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Impact of *Phyllanthus amarus* extract on antioxidant enzymes in *Drosophila melanogaster*

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ABSTRACT

Oxidative stress (OS) leads to deleterious effects in organisms. Main cause for the oxidative stress is the generation of free radicals in the cells. Antioxidants have ability to scavenge and neutralize the free radicals. Therefore, present study has undertaken to test the antioxidant potential of *Phyllanthus amarus* ethanolic extract (PAE) in *Drosophila melanogaster* through biochemical analysis. OS test has been conducted in the PAE supplemented different age grouped flies by exposing to 20 mM acrylamide (ACR). Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH) were measured in different age grouped stressed and non-stressed PAE supplemented flies. OS test results revealed that PAE II treated groups showed significantly increased ACR resistance when compared to PAE I and control groups. All the age grouped PAE supplemented flies in non-stress conditions had significantly high SOD and CAT activities. Highest SOD and CAT activities were observed in PAE II treated 30 and 45 days flies respectively. Under stress conditions, the elevated levels of SOD activities were found in 30 and 45 days and CAT activities in 30, 45 and 60 days aged PAE treated flies. Overall data highlights that there is a dose dependant relationship between the extract concentrations and enzyme activities.

1. INTRODUCTION

Antioxidants are micronutrients that have gained importance due to their ability to scavenge and neutralize the free radicals before causing damage to body cells. Many naturally occurring antioxidant compounds have been identified as free radical scavengers [1]. Antioxidant enzymes play an important role in lowering the reactive oxygen species (ROS) levels and reduces the oxidative stress. The antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase and glutathione reductase (GSH), acts as natural defense mechanism against oxidative stress in biological systems [2]. Medicinal plants are considered to be the best source for antioxidant compounds [3]. As it produces the significant amount of antioxidants to prevent the oxidative stress caused by photons and oxygen, they are considered as potential source of new compounds with antioxidant activity [4]. Secondary metabolites of plants such as flavonoids and polyphenols exhibited a significant biological role due to their antioxidants activity [5, 6]. Phenolic and flavonoid-rich antioxidants were abundantly found in both edible and non-edible plants. *Phyllanthus amarus* is considered as one such plant species used in Indian Ayurveda enriched with polyphenolic compounds [7]. It is a rejuvenative herb belongs to the family Phyllanthaceae. *In vitro* antioxidant activity of *P. amarus* has been reported by few studies [7,8]. The hepatoprotective, antidiabetic, antiulcer and anticancer activities of *P. amarus* are also been reported [9]. The ethanolic extract of *P. amarus* leaves exhibited hypoglycemic activity on alloxan-induced diabetic mice [10].

Recently, we have reported that the ethanolic extract of *P. amarus* species extends the lifespan in *Drosophila melanogaster* [11]. Since the mechanism by which *P. amarus* enhances the lifespan in *Drosophila* is not yet known. Therefore, in order to understand such mechanism, we performed the few antioxidant enzymatic assays in *Phyllanthus amarus* ethanolic extract (PAE) supplemented flies under stress and non-stress conditions.

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2. MATERIALS AND METHODS

Acrylamide (ACR), Nitro blue tetrazolium (NBT), Hydrogen peroxide, Nicotinamide adenine dinucleotide phosphate (NADP), Trichloroacetic acid (TCA), Reduced glutathione, 5, 5'–dithio-bi's (2-nitrobenzoic acid) (DTNB), Bovine serum albumin (BSA) were procured from Himedia, Mumbai, India.

2.1. Extract preparation

Phyllanthus amarus was collected from natural habitat in Mysore, Karnataka, India. The extract was prepared by using the aerial parts of the plant. The powder form of the plants was subjected to Soxhlet unit to get ethanolic extraction. The collected dried extracts were stored and used for the analyses. All the experiments were carried out using two different concentrations of the PAE (dose-I: 1 mg/ml and dose-II: 10 mg/ml).

