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Anti-Oxidative Effect of *Murraya Koenigii* That Prolongs the Lifespan of *Drosophila Melanogaster*

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Abstract **Original Research Article**

The free radicals (ROS and RNS) damage to proteins, DNA, lipids of the cell. These free radicals creates the imbalance in physiological functions and acts as a prevalent cause of various diseases such as cancer, diabetes, cardiovascular diseases, aging, oxidative stress and metabolic syndrome by dysfunction of antioxidant enzyme system of cell. Using the fruit fly, Drosophila melanogaster, as a model we examined the antioxidant properties of *Murraya koenigii* on the life history parameters. We demonstrate a novel physiological interaction between free radicals, oxidative stress and antioxidant enzyme system by using extracts of *Murraya koenigii* in standard diet of the fly. This study describes how this interaction impacts a very early cellular defect associated with ageing and ageing associate diseases. We also describe progressive deficits in flies expressing the superoxide dismutase gene, catalase and lipid peroxidation. Collectively, our work demonstrates that *Drosophila* can be used to study the cellular, physiological and behavioral basis of human ageing related diseases.

Keywords: *Murraya koenigii*, antioxidant enzyme system, superoxide dismutase, catalase and lipid peroxidation.

INTRODUCTION

The chemically unstable free radicals cause damage to proteins, DNA, lipid cells as a result of imbalance between the generation of reactive oxygen species (ROS) and the antioxidant enzymes. Free radicals are part of aerobic life generated at low level during physiological functions. They are known to revoltingly implicate in the pathogenesis of various diseases such as cancer, diabetes, cardiovascular diseases, aging, oxidative stress and metabolic syndrome. These radicals include superoxide anions, hydroxyl, nitric oxide an hydrogen peroxide radicals. Antioxidants of natural and synthetic origin prevent the free radical damage by its protective role such as reacting with them, chelating catalytic metals and by playing as oxygen scavengers [1]. In the intervening time, the ingestion of several synthetic antioxidants such as BHT and BHA has been reported toxic to man. The importance towards natural antioxidant has now drawn attention and different kinds of plants and its parts have already been proved as natural antioxidant sources.

Murraya koenigii plant belongs to the family Rutaceae. It is a shurbaceous plant founds in India and other Asian countries. It is locally dilects known as curry leaf or meetha neem plant due to presence of pleasant aromatic smell, ornament creature and also used as a spicing flavoring agent for various food and home made preparations. The leaves have somewhat pungent, bitter and weakly acidulous taste and these characteristics retained after drying. *M. koenigii* in India used as herb in Ayurvedic medicine preparations. Leaves and roots can be used to cure piles and relieve heat of the body, dehydration, tenderness and itching. It has been reported previously that much valued as an anti-diabetic, antioxidant, antifungal, antimicrobial, cytotoxic properties, anti inflammatory, antitumor promoting, anti-hypercholesterolemic, kidney painrelief, hepatoprotective activities. *Murraya koenigii* is recognized to be the good source of carbazole alkaloids. The other phytochemicals isolated and characterized so far from the leaves are alkaloids such as mahanine, koenine, koenigine, koenidine, girinimbiol, girinimibine, koenimbine, O-methyl murrayamine A, O-methyl mahanine, isomahanine, bismahanine, bispyrayafoline and rich source of iron. It is also reported to contain 5,8-dimethylfuranocoumarin, 1-al, 3[6', 6' dimethyl 5-hexene] carbazole and β-sitosterol [2-4].

In this study we evaluate the anti oxidant effect of *Murraya koenigii* to suppress the effect of oxidative stress in the form of free radicals in the *Drosophila melanogaster* [5].

MATERIAL AND METHODS

Fly strain and culture

The wild type *Drosophila melanogaster* was used for all experiments captured from fruit market of Shimla, Himachal Pradesh (India) and larvae of *D. melanogaster* (wild type) were reared at 21^{O}C on a standard *Drosophila* diet containing agar-agar, maize powder, sugar, yeast, sodium benzoate as antifungal and propionic acid as antibacterial agent. For healthy growth of the organism, additional live yeast suspension was provided.

