts the other crucial anti-oxidative parameters, as discussed above, and thus further decreases the possibility of increase in lipid peroxidation levels.

However at transcriptional level, larvae from 0.5% AB feeding regime have exhibited much higher expression of cncC as well as hsp70 genes in comparison to the larvae in other feeding regimes. The increase in the cncC gene expression has been linked to induced oxidative stress (Sykiotis and Bohmann, 2010). The cncC plays crucial protective role by regulating the proteins that may be involved in various antioxidant machinery (Sykiotis and Bohmann, 2010). Therefore, the evident increase in expression of these genes has been observed in larvae from all feeding regime under H_2O_2 stress.

The hsp70 genes are known to have protective role in stressful situation and this is mainly attributed to their molecular chaperone nature. But this higher fold increase might also possibly be due to an increase in free radical load from Fenton's reaction (as discussed above) which may also produce oxidative stress like environment thus triggering cells to increasing its antioxidant defensive mechanisms. The combined results of monitoring of various indicators of oxidative stress, including molecular expression of genes, indicates that supplementing the diet of *Drosophila* larvae with AB endows the animal with a greater tolerance to H_2O_2 stress.

CONCLUSION

It can be concluded that exposure to H_2O_2 induces oxidative stress in *Drosophila* larvae from all feeding regimes. The catalytic as well as non-catalytic components of the anti-oxidant system of *Drosophila* larvae are enhanced by supplementing the food with AB. The abhrak bhasma is apparently better than the natural antioxidant ASC in conferring anti-oxidant power to *Drosophila* larvae. However, more investigations are still required to comprehend the specific pathways of this action.

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