2.2. *Drosophila* culture

Wild stocks of *D. melanogaster* (Oregon K strain) flies were procured from *Drosophila* Stock Center, Department of Zoology, University of Mysore, Mysore, Karnataka. The flies were cultured and maintained in standard 'Wheat cream agar media' seeded with dry yeast granules. Further, flies were multiplied by subculture and maintained at 22 ± 1º C with relative humidity of 60 - 70%.

Synchronized eggs were collected from the Delcour technique as per the standard procedure [12] and newly eclosed flies were segregated under mild anesthesia. Then, flies were maintained in PAE supplemented culture media and aged them for 15, 30, 45 and 60 days.

2.3. Oxidative Stress (OS) Test

The OS test was carried out by following filter paper disc method [13]. The OS test was conducted by the induction of ACR in the PAE supplemented flies at the age group of 15, 30, 45 and 60 days.

The flies were induced with 20 mM ACR was employed along with 5% sucrose in a vial of size 9 x 3 cm containing 5-6 layers of filter papers in different batches (20 flies /vial). Then mortality rates were recorded until all the flies attain death and the rate of survival was recorded with an interval of 6 hour. Extract supplemented flies were considered as treated groups and only yeast fed flies were considered as control group. Hundred flies were maintained in each group.

2.4. Biochemical Estimations

The activity of few antioxidant enzymes namely, SOD, CAT and GSH were measured in stress and non-stress conditions. The extract supplemented with OS induced flies were considered as stress group and the extract supplemented without OS induced flies were considered as non-stress group.

The enzyme activity was studied in different age grouped (15, 30, 45, and 60 days) PAE treated flies in two concentrations. The whole body homogenate was prepared by using 0.1 M sodium-phosphate buffer (pH7.4). Then the samples were centrifuged at 2,000 rpm for 10 minutes and the supernatant was used for measuring SOD and CAT activities. The enzyme activities were measured in triplicates in each group.

2.4.1. *Superoxide dismutase (SOD)*

SOD activity was measured by using NBT method [14]. Three ml reaction mixture was prepared by adding 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 2 µM riboflavin, 0.1 mM EDTA, 75 µM NBT and 50 µL of enzyme extract.

All the tubes were exposed to 400 W bulbs for 15 min. and then absorbance was read at 560 nm. The 50% inhibition of the reaction between riboflavin and NBT in the presence of methionine was considered as one unit of SOD activity and it was expressed in units/mg of protein.

2.4.2. *Catalase (CAT)*

CAT activity was measured by following the standard protocol [15]. The activity was measured based on the quantity of the H_2O_2 substrate remaining after the action of CAT present in the enzyme extract. To measure this, 0.4 ml of enzyme extract was mixed with 2.6 ml of phosphate buffer along with 30% of H_2O_2. The activity was measured by determining the decomposition of H_2O_2 at 240 nm. CAT activity was calculated by using the millimolar extinction coefficient of 43.6 and expressed in terms of µm/min/mg of protein.

2.4.3. *Reduced glutathione (GSH)*

The activity of GSH was measured by using DTNB method [16]. To measure the enzyme activity, flies were homogenized in ice-cold 10% TCA and 10 mM EDTA solutions (1:1).

Then, homogenate was centrifuged at 5000 rpm. Further, enzyme reaction mixture was prepared by adding 200 µl of supernatant, 0.2M Tris-buffer (pH8.0) and 50 µl of DTNB. The reaction mixture was incubated for 10 min. at room temperature to get yellow coloured complex. The absorbance was read at 412 nm and the activity was expressed as µg/mg protein.

2.4.4. *Estimation of protein*

Protein estimation was quantified as per the standard procedure [17]. For this, 20 µl of the homogenate was mixed with Lowry's reagent and Folin-Ciocolteu's solution. The optical density was measured at 660 nm against blank after incubation. Then calculated the amount of protein in each group with the BSA standard graph.