Plant collection and Extraction procedure

The plant, *Murraya koenigii* personally collected from the plane area of Haryana (India) and were kept for air dry after initial washing with double distilled water on the same day. The dried leaves were ground to fine powder using laboratory blender on medium speed. The 1:20 ratio of powder and solvent (ethanol+ water, 50:50) was transferred into reflux apparatus and maintained for six hours at boiling temperature. The extracted solvent was allowed to evaporate to dryness under vacuum on a rotary evaporator at 40°C. All the extracts were used to filter through a Whattman filter No 1 and were stored at -20°C till usage. The obtained viscous dried extracts were used directly as 2mg/ml, 4mg/ml, 6mg/ml and 8mg/ml in standard food medium.

Survivorship

The antioxidant effect of plants on the life span of adult flies will be studied by feeding the flies (~150 flies from each group) to different diet regimes (control food and different concentration with plant extract) from day 1 of their life cycle (10 flies/vial and 15 vials per group). Flies will be transferred to fresh vials every 10 days and the number of dead flies will be recorded till the last fly's death. Median lifespans were estimated using linear interpolation over scoring intervals.

Developmental Time

Eggs were allowed to grow on different diet regimes (control food and different concentration with plant extract) for 16 hours, then permitted to mature at 25ºC. Time to pupation was scored daily.

Fecundity assay

Fecundity was measured as the mean number of eggs laid per female fly over a 24-hour period. ~18 hours after transferring experimental flies to fresh vials, the number of eggs laid in these new vials was counted. The counting was done by eye under a stereo zoom microscope. Differences in egg laying between conditions were analyzed using a nonparametric Wilcoxon rank-sum test and a significance threshold of $P<0.05$ was used.

Activity of Superoxide Dismutase (SOD) Enzyme

The approximate 100 flies were homogenized in 1 ml cold 0.1 M phosphate buffer (pH-7.4) solution and crushed by using mortar-pestle tissue homogenizer, then, centrifuged at 8000 g for 10 min. The supernatant was collected for enzyme activity. In the method of SOD enzyme estimation, the reduction of NBT (nitroblue tetrazolium) by addition SOD enzyme (tissue homogenized) under aerobic condition was observed. We have made a cocktail of four solutions containing 50µM of sodium carbonate (Na_2CO_3, pH - 10), 96µM of NBT, 0.6% of triton X-100 and 2 mM of hydroxylamine hydrochloride (pH-6), then added 0.1 ml of enzyme supernatant. The enzyme activity was expressed as units/min/mg of protein at A- 560 nm, where one unit of SOD enzyme was expressed as amount of inhibition with rate of reaction by 50%.

Catalase (CAT) Assay

CAT activity in the control and plant extract fed flies will be measured by following the ability of the enzyme to split H_2O_2 within 1 min of incubation time. The experiment will be carried out in 15 mL falcon tube. The assay mixture consisted of 1 mL of 0.01 M sodium phosphate buffer (pH 7.0), 25 μL of sample 10% homogenate. The final volume will be made 1 mL by adding distilled water and the tubes will be vortexed. 500 μL of 0.2 M Hydrogen peroxide (1:3 by volume) will be added followed by 2 mL dichromate acetic acid (5% of K_2Cr2O_7 with glacial acetic acid). Tubes will be vortexed again and will be kept for boiling for 15 min and will be cooled under tap water before measuring the optical density (OD). OD will be measured at 570 nm.

