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Antistress ability of *Myristrica fragrans* (Japatrae) a nutmeg to detoxify reactive oxygen species in stress-induced *Drosophila melanogaster.*

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Abstract

Stress describes a positive or negative condition, which has an impact on an organism's genome, transcriptome, proteome, and phenome well-being. Origin of stress may vary but its effect is deleterious. The anti-stress property of *Myristrica fragrans* (Leaf powder) in combating stress in stress-induced *D. melanogaster* a fruit fly were experimented where four groups of flies were reared simultaneously. The Control flies reared on normal media followed by media containing MTX (Second group), the third group on the media containing MTX and 0.5 gm of japatrae, finally flies on only 0.5 gm of japatrae in media. Then the flies were assayed for stress related marker enzymes like SOD, CAT, and GPx. Reduction in level of ROS by *Myristrica fragrans* in stress-induced fruit flies has increased the ability to scavenge them and lowering the

free radical concentration there by balancing the expression of stress related marker enzymes in the stress-induced flies. Key words: Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx), Methotrexate (MTX), Reactive oxygen species (ROS).

Introduction

Hans Selye termed stress as an inadequate physiological response to any demand. Stress is simply a fact of nature, forces from the inside or outside world affecting the individual. The stress of exhilarating, creatively successful work is beneficial, while that of failure, humiliation, or infection is detrimental (Koolhaas, 2011). The word stress is derived from Latin word "stringi", which means "to be drawn tight". In medical terms stress is described as '*a physical or psychological stimulus that can produce mental tension or physiological reactions that may lead to illness.'* It is related to both external and internal factors. Acute, episodic, and chronic stress are the different types of stress (Lyle and Alma Dell Smith, 2013).

Acute stress is the most common form of stress. Stomach, gut and bowel problems, such as heartburn, acid stomach, flatulence, diarrhea, muscular problems including tension headache, back pain, anxiety are the causes of acute stress. Episodic acute stress is common for people with acute stress reactions to be over aroused, short-tempered, irritable, anxious, and tense. Often they describe themselves as having "a lot of nervous energy." Always in a hurry, they tend to be abrupt, and sometimes their irritability comes across as hostility. Some chronic stresses stem from traumatic, early childhood experiences that become internalized and remain forever painful and present. Some experiences profoundly affect personality. Chronic stress kills through suicide, violence, heart attack, stroke, and, perhaps, even cancer (Melinda Grossman, 2008; Lyle and Alma Dell Smith, 2013).

Myristrica fragrans is a bushy, evergreen and aromatic tree with oblong leaves and pale yellow flowers, followed by round fleshy fruits, containing a brown seed. Nutmeg is a spicy bitter, astringent, and warming herb that is a digestive tonic. It helps to control vomiting and relaxes spasms. Its topical application has anti-inflammatory effects (Herbal encyclopedia). So due to this beneficial property we selected this plant to investigate anti stress property on *D. melanogaster*, which were grown under the condition of stress, where stress induction was done by feeding the fruit flies in the medium containing MTX.

MTX is thought to affect cancer and rheumatoid arthritis by two different pathways. For cancer, MTX allosterically inhibits dihydrofolate reductase (DHFR), an enzyme that participates in the tetrahydrofolate synthesis. The affinity of MTX for DHFR is about one thousand-fold that of folate. DHFR catalyses the conversion of dihydrofolate to the active tetrahydrofolate. Folic acid is needed for the *de novo* synthesis of the nucleoside thymidine, required for DNA synthesis. Also, folate is needed for purine base synthesis, so all purine synthesis will be inhibited. Methotrexate, therefore, inhibits the synthesis of DNA, RNA, thymidylates, and proteins thus causing drug induced oxidative stress (Cronstein, 2005). In view of their abundance as normal by-products of metabolism, ROS (Reactive oxygen species), such as singlet oxygen, superoxide, peroxyl radicals, and peroxynitrite, are considered as probably the main source of spontaneous DNA damage (Evans *et al*., 2004). To prevent ROS from rising to excessive levels, cells are equipped with a variety of antioxidant defense systems. Such systems include the enzymes SOD, CAT, and GPx (Jan Vijg, 2007). SOD an enzyme that catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. CAT a common enzyme found in living organisms exposed to oxygen which has high turnover number of all enzymes. One CAT molecule can convert millions of molecules of hydrogen peroxide to water and oxygen each second. Thus, these enzymes are an important antioxidant defense in nearly all cells exposed to oxygen. Oxidative stress dependent upon superoxide radical can account for a number of acute and chronic disease states. Biological role of GPx is to protect the organism from oxidative damage similar to that of CAT (Weydert and Cullen, 2010).

