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SUPPRESSION OF ELEVATED REACTIVE OXYGEN SPECIES BY *ACORUS CALAMUS* (VACHA) A SWEET FLAG IN *DROSOPHILA MELANOGASTER* UNDER STRESS FULL CONDITIONS

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Keywords:

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ABSTRACT: Reactive Oxygen Species is a phrase used to describe a number of reactive molecules and free radicals derived from molecular Oxygen. The production of Oxygen based radicals is the bane to all aerobic species where its excessive levels intern induces oxidative stress. The present study is to evaluate the anti-stress property of *Acorus calamus (vacha*), where the fruit flies in different groups are assayed for Stress related marker enzymes like SOD, CAT, and GPx. Where its activity in Stress induced flies (MTX treated) has increased compared to that of control flies. After incorporation of the plant sample there is reduction in level of these defensive enzymes there by vacha has increased the ability to scavenge ROS lowering the free radical concentration and reducing the expression of stress related marker enzymes in the stress induced flies. Thus, *vacha* may have anti-stress property i.e ability to reduce the level of ROS.

INTRODUCTION: Reactive oxygen species (ROS) are generated in all aerobic cellular metabolic process.

They include singlet oxygen, superoxide, and hydroxyl radicals [1]. But they are not limited only to these which also includes hydrogen peroxide, Peroxyl radicals, Peroxynitrite which reacts with various intracellular targets, viz., lipids, proteins and DNA [1, 2].

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Although ROS are generated during normal aerobic metabolism in all aerobic organisms from prokaryotic organisms to highly evolved Mammals, the biological effects of ROS on these intracellular targets are dependent on their concentration and increased levels of these species are present during oxidative stress [3]. Increased levels of ROS are cytotoxic and can also result in ROS-induced damage including cell death, mutations, chromosomal aberrations and carcinogenesis [2]. While lower levels are necessary for the regulation of several key physiological mechanisms including cell differentiation, apoptosis, cell proliferation and regulation of redox sensitive signal transduction pathways [4,5]. Detoxification of ROS is paramount to the survival of all aerobic life forms. As such a number of defense mechanisms have evolved to meet this need and provide a balance between

production and removal of ROS. An imbalance toward the pro-oxidative state is often referred to as "Oxidative stress" [6].

Elevated amount of ROS leads to a condition of Oxidative stress. Such type of induction was done by rearing *D. melanogaster* in medium containing MTX. It formerly known as amethopterin is an antimetabolite and antifolate drug [7]. Methotrexate acts specifically during DNA and RNA synthesis, and thus it is cytotoxic during the S-phase of the cell cycle [8]. And may cause drug induce oxidative stress and much ROS is produced [9]. Some of the less reactive of these species (such as superoxide) can be converted by oxidoreduction reactions with transition metals or other redox cycling compounds (including quinones) into more aggressive radical species that can cause extensive cellular damage [10]. The major portion of long term effects is inflicted by damage on DNA [11]. Most of these oxygen-derived species are produced at a low level by normal aerobic metabolism and the damage they cause to cells is constantly repaired. However, under the severe levels of oxidative stress that cause necrosis, the damage causes ATP depletion, preventing controlled apoptotic death and causing the cell to simply fall apart [12, 13]. 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 which antagonizes this ROS [1]. Superoxide dismutase (SOD) catalyzes the conversion of two superoxide anions into a molecule of H_2O_2 and O_2. Thus, they are an important antioxidant defense in nearly all cells exposed to oxygen.

Similarly CAT also catalyzes the decomposition of hydrogen peroxide to water and oxygen. Thus, completes the detoxification initiated by SOD [14]. It is a very important enzyme in reproductive reactions. CAT has one of the highest turnover numbers of all enzymes; one Catalase molecule can convert millions of molecules of hydrogen peroxide to water and oxygen each second [15]. It is concentrated in peroxisomes of eukaryotic cells [16]. GPx is a group of enzymes containing selenium with peroxidase activity whose main biological role is to protect the organism from oxidative damage, where it reduces lipid hydroperoxides and organic peroxides to their co responding alcohols and to reduce free hydrogen peroxide to water [6].