Lipid Peroxidation

Malondialdehyde, a lipid peroxidation end product in fly homogenate, was measured according to the method described by Wills [7] with some minor modifications. A 200 mL aliquot of fly (without head and wing) homogenate was mixed with 2 ml of thiobarbituric acid (TBA)– trichloroacetic acid (TCA) reagent (0.375 and 15%, respectively). The volume was made up to 3 ml with distilled water and boiled on a water bath at 95^{O}C for 20 min. The solution was then cooled under tap water. The reaction product (TBA–MDA complex) was extracted by adding 3 ml of n-butanol to the above solution. The absorbance of the pink colored extract in n-butanol was measured at 532 nm using a spectrophotometer. The amount of MDA was calculated using a molar extinction coefficient of $1.56 * 10^5$ M^{-1} cm^{-1} and expressed as nmol of MDA formed per mg of protein.

RESULTS

Effect of plant extracts on survivorship of Drosophila

The wild type strain of Drosophila melanogaster from Shimla, India was reared at 21^{O}C on standard food as control and different concentration of plant extracts, i.e. 2mg/ml, 4mg/ml, 6mg/ml and 8mg/ml for observation of survivorship. The survival was recorded till the all flies died. In the lifespan of Drosophila, it consists of three phases 1) Health lifespan, there will be no natural death 2) Transition lifespan, it involves slight decline in the adult curve and 3) Senescent lifespan, that lifespan involves the steady decline in the survivorship curve.

Results from the study showed that the control flies have healthy lifespan is about 35 days, 35 to 75 days is about transition lifespan. Using plant extracts of Murraya koenigii in the standard food medium of Drosophila the lifespan extended accordingly the concentration of plant extracts. The maximum lifespan of flies with above mentioned plant extracts concentration were 75days, 78days, 98days and 88days respectively. Hence the results from that study showed that the plant extracts of *Murraya koenigii* successfully extended the median lifespan and maximum lifespan by 23days.

Developmental Delay

The studies from the literature explained about the correlation between the developmental time and physical health. In Drosophila more will be developmental time more will be body mass and body size. The use of plant extract in diet of *Murraya koenigii* at concentration 2mg/ml, 4mg/ml, 6mg/ml and 8mg/ml. There will be a developmental delay, according to respective concentration i.e. from 12day to 16day. Hence the developmental time of fly was positively correlated with the increase in the concentration of plant extract food medium and also physical health span.

Fecundity

The number of eggs laid by a fly is known as fecundity. We observed significant increase in mean fecundity across the concentration gradient of plant extracts.

Biochemical Assay

Effect of plant extracts diet on the superoxide dismutase enzyme (SOD)

That enzyme activity measures the healthiness of the body. Basically SOD deactivates the highly reactive oxygen free radicals (superoxide) to a less reactive hydrogen peroxide. The SOD activity was measured as units/mg protein. In that experiment as per observations during aging process from 7day, 21day and 35day the activity of SOD was going to decrease. But while used the different concentration of plant extracts of Muraya koenigii in the standard food medium, the activity of SOD enzyme was increased within a limit. These observations showed that during the aging process, free radical generation continuously increasing and damages to the activity of the SOD enzyme. The dietary antioxidant of plant extracts in the food medium removes the oxidative stress by stabilization of free radicals and that relaxation gives a chance to antioxidant enzyme system to increase the activity of the SOD enzyme as these enzymes expressed in the early age of the fly. In these experiments the plant extract concentration of 2mg/ml, 4mg/ml, 6mg/ml and 8mg/ml successfully removed the burden of oxidative stress and increased the activity of SOD enzyme.

Catalase

We have evaluated the catalase activity in the three age groups i.e. 7days, 21days and 35days old flies along the concentration gradient i.e. control, 2mg/ml, 4mg/ml, 6mg/ml and 8mg/ml. we have found significant increase in the catalase enzyme activity in all age group. The plant extracts concentration 8mg/ml showed reduction but although it is significant compare to control group.

Lipid Peroxidation

It is the peroxidation of lipids of cell membrane result to the formation of malondialdehyde (MDA). We observed the significant decrease in the peroxidation of lipids. Across the concentration gradient in all three age group MDA formation was significantly decrease which showed the suppression the effect of oxidative stress.