In the present study we have cultured *D. melanogaster* in different groups where in the first group fruit flies are reared on normal rava cream agar media as well as in the medium containing MTX in different concentrations (second group) to induce the stress in the flies, and in the third group flies were reared on medium containing both plant sample as well as different concentrations of MTX. And the flies were cultured in the media containing only plant sample (*Myristrica fragrans*) in the fourth group. The enzymatic assay of

SOD, CAT, and GPx were done for all the groups of flies, and the results of all the groups are compared with each other in order to investigate the ability of the plant sample in reducing stress in stress-induced flies.

The purpose/objective of the present study is to evaluate the anti-stress property of *Myristrica fragrans* on stress-induced fruit flies. In this study we concluded that the plant sample used here has the ability to balance between ROS and antioxidant defense system, which is confirmed by ROS scavenging enzymatic studies. This may open up new avenues of research in a search of plants to combat against environmental stress.

Materials and Methods

Culturing of fruit flies

The *Drosophila* Stock Centre, Department of Zoology, University of Mysore, provided the stocks of wild type of *D. melanogaster*. Further the stocks were cultured in our laboratory at 26 ± 1°C. The flies are grown on a flour-based medium gelled with agar and seeded with baker's yeast and are sub cultured to fresh medium for every 15-20 days (Ashburner and Thompson, 1978).

Stress induction and reduction study

Any alteration in the food creates an environmental stress in an organism. MTX an anti cancerous drug that induces stress in fruit flies was used and mixed along with the media in different concentration in range of 5 ppm, 10 ppm, 15 ppm, and 20 ppm, and 25 ppm in second group of flies over the first group (Control) where fruit flies are cultured in cream rava agar medium without MTX and plant sample. To investigate stress reduction parameter by plant sample 0.5 g of japatrae is added to the medium containing MTX at different concentrations in third group. Finally fruit flies are cultured only in the medium with 0.5 g of plant sample in fourth group. Later the stress induction and reduction parameters were found by the estimating antioxidant defense enzymes in every group of flies.

Enzyme collection

Different groups of flies were taken in different eppendorf tubes as MTX flies of different concentrations from 5 ppm - 25 ppm and also the stress-induced flies along with plant sample. These were fully homogenized in 200 microlitres of fresh phosphate buffer of 50 mM for CAT assay and GPx assay of pH 7.0. For SOD assay 250 mM phosphate buffer of pH 7.8. These were homogenized with the help of tissue homogenizer, which was kept in ice cold condition and centrifuged at 8000 rpm for 20 min in a cooling microfuge. After centrifugation supernatant was transferred to a fresh eppendorff tube, and 100 microlitres of this supernatant serves as enzyme source for SOD, CAT, and GPx enzymatic assays.

SOD assay

SOD enzyme (EC 1.15.1.1) was assayed using a slightly modified procedure originally described by Beauchamp and Fridovich (1971). Mix 3 ml of cocktail solution containing 250 mM Phosphate buffer pH 7.8 (0.8 ml), 100 mM Methionine (1 ml), 100 mM Riboflavin (0.5 ml), 5 mM EDTA (0.1 ml), 750 µM NBT (0.5 ml), Enzyme extract (0.1 ml) total volume is 3 ml. A blank was set without the enzyme and NBT to calibrate the spectrophotometer. Another control was prepared having NBT but no enzyme and is taken as a reference control. Then all the tubes were exposed to 400W bulb for 15 minutes and these colored solutions absorbances were read at 560 nm immediately to know the activity, and later on to know the specific activity. Protein estimation is done by the method described by Lowry (1951) and is expressed in units/mg of protein.

CAT assay

CAT enzyme (EC.1.11.1.6) is assayed by following the method of Beers and Sizer (1952). 0.1 ml of crude Enzyme extract was mixed with 2.9 ml of 30% of hydrogen peroxide (freshly prepared using 50 mM phosphate buffer). The absorbance was measured by spectrophotometer at 240nm. Decrease in the absorbance indicates the action CAT on hydrogen peroxide. Protein estimation is done by method described by Lowry (1951) in order to determine specific activity and the same is expressed in units/mg of protein.

GPx assay

GPx (EC.1.11.1.9) is assayed according to the procedure of Rotruck *et al*. (1973) with some modifications. The reaction mixture consisting of 0.4 ml of 0.4 M sodium phosphate buffer (pH 7.0), 0.1 ml of 10 mM sodium azide, 0.2 ml of 4 mM reduced glutathione, 0.1 ml of 2.5 mM H_2O_2, 0.2 ml of distilled water and 0.1 ml of enzyme was incubated at 37ºC for 15 min. The reaction was terminated with 0.5 ml of 10% TCA and after centrifugation, 2 ml of the supernatant was added to 3 ml of phosphate buffer and 1 ml of DTNB (5,5-dithiobis 2-nitrobenzoic acid) reagent (0.04% DTNB in 1% sodium citrate). The color developed was read at 412 nm and the activity is calculated by determining the amount of glutathione utilized. Protein estimation is done by the method described by Lowry (1951) in order to determine specific activity and the same is expressed in units/mg of protein.