2.5. Statistical Analysis

All the enzyme activities were statistically analyzed using the SPSS software, version 20.0 and the values were expressed in mean ± SE. The significance differences among the analyzed groups were compared by one-way ANOVA followed by DMRT. Values of $p < 0.05$ considered as statistically significant.

3. RESULTS

3.1. Effect of PAE on ACR resistance in *D. melanogaster* flies

The PAE supplemented flies of age group 15, 30, 45 and 60 days were exposed to ACR for the induction of OS. The result obtained from OS test of different aged flies were represented in figure 1. The resistance ability was significantly increased in the 15 days age group of both PAE I and PAE II supplemented flies and 45 days of PAE II supplemented flies when compared to control group. However, the pattern of stress induction was same in 30 days and 60 days aged PAE treated and control groups. Further, it reveals that PAE II treated groups showed high ACR resistance ability when compared to PAE I and control groups.

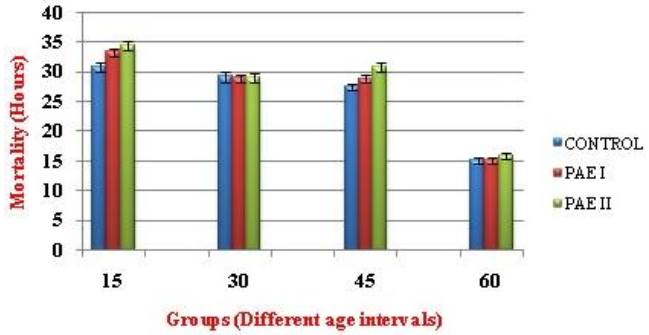

Fig. 1: Effect of PAE on ACR-induced mortality in *D. melanogaster*.

3.2. Biochemical Estimations

3.2.1. SOD

The SOD activity was measured in stress and non-stressed group of PAE fed flies are compiled in figure 2. The activity was measured in the flies with different age intervals.

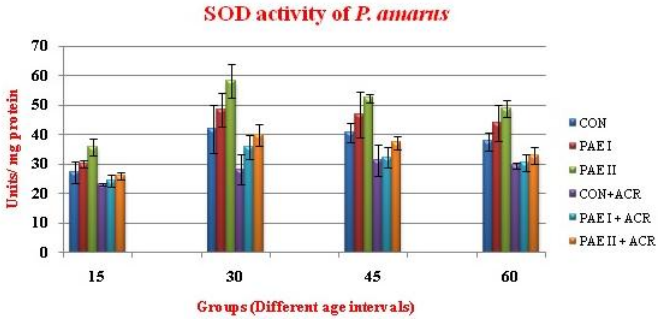

Fig. 2: SOD activity in control and PAE prefed flies before and after exposre to ACR.

The enzyme activity in non-stress state of all the analyzed group was comparatively lower at 15 days aged flies, shows maximum at 30 days, then gradually declined in 45 and 60 days age grouped flies. It was found that all the age groups of PAE treated flies of both the dose treatments had significantly higher enzyme activity than control groups. PAE II has maximum SOD activity in all the age groups. The maximum enzyme activity of PAE II was observed at the age group of 30 days (58.10 units/mg of protein). Under stress condition, ACR induced control flies of all the age group has reduced the SOD activity than PAE treated groups. In both the doses of PAE supplemented stressed fly groups, the enzyme activity was comparatively lower at 15 days and increased to maximum at 30 days. Further, SOD activity was suddenly decreased in PAE I and gradually decreased in PAE II treated stress group at 45 and 60 days. Similar to non-stress groups, PAE II supplemented stress group has maximum enzyme activity at the age of 30 days (39.82 units/mg of protein). Further result reveals that in both stress and non-stress conditions, the PAE II showed maximum SOD activity in all the age groups.