Acorus calamus, commonly known as Sweet Flag or Calamus [17]. And is a tall perennial wetland monocot of the Acoraceae family. Calamus has many traditional uses. Native Americans used the plant to soothe toothaches and headaches. Chinese medicine used the plant in treatment of deafness, epilepsy and vertigo. Ayurvedic medicine considers Calamus to be a rejuvenator of nervous system. It eases digestion and enhances the production of stomach juices. Calamus extract is beneficial in cases of asthma, bronchitis, coughs, but also anorexia, intestinal colic, gastritic ulcers and gastritis. Sweet flag has a very long history of medicinal use in Chinese and Indian herbal traditions. It is widely employed in modern herbal medicine as its sedative, laxative, diuretic, and carminative properties [18]. Therefore, a rational approach was made to evaluate its anti-stress properties in stress induced flies.

In the present study, we have cultured *D. melanogaster* in different groups where in first group fruit flies are reared on a flour-based medium as well as in the medium containing MTX in different concentrations (second group) to induce the stress in the flies, and in third group flies were reared on medium containing both plant sample as well as MTX. And the flies were cultured in the media containing only plant sample (*Acorus calamus*) in fourth group. The enzymatic assay of SOD, CAT and GPx were done for all the group of flies and comparative study was carried out to investigate the ability of the plant sample in reducing stress in flies.

The purpose/objective of the present study is to evaluate the anti-stress property of *Acorus calamus* 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 avenue of research in a search of plants to combat against oxidative stress.

MATERIALS AND METHODS:

(i) **Culturing of fruit flies [19]:** 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 $26^oC \pm 1^oC$. 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.

(ii) **Stress induction and reduction study:** Any alteration in the food creates an environmental stress in an organism. MTX an anti-cancerous and hepatotoxic drug that induces oxidative stress in fruit flies is used to induce the stress by the method described earlier [20]. To investigate stress reducing action by plant sample, 0.5g of vacha is added to third and fourth group of flies. Later the stress induction and reduction parameters were found by the estimating antioxidant defense enzymes in every group of flies.

(iii) **Enzyme collection:** All four groups of flies were taken in different eppendorf tubes (4 flies in each tube). They were homogenized in a 200µl of 250mM phosphate buffer (pH 7.8), 50 mM (pH 7) & 0.4M (pH 7) for SOD, CAT, & GPx assay respectively, and are centrifuged at 8000 rpm for 20 min in a cooling microfuge. 100µl of this supernatant serves as enzyme source for SOD, CAT and GPx enzymatic assays.

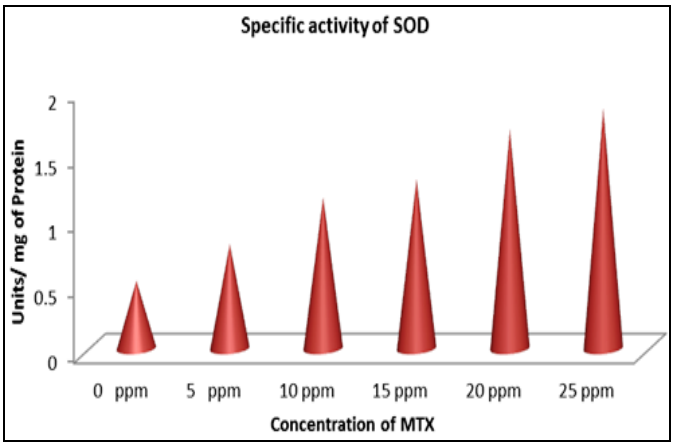
(iv) **Assay of SOD activity:** SOD enzyme (EC.1.15.1.1) is assayed by the method described earlier [20]. Using a slightly modified procedure originally described by Beauchamp and Fridovich (1971) [21]. Protein estimation is done by method described by Lowry O.H (1951) [22]. Specific activity is expressed in Units/mg of protein.

