Protective Effect of *Diploschistes ocellatus* Against Heat Shock-Mediated Defects on Function of Reproductive Organs in *Drosophila melanogaster*

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Abstract

**Introduction:** Repeated heat shock (HS) stresses reduce the reproduction rate of *Drosophila* flies. Heat shock proteins (HSPs) protect cells against irreversible damages induced by heat-induced. Oxidative stress declines protective function of HSPs. *Diploschistes ocellatus* lichen aqueous extract possesses a strong antioxidant potential in vitro. Antioxidants can preserve HSPs function. Therefore, the present study for the first time investigated the cytoprotective effects of *D. ocellatus* aqueous extract against HS-mediated deleterious effects on reproductive function in *Drosophila melanogaster*.

**Methods:** Three different types of culture media including control, 30% lichen extract, and 60% lichen extract were prepared. Adult *D. melanogaster* flies were placed on Delcour medium and allowed to lay eggs for 2 hours. Then the eggs were equally distributed between the culture media. After flies completed their life cycle, the adult enclosed flies were exposed to HS. To assess reproductive function, the newly emerged adult flies were transferred to the freshly prepared regular culture medium every three days for 3 times and finally adult offspring born to these flies were enumerated.

**Results:** HS negatively affected the reproduction rate in flies in control group. Quantification of adult enclosed flies born to the *D. ocellatus* extract treated flies showed that lichen extract could negate the deleterious effects of HS on reproduction function of *D. melanogaster* in a dose-dependent manner.

**Conclusion:** *Diploschistes ocellatus* aqueous extract attenuated the harmful effects of HS stress on reproductive function of *D. melanogaster*. The secondary metabolites present in *D. ocellatus* can be considered as a bona fide candidate in novel drug development to target reproductive diseases in which oxidative stress is involved. Moreover, it can be concluded that *D. melanogaster* is an ideal model organism to induce cellular stress in vitro and study therapeutic potential of lichen extracts.

**Keywords:** *Drosophila melanogaster*, *Diploschistes ocellatus*, Heat shock proteins, Oxidative stress, Reproduction.
Antioxidant compounds attenuate free radical-induced damages by affecting electron transport chain function and inhibiting oxidation of lipids and other macro molecules.8 HSPs possess antioxidant and anti-inflammatory potentials. These proteins assist the primary and secondary folding of proteins and thus protect the cells against damages and apoptosis.9 It has been reported that elevation of cellular HSP level can cause resistance against different stresses.10 HSPs along with antioxidant molecules scavenge reactive oxygen species (ROS) in oxidative stress.11,12 Moreover, cellular antioxidants lead to a lower need for HSP expression to re-assemble the denatured proteins.13

Heat has severe effects on physiology and fitness of insects. For Drosophila melanogaster, extreme temperature is extremely harmful and even fatal. Due to lack of physiological processes for temperature balancing in these insects, they adjust their body temperature via different behavioral responses.14

In Drosophila, reactions to intense heat stress have been estimated as survival rate, sedation time or recovery from sedation, which are characteristics in which locomotor capacity is involved.15,16 However, to assess the unpleasant impacts of naturally occurring thermal stress, it is also possible to concentrate on reproductive features including fecundity, fertility, and hatchability. These reproductive features are regularly influenced by temperature changes which may be even evolutionarily more substantial.17 Survival and reproduction are two major parameters of fitness. Some studies have reported the negative impacts of HS on reproduction capacity of D. melanogaster. HS may introduce the cells to oxidative stress and thus antioxidants can ameliorate the deleterious effects of HS on reproductive health of fruit flies.

Lichens are distinctive types of living organisms composed of two elements, a fungus (namely, mycobiont) and an algae or cyanobacterium (called as photobiont). These two eukaryotic components live in symbiotic relationship.18-20 The fungi produce the thallus structure (the main body of lichen) that hosts the algae or the cyanobacterium, and in addition to protection, provides optimal conditions for photosynthesis of the photobionts. This procedure produces sugars and other nutrients required for growth of the fungus.20 As with plants, lichens are also able to synthesize secondary metabolites, and thus are used in folk medicine in many parts of the world. Most of these bioactive metabolites possess antioxidant, anti-bacterial, anti-inflammatory, and anti-cancer properties.