3.2.2. CAT

The CAT activity was measured in PAE prefed flies of stress and non-stressed groups are represented in figure 3. The activity was expressed as µm H_2O_2 consumed/min/mg of protein. The enzyme activity of PAE treated groups was gradually increased from 15 to 30 days aged flies and showed maximum activity in 45 days, then declined in 60 days aged flies.

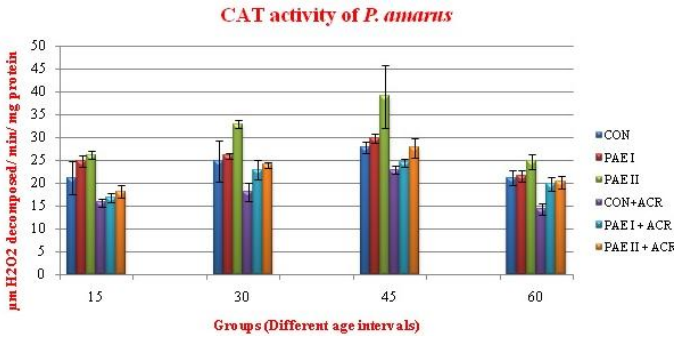

Fig. 3: CAT activity in control and PAE prefed files before and after exposure to ACR.

It was found that the enzyme activity in all the age groups of PAE treated flies was significantly increased compare to control group. Further, it reveals that PAE II treatment groups had highest CAT activity in all the age groups, with the maximum activity at 45 days (38.93µm H_2O_2 consumed/min/mg of protein). Similar observation was found in ACR induced stress groups, where PAE treated groups had higher CAT activity compared to ACR with control. In both the doses of PAE treated stress groups, enzyme activity was gradually increased from 15 to 30 days and reached maximum in 45 days. Whereas in both the doses of PAE treated 60 days stress groups, enzyme activity was declined. Similar to non-stressed conditions, the PAE II has maximum CAT activity in all the age groups except in 60 days in stress conditions.

3.2.3. GSH

The GSH activity was measured in PAE prefed flies of stress and non-stressed groups are shown in figure 4. The data reveals that GSH activity of the PAE I in non-stressed groups was increased significantly in 15 and 45 days when compared to control group. PAE II supplemented flies have significantly increased GSH activity in all the age group flies except in 60 days, where activity was declined. However, the maximum enzyme activity was observed in PAE II at 45 days (77.77 units/mg of

protein). On ACR treatment, the GSH activity of PAE treated flies was found to be lower when compared to control except in PAE II treated 60 days flies where the activity was significantly increased.

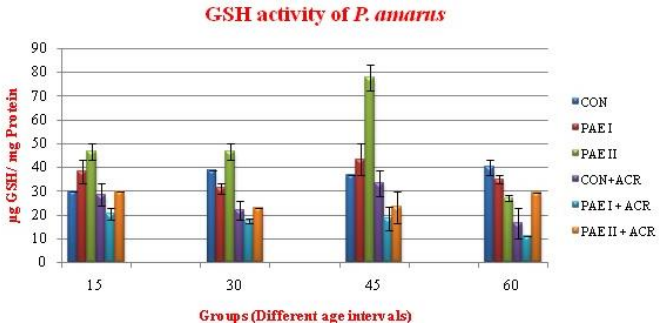

Fig. 4: GSH activity in control and PAE prefed files before and after exposure to ACR.

4. DISCUSSION

Oxidative stress leads to deleterious effects on the physiology of organisms. Main cause for the oxidative stress is the generation of reactive oxygen species in the cells and later they are converted into free radicals [18]. It has been reported that the cranberry diet reduces the oxidative stress and extend the lifespan [19]. ACR is commonly used as a toxicant to generate oxidative stress in model organisms [13]. In the present study, OS test was carried out for induction of stress by using ACR. PAE supplemented flies when undergone OS, showed prolonged survival time than control flies. The resistance ability for ACR was increased in PAE II by 11.36% in 15 days age grouped flies and by 12.66 % in 45 days age grouped flies. The PAE II supplemented flies showed more resistance ability with high degree of survival time than PAE I treatment. Thus, oxidative stress resistance ability of PAE was validated from the current study.