Results

Activity of SOD, CAT, and GPx activity in flies exposed to MTX

The normal flies were taken as control files and their enzymatic assay was done so as to compare with that of stress-induced flies by different concentrations of MTX where the specific activity of the SOD, CAT, and GPx increases gradually with the increase in the concentration of MTX over the control flies (Figures 1, 2, 3).

Enzyme activity in the stress-induced flies treated with plant sample

Addition of plant sample to the medium containing different concentrations of MTX has shown a considerable decrease in the level of antioxidant enzymes by suppressing the elevated level of ROS even though it is not equivalent to that of control flies (Figures 4, 5, 6; Table 1).

Variation of enzyme activity in normal flies treated with plant sample alone

Comparative study of the fruit flies grown in the media containing only 0.5 g of plant sample with that of control is carried out. The enzyme activity is different in the fourth group of flies reared on the media containing only 0.5 g of the plant sample (Table 2). There is increased CAT activity in the fourth group flies grown on medium containing only 0.5 g of japatrae over the control flies (Figure 8), and the activity of SOD and GPx is found to be decreased in the same flies grown on medium containing only 0.5 g of japatrae to that of control flies (Figure 7, 9; Table 2). The above result shows that CAT, SOD, and GPx activity has increased when MTX is added and the plant sample is found to reduce the stress.

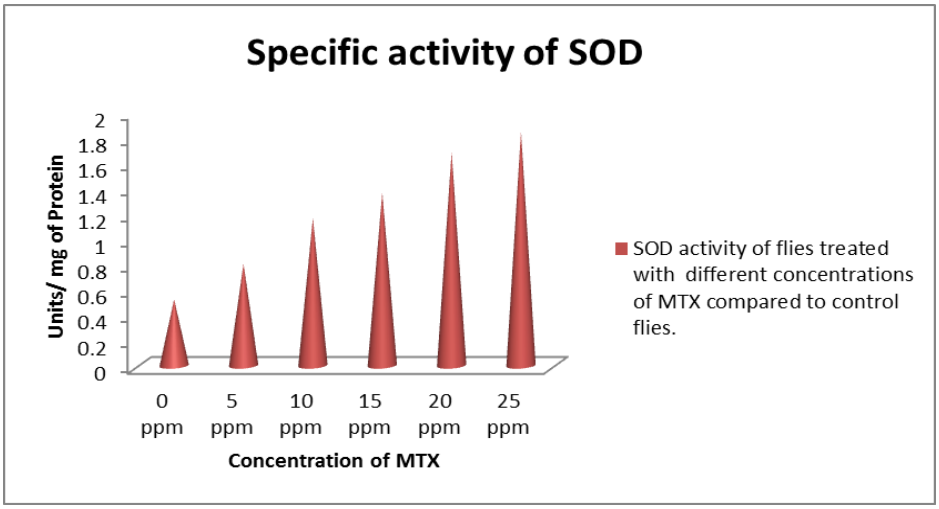

Figure 1. Activity of SOD (Second group) increases gradually in the flies reared on media containing increased concentrations of Methotrexate when compared with control flies.

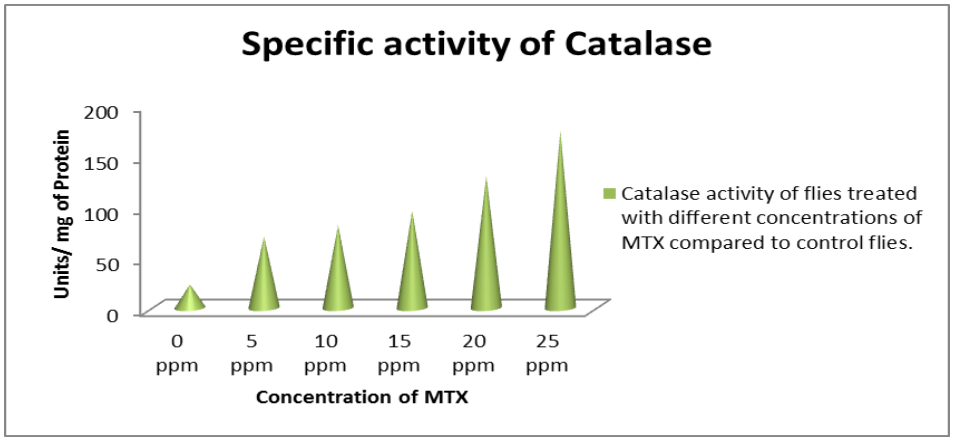

Figure 2. Activity of CAT (Second group) increases gradually in flies reared on media containing increasing concentrations of Methotrexate when compared with control flies.

