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Cytoprotective properties of Hemidesmus indicusagainst H_2O_2on Drosophila melanogaster

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ABSTRACT

Reactive oxygen species, including hydrogen peroxide (H_2O_2), singlet oxygen, hydroxyl and superoxide radicals, have positive role in energy production in vivo systems, phagocytosis, intercellular signalling, and regulation of cell growth. At high concentrations, H_2O_2 acts as a toxin which has scavenging properties. Avariety ofdietary plantsincludinggrams,legumes,fruits,vegetables,tea,wine etc.are rich inantioxidants and scavenge the free radicals and reduces the oxidative stress. Hemidesmus indicus (H. indicus) being one of the indigenous plant known for its antioxidant and scavenging properties. Drosophila melanogaster is an excellent model organism to evaluate lethal concentration and the effect of different chemical or bioactive substances due to its short generation time and lifespan. The present study deals with control of H_2O_2 production by H.indicus using drosophila as model organisms. Drosophila species were treated with different concentrations of H_2O_2 and H. indicus. The present study reveals that prolonged exposure to H_2O_2 at higher concentration causes toxicity which in turn affects the hatching and life cycle of drosophila. The study reveals that the supplementation of H. indicus increases the resistance ability against oxidative stress generated by hydrogen peroxide. It acts a cyoprotective molecule thereby significantly reducing the levels of antioxidant enzymes such as catalase and superoxide dismutase generated by hydrogen peroxide in drosophila. Also, it helps to maintain the GSH levels in drosophila both larval and adult forms by restoring the glutathione peroxidase and glutathione reductase levels. Thus, H. indicus is one of accessible source of antioxidant in pharmaceutical industries.

Keywords : Hemidesmus indicus, Hydrogen Peroxide, Drosophila, LC_{50}, Glutathione

I. INTRODUCTION

H_2O_2 (Hydrogen peroxide) in biological system can pass through biological membranes easily hence can react with other biological molecules and generates potent ROS. It target cell externally and internally causes oxidative stress leading to aging. Some of the reactive oxygen species, including hydrogen peroxide, has positive role in energy production in vivo systems, phagocytosis, intercellular signalling, and regulation of cell growth (Packer et al., 2008). Along with that it also affects the DNA and membranes stability by attacking the lipids, proteins, and carbohydrates in cell membrane and tissues and nucleic acid bases (Jung et al., 2009).

Medicinal plants have been used as an exemplary source for centuries as an alternative remedy for treating human diseases because they contain numerous active constituents of therapeutic value (Nostro et al., 2000). Hemidesmus indicus one of the indigenous plant also known as ANATMOOL or sativa. It is found in tropical region of India. It is rich in hemidesmol, glycoside, tanin, β-amyrins,flavenmin 1, hemidesmin 2, steroids, 2 – hydroxy, 4-methoxy Benzoic acid,saponins ,

Phenols, and etc. They possess bioactive compound 2 –hydroxy, 4- methyl benzoic acid, acts as antioxidant. Gayatri et al (2008) had shown that H.indicus have hepatoprotective properties in rat. Along with antioxidant properties it also possesses anti venom against viperarusseiiand antimicrobial properties (Baheti et al., 2006). It is use in Ayurveda to cure high blood pressure, Arthritis, increase semen also. The antioxidant present in the H. indicus would act as a potent inhibitor of free radicals generates into the body for example- H_2O_2, OH^-, O_2^-.The fruit fly Drosophila melanogaster is a eukaryote widely used in genetics. It requires simple facilities, inexpensive culture media, it has a short generation time (appox. 10 days at 25ºC), it breeds a large number of individuals per generation, and in vivo assays can be done easily.

In the absence of systematic studies in literature, the present study is aimed to evaluate the role of Hemidesmus indicus root extract in hydrogen peroxide induced oxidative stress in drosophila.

II. METHODS AND MATERIAL

A. Plant Material

The root powder of *Hemidesmus indicus* were directly purchased from Punervasu pharmacy, Pune.