(v) **Assay of CAT activity:** CAT enzyme (EC.1.11.1.6) is assayed by the method described earlier [23]. 0.1ml of crude Enzyme extract is mixed with 2.9ml of 30% of hydrogen peroxide. Decrease in the absorbance at A240nm indicates the action of CAT on H_2O_2. Protein estimation is done by method described by Lowry O.H (1951) [22]. Specific activity is expressed in units/mg of protein.

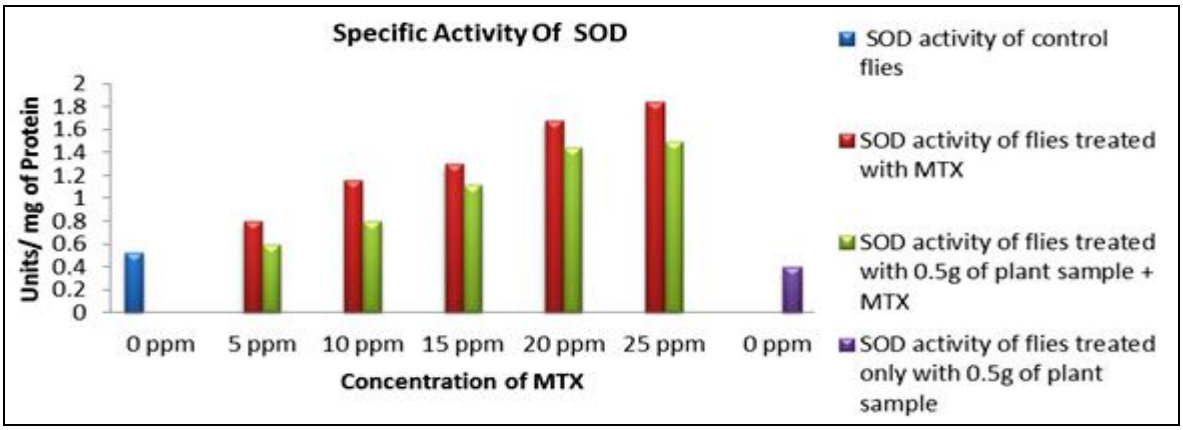
(vi) **Assay of GPx activity:** GPx (EC.1.11.1.9) is assayed according to the method described earlier [24] with minor modifications. The reaction mixture consisting of 0.4 ml of 0.4 M sodium phosphate buffer (pH 7.0), 0.1 ml of 10mM sodium azide, 0.2 ml of 4 mM reduced glutathione, 0.1 ml of 2.5 mM H_2O_2, 0.2ml of water and 0.1 ml of enzyme is incubated at 37°c for 15 min. The reaction is terminated with 0.5 ml of 10% TCA and after centrifugation, 2 ml of the supernatant is added to 3 ml of phosphate buffer and 1ml of DTNB(5,5-dithiobis 2-nitrobenzoic acid) reagent (0.04% DTNB in 1% sodium citrate). The color developed is read at 412 nm and the activity is calculated by the determining the amount of glutathione utilized. Protein estimation is done by method described by Lowry O.H (1951) [22]. Specific activity is expressed in units/mg of protein.

RESULTS:

1. **Change in physiology of SOD activity:** Culturing of flies in culture media along with MTX - a stress inducer increased the activity of SOD – stress marker enzyme. The activity is further increased in increase concentration of MTX from 5ppm to 25ppm in the flies in concentration dependent manner when compared to that of control flies "**Graph 1" & "Table 1**". There is a considerable decrease in the activity of SOD in third group of flies by the introduction of 0.5g of plant sample "**Graph 2**" & "Table 1". The activity is further reduced in fourth group of flies over the second group, "Graph 2".