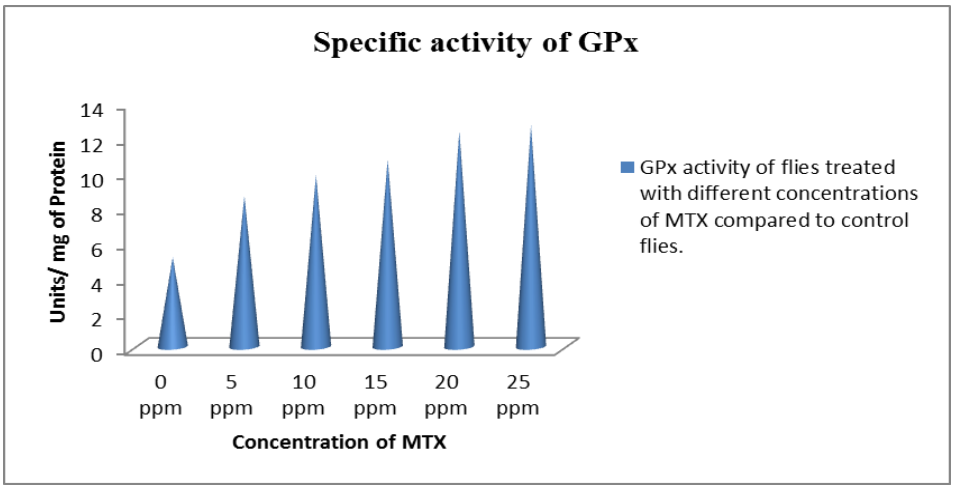

Figure 3. Activity of GPx (Second group) increases gradually in flies reared on media containing increasing concentrations of Methotrexate when compared with control flies.

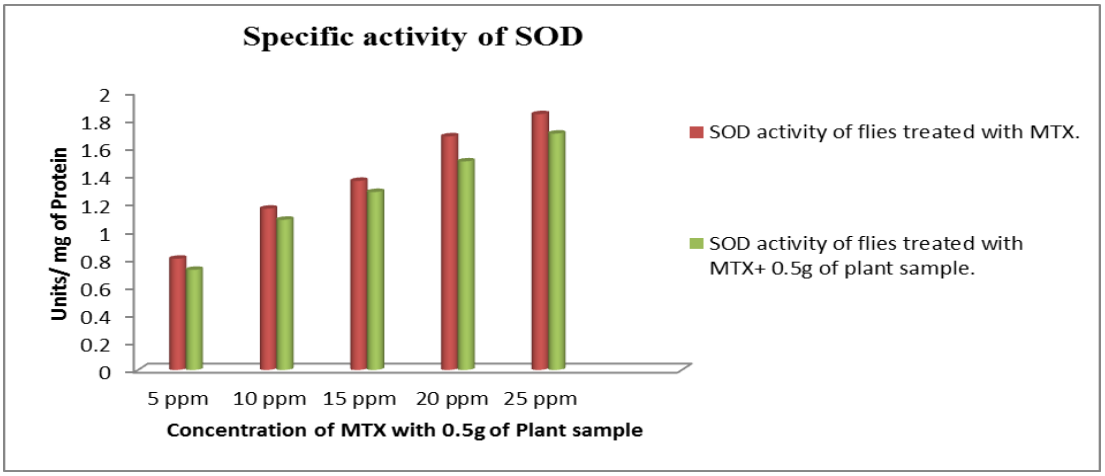

Figure 4. Activity of SOD (Third group) increases gradually in flies reared on media containing increasing concentrations of Methotrexate and reduction of activity in the presence of 0.5 g of plant sample.