B. Preparation of Plant extract: Different concentrations (0.25 – 7 mg/L) of *Hemidesmus indicus* root powder was prepared in autoclaved distilled water.

C. Preparation of Hydrogen peroxide: Different concentrations (0- 30 mg/L) of hydrogen peroxide (Himedia, USA) were used for the experiment.

D. Drosophila Culture and LC_{50}: The wild-type *Drosophila melanogaster* strain was maintained in the laboratory on a standard cornmeal, yeast, dextrose, and agar medium at 25°C (Ford et al., 2007). Eggs were collected from these flies by shaking them without anesthesia into bottles containing an approximately 2cm layer of fermenting fresh baker's yeast supplemented with sucrose The egg collection bottles were then kept undisturbed in the dark for 8 h at 25ºC. After removing the parental flies, the egg collection bottles were taken back to 25ºC where they remained at a relative humidity of 65% for the rest of their development. Three days later, the 72 h larvae were collected by washing them out the bottles with tap water at room temperature through a fine-meshed stainless steel strainer. They were thoroughly washed free of yeast with tap water while still in the strainer.

2.5 Estimation of sub lethal toxicity (LC_{50}): The larvae were transferred to vials (20 larvae/vial) containing 0.5 g of Drosophila Instant Medium (Carolina Biological Supply Co, NC, USA) prepared with the solutions of the test compounds, hydrogen peroxide at 0 to 30 mg/L. Five replications were made for each concentration in five independent experiments for each hydrogen peroxide. The treatment vials were kept at 25ºC and at a relative humidity of 65%. The surviving flies were collected from the vials on days 10 to 12 after egg laying and shaken into a flask containing 70% ethanol to quantify mortality. The LC50's for each strain and hydrogen peroxide were calculated using logistic regression with all five replications of every concentration. LC50's obtained from the five experiments were analysed with a two-way ANOVA (one factor being the strain, the other the treatment).

2.6 Measurement of antioxidant enzymes: Catalase (CAT, *EC 1.11.1.6)* activity was determined by the method described by Aebi (1984). Superoxide dismutase (SOD, *EC 1.15.1.1)* was assayed according to Beauchamp and Fridovich (1971). Glutathione peroxidase (GPx, *EC 1.15.1.9*) activity was measured by the method of Lawrence and Burk (1976). Glutathione Reductase (GR, EC 1.8.1.7) activity was determined by the protocol of Goldberg and Spooner (1983).

2.7 Statistical analyses

All experiments were repeated at least five times and data presented is average of these replicates. One-way analysis of variance (ANOVA) test associated

with the Tukey's test was used to determine the statistical significance of the differences among experimental groups. All the statistical analyses were done using SPSS 17.0 software. A logarithmic trend line was used to calculate the LC_{50} values.

III. RESULTS AND DISCUSSION

Drosophila melanogaster is considered to be the best known multicellular eukaryote model organism as we can study the interactions between genes and environmental conditions simultaneously. Recently, there have been successful attempts to use this species to investigate the effect of a certain type of diet on viability and lifespan (Khan et al, 2012; Li et al., 2007; Alberto et al., 2010; Soh et al., 2012).Drosophila use as an excellent model system to evaluate lethal concentration and their effect of different chemical or bioactive substances such as, growth and moulting disruption effects of azadirachatin against drosophila melanogaster (Diptera: Drosophilidae) by RadiaBezzar-Bendjazia et al, 2015. Other studies on Drosophila melanogaster have shown the effect of Asprin and acetaldehyde on longitivity and metamorphosis duration (Duygukeser and AylaKaratas, 2012).

Presently 80 percent of the world population relies on plant derived medicines and serves as first line of defense in maintaining health and combating many diseases (Veale et al., 1992). H. indicus serves as an alternative tonic, demulcent, diaphoretic and traditionally been used to treat venereal diseases, skin diseases, urinary infections, negative emotions and impotence (Jain et al., 2003).

A. To measure and evaluate the median lethal concentration (LC_{50})

LC_{50} is defined as the lethal concentration at which 50 % of the population if killed in a given period of time. There can be wide range of tolerance to toxic agents among different population of a species which should be taken into account.