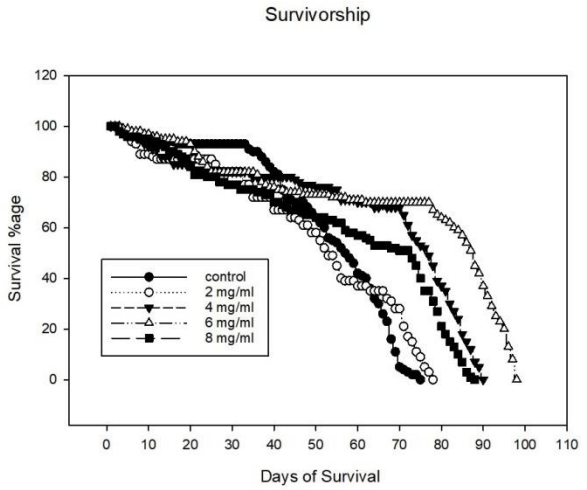

Fig-1: It shows the survivorship curvefor flies reared in different plant extract gradient i.e. 0mg/ml, 2mg/ml, 4mg/ml, 6mg/ml and 8mg/ml

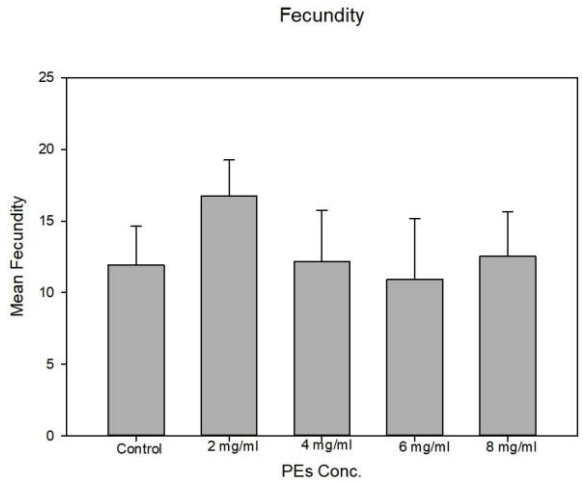

Fig-2: The mean fecundity measured per day egg laid by the fly. It explains the effect on fecundity along the plant extract concentration gradient as mentioned earlier

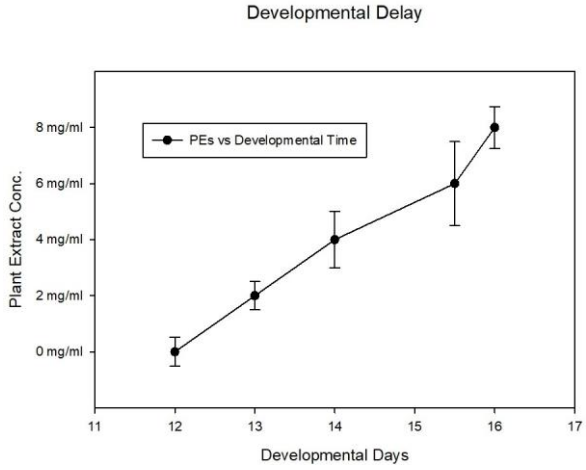

Fig-3: It represents delay in the developmental time i.e. egg to adult. It expresses developmental time versus plant extract gradient

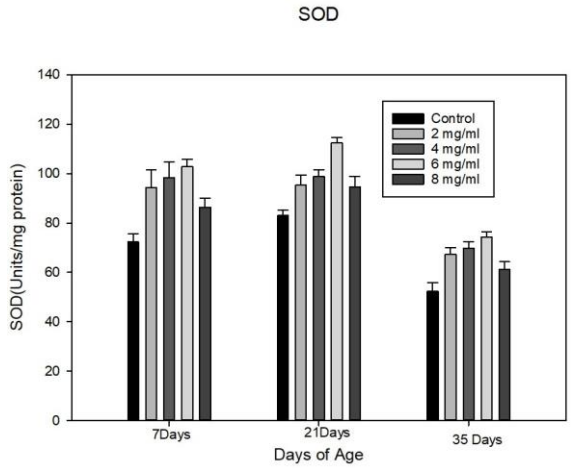

Fig-4: It shows the SOD (Superoxide dismutase) enzyme activity in three age group i.e. 7days, 21days and 35days along with different plant extract gradient concentration