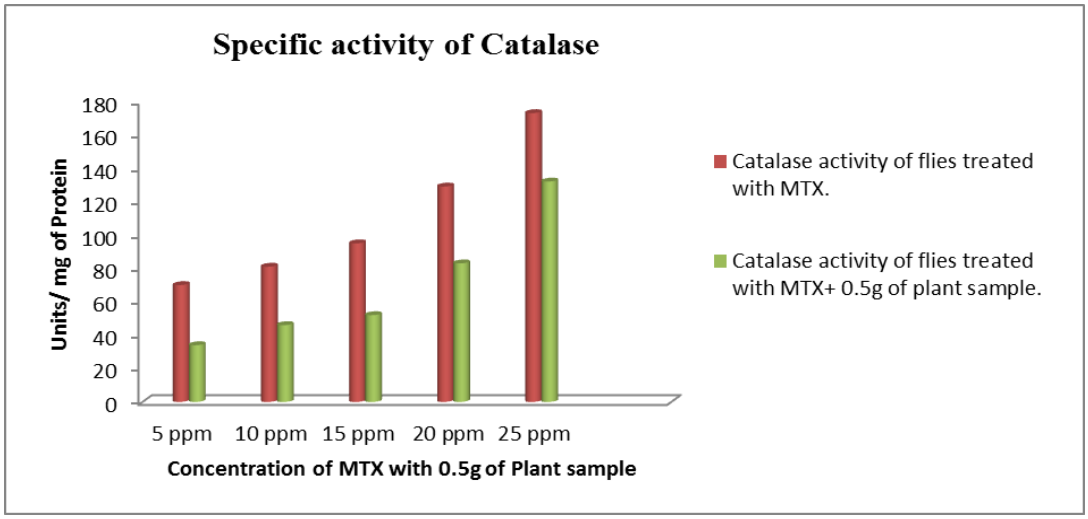

Figure 5. Activity of CAT (Third group) increases gradually in flies reared on media containing increasing concentrations of Methotrexate and reduction of activity in the presence of 0.5 g of plant sample.

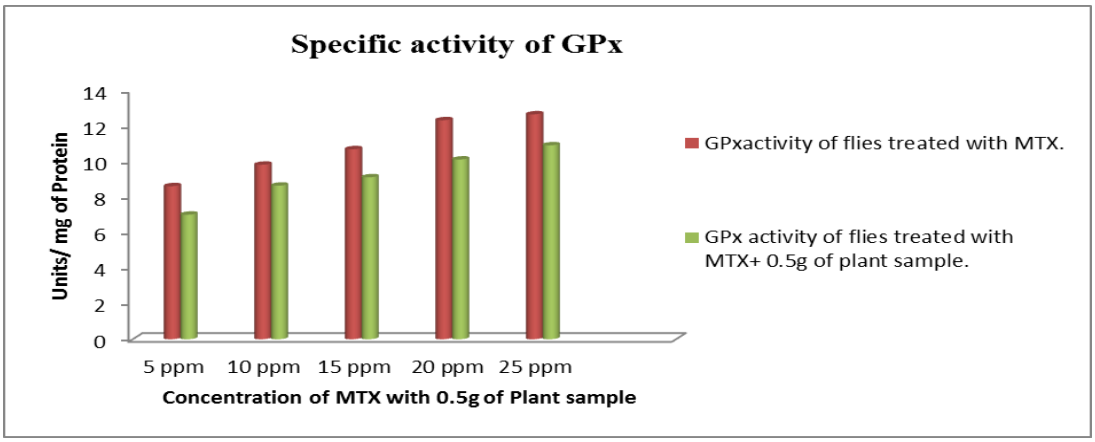

Figure 6. Activity of GPx (Third group) increases gradually in flies reared on media containing increasing concentrations of Methotrexate and reduction of activity in the presence of 0.5 g of plant sample.

Table 1. Comparison table of SOD, CAT and GPx activity of stress induced (MTX) flies (Second group) and 0.5 gm of plant sample + MTX at different concentrations (Third group).

	Concentration of MTX	5 ppm	10 ppm	15 ppm	20 ppm	25 ppm
SOD activity (units/mg protein)	MTX alone	0.80	1.16	1.36	1.68	1.84
	MTX+0.5 gm plant sample	0.72	1.08	1.28	1.5	1.7
Catalase activity (units/mg protein)	MTX alone	70	81	95	129	173
	MTX+0.5 gm plant sample	34.5	46	51.7	82.5	132
Gpx activity(units/mg protein)	MTX alone	8.6	9.81	10.68	12.31	12.64
	MTX+0.5 gm plant sample	7.00	8.63	9.10	10.1	10.9