In the following experiment the drosophila was treated with different concentrations of hydrogen peroxide ranging from 0 to 30 mg/L. At the different concentrations of H_2O_2 viz 0, 5, 10, 15, 20, 25 and 30 mg/L percent mortality were 3, 14, 28, 37, 52, 69 and 85 respectively. Figure 1 shows the mortality rates and LD50's for hydrogen peroxide was 19.64 mg/L (Figure 1). On the other hand, the ANOVA results show significant differences between control ($F = 41.21$, $p = 0.00003$) and hydrogen peroxide interaction ($F = 9.62$, $p = 0.0032$). Results revealed an increase in mortality rates directly proportional to increase in different concentrations of hydrogen peroxide (Figure 1). The similar resultwere observed by insecticide beta cyfluthrin on drosophila studeis (GireeshNaada et al., 2005). The crucial element of drosophila ethanol also showed the concurrent result (You et al., 2004).

H. indicus (anatmool) was also used to test its lethal concentration on drosophila with different concentration such as 0.05 to 7 mg/L. At higher (7 mg/L) concentration of H. indicus approximately 25 % mortality was observed.Thus drosophila treated with different concentration ofH. indicus does not showed any significant percent mortality (Table 1).

In the further experiments three concentrations (10, 15, 20 mg/L)of H_2O_2 were used in combination with (0.5 mg/L)H. indicus.

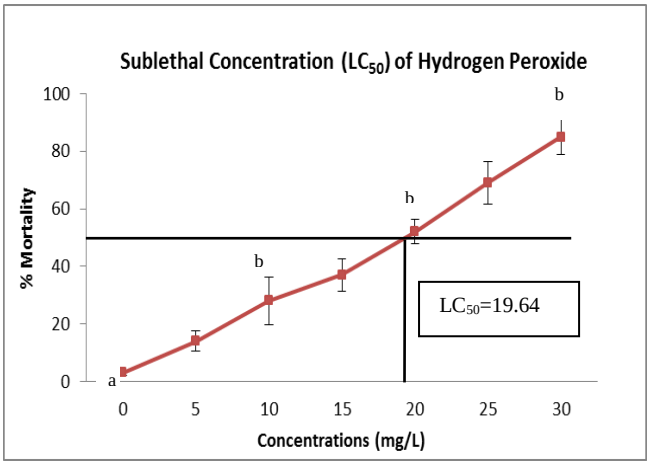


Figure 1. Median Lethal Concentration (LC_{50})at different concentrations of Hydrogen Peroxide

Dissimilar alphabets a and b in superscript in the figure indicate statistically significant difference at 0.05 level.

Table 1. Percent Mortality of flies at different concentrations of H_2O_2 and (0.5 mg/L)of H. indicus

Different Conditions	% Mortality
Control	9 ± 1.2^a
(0.5 mg/L) H. Indicus	14 ± 2.3
(10 mg/L)H_2O_2	14 ± 1.5
(10 mg/L)H_2O_2 + (0.5 mg/L) H. indicus	9 ± 1.1
(15 mg/L)H_2O_2	42 ± 2.3^b
(15 mg/L)H_2O_2 + (0.5 mg/L)of H. indicus	14 ± 1.3^c
(20 mg/L)H_2O_2	52 ± 3.4^b
(20 mg/L)H_2O_2 + (0.5 mg/L) H. indicus	19 ± 1.8^c

Control, H. indicus:Hemidesmus indicus, H_2O_2: Hydrogen peroxide. Dissimilar alphabets a and b in superscript in the figure indicate statistically significant difference at 0.05 level.