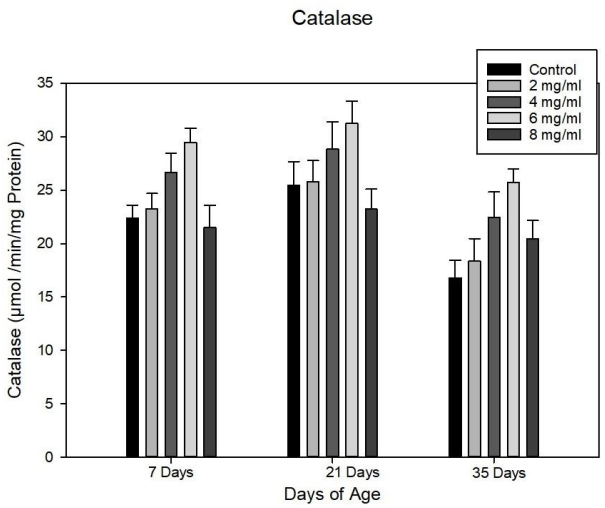

Fig-5: It shows the Catalase enzyme activity in three age group, i.e. 7days, 21days and 35days along with different plant extract gradient concentration

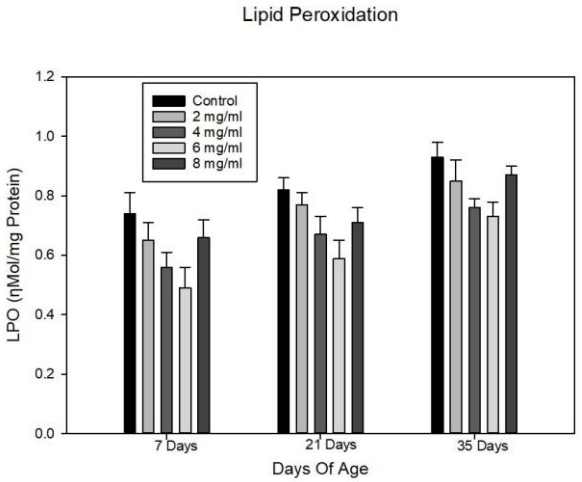

Fig-6: It represents the quantification of LPO (Lipid Peroxidation) that measures the malondialdehyde (MDA) in three age group i.e. 7days, 21days and 35days along with different plant extract gradient concentration

DISCUSSION AND CONCLUSION

Many studies have evaluated number of natural antioxidants, neutraceuticals and plants which have been identified as free radical scavengers. As stated by free radical theory of aging (FRTA) [8, 9]. The production of free radicals is unavoidable created by livings in anaerobic respiration. Researches on these plant neutraceuticals are of interest to the scientific community. To better understand their antioxidative property it is essential to identify the active ingradients of plant species by both in-vivo and ex-vivo [10-13].

The plant Murraya koenigii has richest source of carbazole alkaloids that has been reported as pharmalogical use as anti-tumor, anti-inflammatory, anti-diabetic and also antioxidant activies [14].

In this study we have successfully evaluated the antioxidant capacity of *Murraya koenigii* in the

Drosophila melanogaster. The days of survival and fecundity was significantly increased. The oxidative stress markers SOD, Catalase and Lipid Peroxidation also showed the significant result towards the suppression in the formation of free radicals.

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Conflicts of Interest: The author declares that there is no conflicts of interest regarding publishing this paper.

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