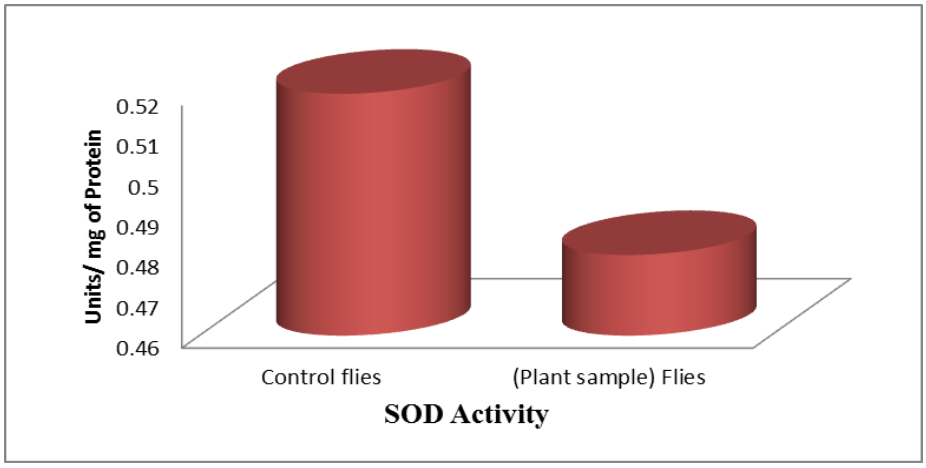

Figure 7. SOD activity of the fruit flies in Control (First group) and plant sample (Fourth group) comparative study.

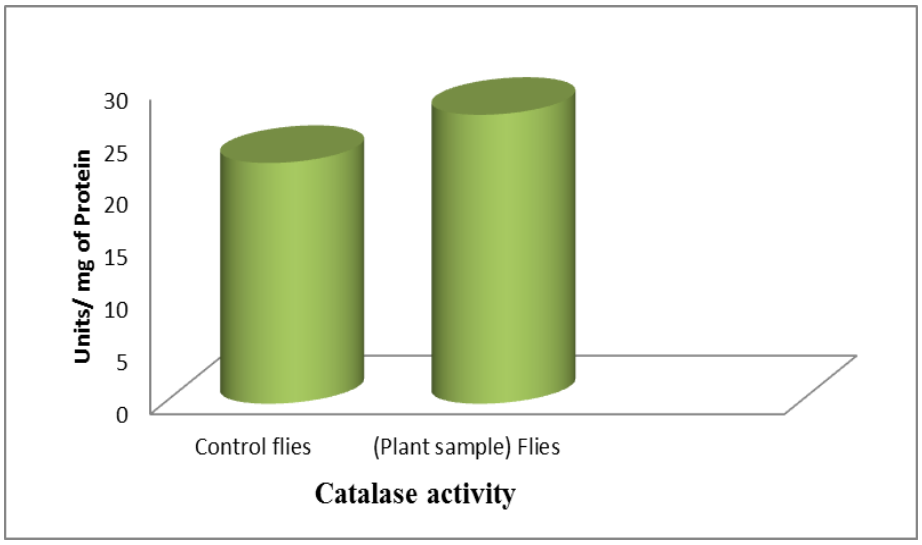

Figure 8. CAT activity of the fruit flies in Control (First group) and plant sample (Fourth group) comparative study.

Table 2. Variation of enzyme activity in normal *D. melanogaster* (First group) and the flies treated only with plant sample (Fourth group).

	SOD activity in units/mg of protein	Catalase activity in units/mg of protein	Gpx activity in units/mg of protein
Control flies	0.52	23	5.1
Flies treated with plant sample	0.48	27.6	4.9

Discussion

Stress is defined as a condition that disturbs the normal function of the biological system or a condition that decreases fitness. It was a physical or psychological stimulus that can produce mental tension or physiological reactions that may lead to illness. It is a well-known fact that stress of any nature produces a

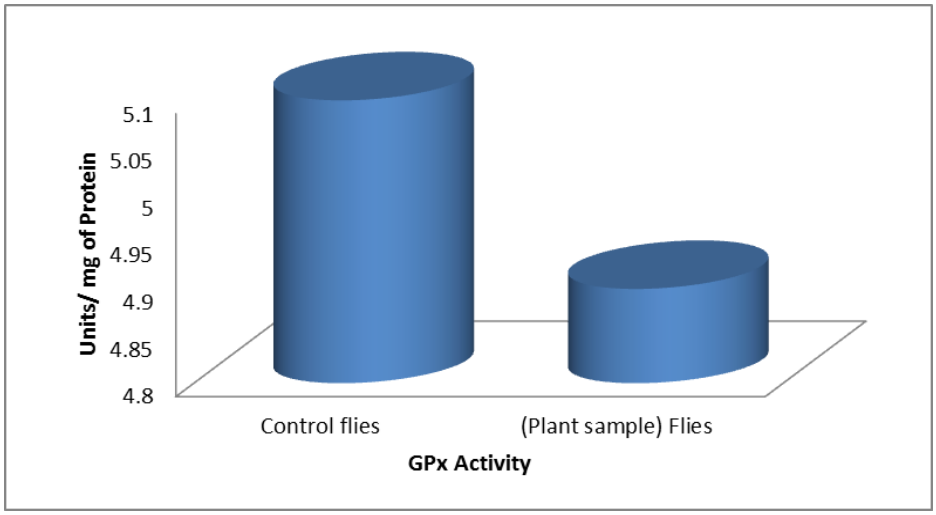

Figure 9. GPx activity of the fruit flies in Control (First group) and plant sample (Fourth group) comparative study.