D. ocellatus is a wide-spread species of lichen growing on calcic rocks and soils. Its numerous thallus structures are powdery light grey to white. Each thick thallus encloses a black apothecium which is a disc-like composition on the exterior of the thallus and is used in sexual reproduction of the lichen.21

Lichens have been used as valuable biological resources since ancient times. People in different countries use the lichens as remedies, foods, and natural dyes.22,23 Lichens are also considered as natural therapeutics in pharmaceutical industry due to their unique bioactive ingredients.24 Lichens possess secondary metabolites with very strong antioxidant potential that are capable of scavenging free radicals.25 In the present study, the cytoprotective effects of Diploschistes ocellatus aqueous extract were evaluated against deleterious actions of thermal stress on reproductive function of D. melanogaster for the first time.

Materials and Methods
Fly Stock and Husbandry
Wild type of D. melanogaster, Oregon K strain was obtained from Drosophila Lab, Department of Biology, University of Zabol, Zabol. Flies were raised, amplified, and maintained on standard wheat cream agar medium supplemented with dry yeast granules at 25±1°C and 50%–60% relative humidity in a vivarium. Synchronized adults were used in all the experiments.

Lichen Aqueous Extract Preparation
The D. ocellatus lichen was collected from local environment. Taxonomy of the lichen was confirmed by a botanist. The vegetative parts were removed and washed. Samples were air dried and powdered. A suspension was prepared in 100 mL distilled water and kept in shaker incubator at 37°C overnight. Finally, the solution was filtered using Whatman filter paper grade 1 and used for the experiments.

Evaluation of Antioxidant Potential, Toxicity, and Effective Dose of Diploschistes ocellatus extract
The antioxidant activity D. ocellatus aqueous extract was assessed by DPPH radical scavenging assay as described by Brand-Williams et al.26 with minor modifications.

To determine possible lethality of D. ocellatus extract, different fly culture media were prepared with 10%, 50%, and 90% D. ocellatus extract concentrations. A total of 20 five-day-old adult flies were transferred to each medium vial and were monitored for 3 weeks. Flies were shifted to a new medium vial with same preparations every 4 days. Number of dead flies was recorded each day.

Diploschistes ocellatus Extract Treatment
Following proliferation of flies, virgin females and single males were isolated and kept in separate culture vials. On the day 5, 50 males and 30 females were transferred to culture bottles containing 3 different media, 60% (v/v) D. ocellatus extract, 30% (v/v) D. ocellatus extract, and control media (without extract). The flies were allowed to mate and reproduce for 3 days and then the adult flies were discarded. The emerging offspring were taken for HS experiments.
Heat Shock Exposure
The newly enclosed adults were separated based on their gender and transferred to freshly prepared culture media vials. Two-day-old virgin female flies and single males were transferred to agar media vial to avoid lethality due to desiccation and then kept in oven at 37°C for 90 minutes. After a 16-hour rest in regular medium in vivarium, the flies were added to agar culture vials and again were exposed to 40°C for 60 minutes.

Reproductive Success Assessment
Following a 3-hour rest interval after HS exposure, the 30 males and 20 female flies were transferred to normal culture bottles and were allowed to copulate and lay eggs for 3 days. Then adult flies were discarded. The culture bottles were carefully monitored for nine days to quantify the egg-to-adult number of flies. Indeed, the number of hatched larvae that could go for pupation was included by counting emerging adult flies. This procedure was done repeatedly every 3 days.

Statistical Analysis
The data was presented as mean ± standard error (SE) and analyzed by independent samples student t-test and one-way ANOVA followed by Dunnet test in PASW (predictive analytics software) version 19.0. The P value of <0.05 was considered as significance level.

Results
Diploschistes ocellatus Aqueous Extract Antioxidant Activity, Toxicity, and Dose Selection
The antioxidant activity percentage (AA%) of D. ocellatus aqueous extract was found to be 84 (inhibition %) according to the DPPH scavenging activity of ascorbic acid. This value indicates the high antioxidant potential of the lichen extract.

The extract was not toxic and caused no lethality at any of the doses used. Therefore, two v/v concentrations (30% and 60%) of D. ocellatus aqueous were chosen for the main experiments as effective doses.