B. Morphological Changes in Drosophila Life cycle

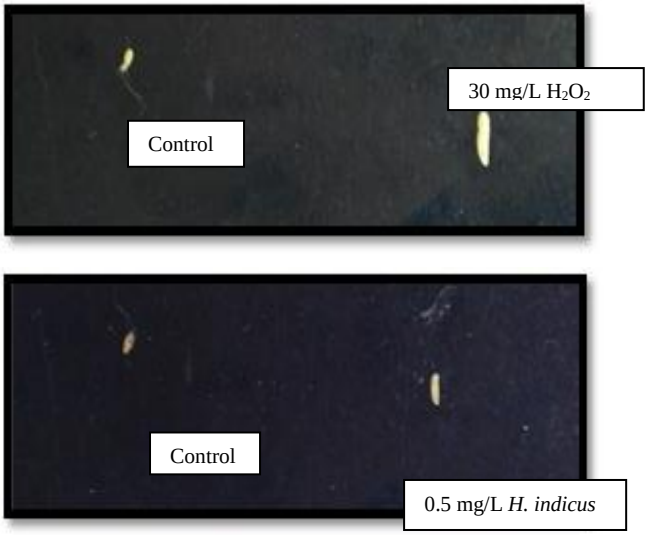
Drosophila exposed at different concentrations of H_2O_2 ranging from 5 to 30 mg/L showed significant morphological changes in its life cycle. When drosophila exposed at different concentration of H. indicus no morphological changes were observed in the emerged out larval forms. Following Observations (Slide 1) were made under different treatments:

1. Control and H. indicus (0.5 mg/L) showed no significant change in the morphology and percent mortality.
2. H_2O_2 (15 mg/L and 20 mg/L) showed significant mortality and the size of emerged larva was almost double compared to control larva.
3. In combination (10 mg/L H_2O_2 + 0.5 mg/L and 15 mg/L H_2O_2 + 0.5 mg/L H.indicus) significantly restored the size of larva as control larval forms.
4. At 20 mg/L of H_2O_2 the larval size was almost 3 times than the control larval forms. Also, the delay in the emergence of larval forms was observed compared to control. The addition of extract (0.5 mg/L) significantly restored the larval size of drosophila.

Interestingly at 30 mg/L of H_2O_2 the life cycle of drosophila was ceased at the stage of eggs which was observed till 15 days.

This may be due to inhibitory effect of hydrogen peroxide on gonadal development (Pratt,1980) The inhibition of oviposition is may be a result of imbalanced endocrine system or inhibition of ovarian development or deformities in oviposition organs (Asai et al., 1985). The reduced fecundity rate was observed by the effect of beta -cyfluthrin, (GireeshNadda et al., 2005). Spodopteralittoralis as a sub lethal pyrethroid insecticidal was reported (Radwan 1984) and besides that the life cycle was delayed by 5-6 days as compared to control. i.e prolongation in life cycle. This may be due some cytotoxicity of H_2O_2 or over production of growth hormone.

At the concentration 30 mg/L of H_2O_2 the eggs were not able to hatch from the egg this is may be due to direct impact of H_2O_2 various tissue such as trophocytes, perifollicular tissue, follicular epithelium and oocyte themselves (Kaur et al., 1993) or hormonal imbalance (GireeshNadda, 2005). In the present study it was observed that the egg hatching process and ovicidal action was decreased due to accumulation of H_2O_2 in eggs resulted in their direct death. The eggs which laid but do not hatched, are may be result of inappropriate incorporation of the yolk so that the embryo failed to complete metamorphosis.(Kaur et al., 1993) or may be due antifeedant effect of H_2O_2 resulted in weak and non-viable egg n drosophila species (Moore, 1980; Kumar and chapman, 1984).



Slide 1. Morphological changes observed in drosophila (3 instar larva) under different conditions

C. Evaluation of Antioxidant Enzymes

The H_2O_2 is one of the potent ROS generator .which induces oxidative stress use in experiment to generate oxidative stress. This oxidative stress can be reduced by bioactive compound obtained from plant (Sinha et al., 2006). Hemidesmus indicus is also one of the plant possess bioactive compounds can act as antioxidant. 2 – hydroxy ,4-methoxy Benzoic acidact as a hepatoprotective compound in rat (Gayatri et al, 2008) i.e act as antioxidant which scavenge the free radical.