non-specific state in the organism, *i.e*., the state of stress or "stress syndrome", which was characterized by adrenal hypertrophy, depletion of adrenal ascorbic acid and cortisol, and a decrease in the size of lymphoid tissue (Singh *et al*., 1978). Any damaging or potentially damaging stimulus (stressor) besides having its own specific effects induces the secretion of adrenal corticosteroids and catecholamines, cardiovascular alterations, and gastrointestinal lesions. The change observed in the stress syndrome has been explained on the basis of activation of hypothalamo-hypophysealadrenal axis. The corticoids, thus released, help animals in combating stressful situation (Selye, 1938).

Oxidative stress is a condition characterized by elevated levels of intracellular ROS. Either are, or break down to form, free radicals. ROS include superoxide anion (O2-), singlet oxygen (O2), hydroxyl radical (OH-), and hydrogen peroxide (H_2O_2), that are capable of reacting with, and damaging not only DNA, even proteins, and lipids as well (Jan Vijg, 2007).

The results of the current study demonstrated an increase in the activity of the stress related marker enzymes in stress-induced fruit flies viz., SOD, which dismutases the highly reactive superoxide anion to the less reactive species H_2O_2 (Chelikani *et al*., 2004), CAT, a haeme containing enzyme, and GPx, which scavenges hydrogen peroxide or tert-butyl hydroperoxide into water and molecular oxygen (Christine *et al*., 2010). Under normal conditions, ROS are cleared from the cell by the actions of SOD, CAT and GPx. low level of intracellular ROS have been identified as second messengers in signaling pathways and implicated in transcriptional regulation to promote cell growth, but higher doses of ROS result in growth arrest and cell death. Oxidative damage to proteins plays a crucial role in ageing, because oxidized proteins lose catalytic function and are preferentially hydrolyzed (Jan Vijg, 2007).

The activity of SOD, CAT, and GPx increases significantly in a concentration dependent manner after inducing stress by MTX. One possible reason is that the stress inducing agent MTX an anticancerous drug, which acts by inhibiting the metabolism of folic acid where it is needed for the *de novo* synthesis of nucleocides, may cause drug induced oxidative stress and much ROS is produced. In order to antagonize ROS, the level of the defensive enzymes such as CAT, SOD, and GPx increases under stressful condition (Figures 1, 2, 3) (Maehly and Chance, 1954). Similar result of increase in the antioxidant enzyme levels in the medium fed with same concentration of MTX is obtained by Deepthi and Sathish (2011). There are considerable studies on free radical mediated changes to biological systems due to stress created by surrounding environment. Environmental stress causes generation of free radicals in higher concentration that would cause irreversible mutations in DNA. But, however, the expression of these defective genes can be buffered by the action of Hsp90 a molecular chaperone (Queitsch *et al*., 2002; Rutherford, 2003). The action of chaperones camouflages the adverse effect of polymorphic variants or accumulated somatic mutations that

would normally result in protein folding defects. Although Hsp90 is highly abundant and can be further induced by heat stress, it can be overwhelmed when more and more proteins are destabilized when oxidative stress is more as a part of aerobic life and metabolism under stress (Jan Vijg, 2007).

As per our results, the plant powder used in this study is found to reduce the stress induced by MTX. This was confirmed by comparing the activity of antioxidant enzymes in second group of flies with that of third group flies containing both MTX as well as 0.5 g of plant sample (Figures 4, 5, and 6; Table-1). Similarly, *Convolvulus pluricaulis*, *Glycyrrhiza glabra*, and *Rauwolfia serpentine* have reduced the level of antioxidant enzymes in stress-induced fruit flies treated with respective plant sample in different concentrations (Arun and Sathish, 2010; Sowmya and Sathish, 2010; Deepthi and Sathish, 2011). The antioxidant enzyme activity in the third group of flies was found to be decreased slightly, indicating that the plant sample is effective in suppressing the stress. Herbs are not second best to "chemical medicine" in helping to fight stress. On the contrary, herbs are much more intelligent, have many more components that all work together, and of course, they have evolved together with our likewise immensely complicated human bodies, side by side.