The endogenous antioxidant system can scavenge the excess amount of ROS. The major antioxidant enzymes such as SOD, CAT and GSH are serving as primary endogenous antioxidants to deactivate the ROS [20]. The most common ROS is O_2 which is easily dismutated either through non-enzymatically or by enzymatically to H_2O_2. The H_2O_2 is generated in the cells under normal as well as wide range of stressful conditions. H_2O_2 has no unpaired electrons, unlike other oxygen radicals. It can readily cross biological membranes and consequently can cause oxidative damage. Further, H_2O_2 is converted to H_2O by CAT or GSH. As organism get aged or under oxidative stress conditions, the scavenging capacity of the body is enormously decreased and thus could attack the intracellular molecules and ultimately lead to oxidative damage [21, 22].

The antioxidants found in many medicinal plants and herbs are capable of augmenting the antioxidant enzyme activities, their by it scavenges the free radicals. The dietary consumption of Hawthorn extracts showed beneficial impact on endogenous antioxidant enzymes [23]. It has been shown that the antioxidant activity of *Emblica officinalis* increases SOD, CAT and glutathione peroxidase (GPx) in rat [24]. *Tinospora cordifolia* is a well-known antioxidant rich medicinal plant used in Indian Ayurveda. The root extract of *T. cordifolia* increases SOD, GSH and CAT activities in diabetic rats [25]. *Syzygium cumini* an antioxidant rich plant increases GSH, SOD and CAT activity in rat [26]. Further, it has been proved that curcumin of *Curcuma longa* is capable of scavenging free radicals by increasing SOD, CAT and GPx [27].

In the present study SOD, CAT and GSH activities were measured in different age grouped PAE extract supplemented flies. The activities were measured in stressed and non-stressed state. Enzyme activities were gradually increasing, then decreased as the age increased.

The SOD activity was reached peak at 30 days and the CAT activity reached peak at 45 days in both stress and non-stress conditions of all the analyzed groups. Further, it was observed that PAE treated flies have high SOD and CAT activities in all the age grouped flies. Similar such observation was noticed even in extract fed flies induced with ACR. The data on GSH enzyme study summarizes that only 45 days aged non-stressed PAE II has significant amount of enzyme activity and there was no significant observation was exist in GSH activity of rest of the age group flies. Based on these result it indicates that the antioxidants of PAE acts as free radical scavenging molecules by increasing SOD and CAT.

Increasing the activity of antioxidant enzymes is also expected to increase longevity. It has been shown that the anthocyanins of Xinjiang black mulberry fruit prolongs the lifespan in *Drosophila* with increased activities of GPx, SOD and CAT [28]. Further, it has been reported that the activities of antioxidant enzymes might be one of the reasons for prolonging the lifetime of *D. melanogaster*. The supplementation of apple polyphenol increases the lifespan of *D. melanogaster* by regulating SOD and CAT genes [29]. In our earlier report [11], we have shown that PAE II prolongs the life span of *D. melanogaster* by 75%. In the present investigation, we have demonstrated that the supplementation of PAE is associated with the elevation of SOD and CAT activities. Thus, PAE extends the life span of fruit fly by increasing SOD and CAT activities. Present result is similar to the findings of earlier studies conducted on anthocyanins of black mulberry and apple polyphenols on *Drosophila* life span extension [28, 29].

In conclusion, the studies reveal that the supplementation of PAE increases the resistance ability against oxidative stress molecule. It increases the SOD and CAT activities even under stress conditions, thus it prolongs the longevity in *D. melanogaster*. Therefore, PAE may present a viable and potentially powerful therapy for aging and age-related diseases.

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