Effect of Heat Shock on Flies’ Reproduction Success
To test the protective potential of D. ocellatus aqueous extract against reproduction failure following HS in flies, the number of enclosed adult offspring of parents under different culture conditions was determined. As shown in Table 1, HS stress adversely affected the reproduction rate in control group (i.e. the flies that had not been treated with D. ocellatus aqueous extract). Though the number of offspring dramatically reduced over the 9-day screening, the most severe effect was found to be in the first 3 days after HS stress.

Effect of Diploschistes ocellatus Aqueous Extract on HS-Induced Reproduction Defects
Quantification of enclosed adult flies born to D. ocellatus treated parent flies showed that D. ocellatus could suppress the deleterious effects of HS on reproduction success. As indicated in the Table 2, D. ocellatus extract exhibited a dose-dependent protective effect on reproductive success (i.e. the effects were more pronounced in 60% D. ocellatus extract compared to the 30% D. ocellatus extract).

In the absence of HS stress, lichen extract treatment alone did not change reproduction rate of flies. Comparison between control group and sham group who were treated with D. ocellatus extract but were not exposed to HS stress revealed no significant differences in the number of offspring born to D. ocellatus treated and control parent flies (P = 0.63, Table 3).

Discussion
The present study is the first report on the inhibition of the HS stress-induced cellular and molecular damages and restoration of their reproductive success to the normal level by the aqueous extract of D. ocellatus lichen. The fecundity, fertility, hatchability, and adult enclosure were found to be the main parameters affected by HS stress in this study. The number of enclosed adult flies that were able to reproduce was also determined. It was evident that D. ocellatus remarkably attenuated reproduction success of HS exposed flies.

Assessment of resistance to environmental stresses may not always be an appropriate strategy in terms of survival and reproduction.16,17 But in Drosophila species that are thought to have a short normal life span,26,27 even reversible damage may affect the reproductive success of the insect.

Temperature is an important factor in Drosophila life cycle and many physiological functions.29 There are reports on declined reproduction success in male fruit
flies exposed to prolonged high (29°C) temperature. However, female flies are more sensitive to HS, and thermal stress can affect their adaptive parameters like mating tendency, fecundity, and fertility.

High temperature and oxidative stress primarily induce HSPs expression. Moreover, severe oxidative stress impairs HSPs function. Association of these factors in vivo has been definitely confirmed. In addition to lacking complex physiological mechanisms to maintain body temperature in these tiny insects, simple simulation circumstances for environmental stress factors affecting Drosophila life in laboratory conditions facilitate systematic study of cellular responses to stressful stimuli.

With regards to the association between HSPs and oxidative stress, it is advisable to study cytoprotective potential of natural antioxidants, which are able to ameliorate such damages to cells. In addition, the role of secondary metabolites of lichens has not yet been extensively studied. These agents can have potential therapeutic properties due to their strong antioxidant capacities. Therefore, in the present study, the protective effects of D. ocellatus aqueous extract against functional damages induced by HS on reproduction rate of the fruit flies were investigated. The findings were in favor of protective role of D. ocellatus extract through restoration of reproduction of the flies exposed to HS.

Though antioxidant action of D. ocellatus could be the main reason for its cytoprotective impacts, molecular investigations on interaction between lichen extract components and HSPs are highly needed. Other therapeutic actions of the lichen, such as anti-inflammatory, anti-cancer, and antibacterial, can be investigated in future investigations to extend limited knowledge about cellular mechanisms through which the D. ocellatus extract overcomes the external cell stressors.

**Conclusion**

Given the involvement of environmental factors and oxidative stress in development of wide spectrum of human diseases, and disease condition modeling in fruit flies, special attention can be directed to lichen secondary metabolites in future drug development studies. Nevertheless, aqueous extract of the D. ocellatus could be used as a solution containing numerous antioxidant compounds. Thus, characterization of molecules present in this solution is highly recommended. Moreover, recently characterized molecules will maintain the main status in drug development in future.

**Competing Interest**

The authors declare that they have no competing financial, professional, or personal interests that might have influenced the performance or presentation of the study described in this manuscript.

**Ethical Approval**

Not applicable.

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