In fourth group the flies were reared in the media containing only 0.5 gm of plant sample where the specific activity of the SOD and GPx is less than that of control (Figures 7 and 9). The hypothetical reason behind this is may be the plant molecules are reducing the level of ROS much less to that of normal level which in turn reduces the antioxidant enzyme levels. Whereas the fruit flies reared in the medium containing *Convolvulus pluricaulis* and *Glycyrrhiza glabra* the activity of SOD in control and fourth group of flies were almost similar to that of control (Arun and Sathish, 2010; Sowmya and Sathish, 2010).

In the current study specific activities of CAT in both control and fourth group flies were almost the same. But the specific activity of CAT has decreased in flies reared on medium containing *Glycyrrhiza glabra* and there is a slight increase in CAT activity in the flies with *Convolvulus pluricaulis* to that of control flies (Arun, and Sathish, 2010; Sowmya and Sathish, 2010). Especially when it comes to reducing the effects of stress on the body - which is so more than "just a chemical reaction"! - herbs are a perfect solution to reduce stress related build ups of toxins, to calm the overactive mind, to help break down adrenaline, to strengthen the heart and breathing systems, all of which are under attack by ongoing stress (Wang *et al*., 2005).

The stress induction in *Drosophila* was confirmed by the increased activity of cellular defensive enzymes like SOD, CAT, and GPx. As per the results plant powder is found to reduce the stress-induced by MTX in fruit flies. This may open up a new avenue of research in identifying the plants which possess anti-stress property and exploiting its action by using *D. melanogaster* as model organism.

Conclusion

The plant sample used as an anti-stress agent can be used to combat stress related disorders. The anti-stress property was confirmed by employing the SOD, CAT, and GPx activity assay, compared to the stressor induced group. The stressor group treated with plant sample showed decreases in the level of ROS thereby reducing antioxidant enzyme activity. Thus, *Myristrica fragrans* (japatrae) tends to balance between ROS and a variety of enzyme systems that can deactivate ROS, thereby it aids in improving and maintaining the health of *D. melanogaster* even under stressful conditions. This experiment has a profound implication for the broad scope of applications of anti-stress molecules to humans before which fruit flies can be used as models to study its power of action.

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Occurrence of the genus *Zygothrica* (Diptera, Drosophilidae) in a high-altitude forest in northeastern Brazil.

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Introduction

The genus *Zygothrica* includes drosophilids native to the Neotropical region, apart from some species found in Africa and in the Indo-Pacific region (Prigent and Toda, 2006). From the ecological standpoint, representatives of this genus have traditionally been associated with fungi (Malogolowkin, 1952; Grimaldi, 1987, 1990), though some species use flowers as food resources (Grimaldi, 1987).

In the Neotropical region *Zygothrica* is the second genus in the Drosophilidae family in terms of diversity, after *Drosophila* (Bächli, 2014). The taxonomic history of the genus dates back to 1830, when C.R.W. Wiedemann described the type species *Zygothrica*, *Z. dispar* (Wiedemann 1830), as a subgenus of *Achias* (Platystomatidae). Almost one hundred years later, in a pioneering description of several *Zygothrica* species, Sturtevant (1920) enlarged the taxonomic knowledge on this genus. Following this line of study, Duda (1952) and Burla (1956) collected specimens in Costa Rica and in Brazil, respectively, and described a large number of species in the genus. Subsequently, new species were later described and reviewed by Grimaldi (1987, 1990). Today, *Zygothrica* comprises 124 species (Bächli, 2014), 54 of which occur in Brazil (Gottschalk *et al.*, 2008). In spite of that, Grimaldi (1987) believes that only half of the total number of estimated *Zygothrica* species has been described.

In Brazil, representatives of the *Zygothrica* genus have been increasingly captured in recent years (De Toni *et al.*, 2007; Döge *et al.*, 2007; Gottschalk *et al.*, 2007; Schmitz *et al.*, 2007; Döge *et al.*, 2008; Mata *et al.*, 2008; Gottschalk *et al.*, 2009; Bizzo *et al.*, 2010; Hochmüller *et al.*, 2010; Garcia *et al.*, 2012; Poppe *et al.*, 2012; Roque *et al.*, 2013; Poppe *et al.*, 2014). However, in some of the country's regions, such as the northeast, there is a paucity of information on the genus (Gottschalk *et al.*, 2008). In northeastern Brazil, north of the São Francisco River, two subregions of the Atlantic Forest were outlined, *Pernambuco* and *Brejos de Altitude*. The latter is characterized by wet forest islands surrounded by *Caatinga*, a semiarid biome. These areas are located in plateaus between 500 and 1,000 m above sea level, where orographic rainfall ensures