At the concentration of 10 mg/L and 20 mg/L of H_2O_2 the specific activity of catalase was significantly increased in larval (178 ± 0.78 U/mg; 225.2 ± 2.7 U/mg; $p<0.05$) and adult forms of drosophila (123.2 ± 1.0; 155.7 ± 0.5 U/mg; $p<0.05$) compare to control larval and adult forms. When the flies were treated on combination with 0.5 mg/L H. indicus the catalase activity was restored back in larva (51.20± 0.9 U/mg; 92.1 ± 2.9 U/mg; $p<0.05$) and in adult (32.8 ± 1.3 U/mg; 66.1 ± 0.8 U/mg; $p<0.05$), compared to control adult and larval form (178 ± 0.78 U/mg; 225.2 ± 2.7) (Figure 2).SOD one of the cytoplasmic catalytic enzymes which scavenge the superoxide ions. At the concentration of 10 mg/L and 20 mg/L of H_2O_2 the specific activity of SOD was significantly increased in larval (123.18 ± 1.1 U/mg, 179.56 ±1.5 U/mg; $p<0.05$) and adult (102 ± 4.778 U/mg; 121.29 ± 7.61 U/mg; $p<0.05$) forms of drosophila compare to control larval and adult forms. When the flies were treated on combinations with 0.5 mg/L H. indicus the catalase activity was restored back in larva (72.47 ± 1.448; 108.23 ± 4.100U/mg; $p<0.05$) and in adult (63.71 ± 0.5567 U/mg; 72.76 ± 9.07 U/mg; $p<0.05$).compare to control larval and adult forms.

Glutathione reductase is an enzyme that reduces glutathione disulphide to sulphydryls from GSH, which is an important cytoplasmic antioxidant activity. At the concentration of 10 mg/L and 20 mg/L of H_2O_2 the specific activity of GPx was significantly decreased in larval (0.83 ±0.07 U/mg; 0.55±0.30 U/mg) and adult (forms of drosophila 0.52 ±0.39; 0.62.±0.02 U/mg) compared to control larval and adult forms. When the flies were treated on combinations with 0.5 mg/L of H. indicus the GPx activity was restored back in larval forms(0.93 ± 0.03 U/mg; 0.86 ± 0.04 U/mg; $p<0.05$) and in adult (0.92 ± 0.06 U/mg; 0.97 ± 0.06 U/mg; $p<0.05$) compared to control larval and adult form.At the concentration of 10 mg/L and 20 mg/L of H_2O_2 the specific activity of GR was significantly decreased in larval (0.45 ± 0.03 U/mg; 0.24 ± 0.03 U/mg; $p<0.05$) and adult (0.37 ± 0.02 U/mg ; 0.33 ±0.05 U/mg ; $p<0.05$) compare to control larval and adult forms. When the flies were treated on combinations with of 5 mg/L of H. indicus the GR activity was restored back in larva (0.51 ±0.01 U/mg;0.45 ± 0.04 U/mg; $p<0.05$) and in adult (0.43 ± 0.02 U/mg; 0.41 ± 0.01 U/mg; $p<0.05$) compare to control larval and adult forms.

The present study when drosophila's were treated with different concentrations of hydrogen peroxide significant increase in the levels of antioxidant enzymes namely CAT, SOD, GPx and GR as observed. Simultaneous treatment with H. indicus protected and restored the levels of these antioxidant enzymes. Concurrent results were observed by N. Mansa and J.S. Ashadevi (2015). This can be due to increase in activities GPx, SOD and CAT which results in increase in longevity. The

relevant results have been reported on Xijnjiang black mulberry fruit on delaying aging (Jiang et al., 2010).

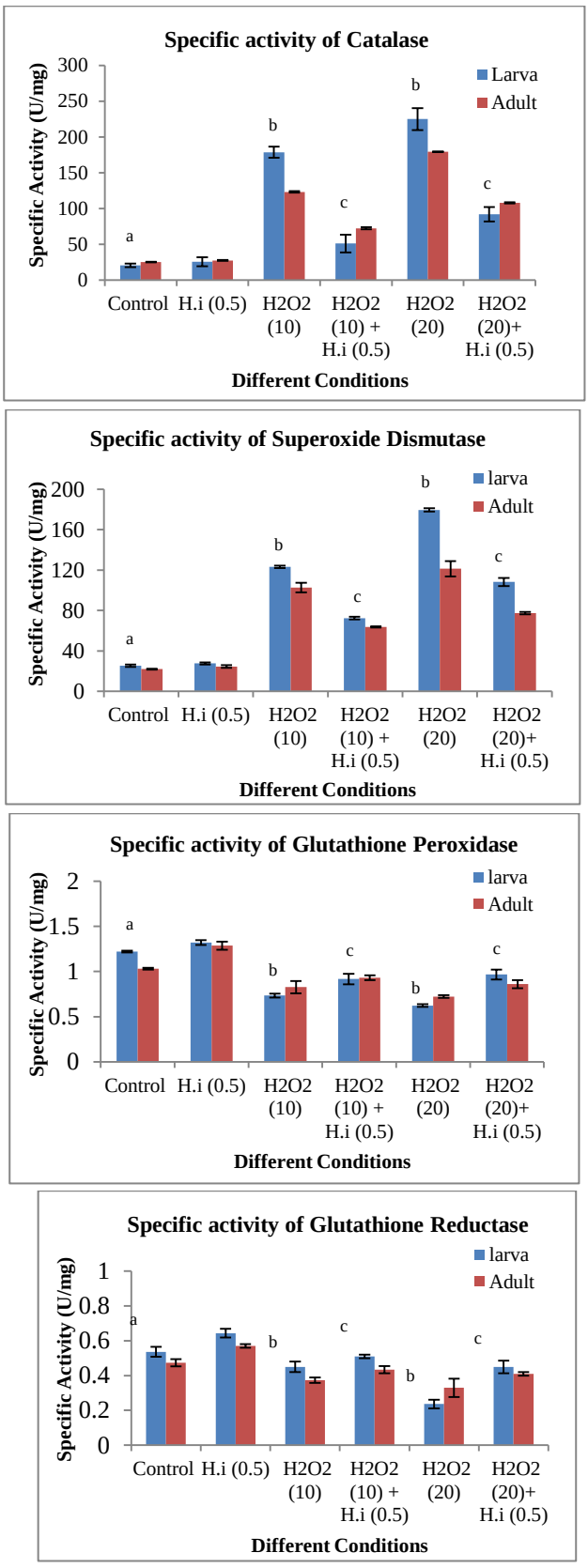


Figure 2. Specific activities of different antioxidant enzymes at different conditions

H.i (0.5): 0.5 mg/L of H. indicus; H202 (10): 10 mg/L of hydrogen peroxide; H202 (10)+ H.i (0.5): 10 mg/L of hydrogen peroxide and 0.5 mg/L of H. indicus ;H202 (20): 20 mg/L of hydrogen peroxide; H202 (20) + H.i (0.5): 20 mg/L of hydrogen peroxide and 0.5 mg/L of H. indicus. Dissimilar alphabets a, b and c in superscript in the figure indicate statistically significant difference at 0.05 level.

IV. CONCLUSION

The studies reveals that the supplementation of H. indicus increases the resistance ability against oxidative stress generated by hydrogen peroxide and thus being a cytoprotective drug. It reduces the oxidative stress generated by hydrogen peroxide in drosophila. Its acts a cytoprotective molecule by reducing the levels of antioxidant enzymes such as catalase and superoxide dismutase. Also, it helps to maintain the GSH levels in drosophila both larval and adult forms by restoring the glutathione peroxidase and glutathione reductase levels. Thus, H. indicus is one of accessible source of antioxidant in pharmaceutical industries. However, Disruption of growth and development in flies and other insect species under Hydrogen peroxide treatment have yet to be fully investigated.

V. ACKNOWLEDGMENTS

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VI. CONFLICT OF INTEREST

Authors declare no conflict of interest.

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