GRAPH 1: ACTIVITY OF SOD INCREASES GRADUALLY IN THE FLIES (SECOND GROUP) REARED ON MEDIA CONTAINING INCREASED CONCENTRATION OF MTX

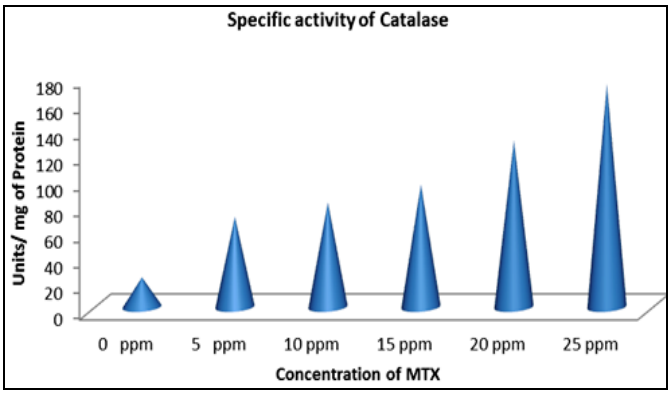


GRAPH 2: ACTIVITY OF SOD INCREASES GRADUALLY IN FLIES REARED ON MEDIA CONTAINING INCREASING CONCENTRATION OF MTX (SECOND GROUP) AND REDUCTION OF ACTIVITY IN THE PRESENCE OF 0.5g OF PLANT SAMPLE (THIRD GROUP).

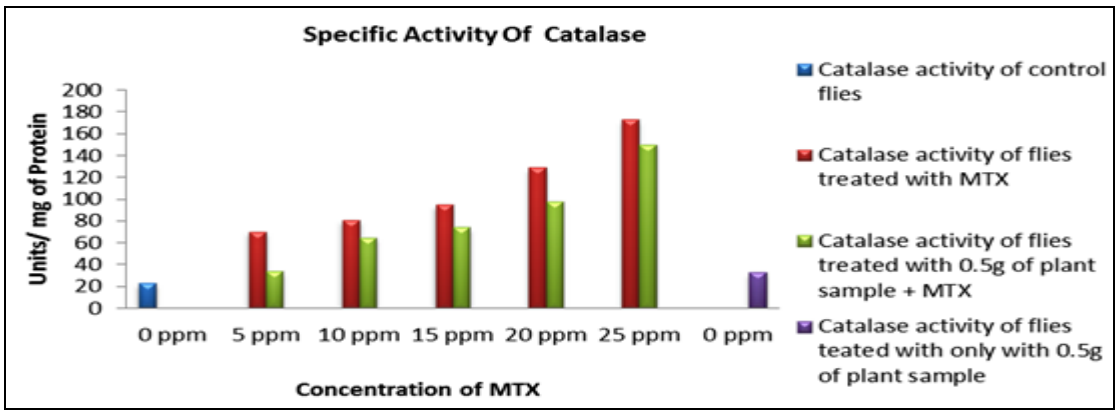
TABLE 1: COMPARISON TABLE OF SOD, CAT AND GPx ACTIVITY OF STRESS INDUCED FLIES (SECOND GROUP) AND MTX+0.5 gmOF PLANT SAMPLE AT DIFFERENT CONCENTRATIONS (THIRD GROUP)

	Concentration of MTX	5ppm	10ppm	15ppm	20ppm	25ppm
CAT	**MTX Alone**	70	81	95	129	173
	MTX + Plant sample	34	65	74	98	150
SOD	**MTX Alone**	0.80	1.16	1.3	1.68	1.84
	MTX + Plant sample	0.60	0.80	1.12	1.44	1.50
GPx	**MTX Alone**	8.36	9.81	10.68	12.31	12.64
	MTX + Plant sample	6.5	7.3	8.2	11.12	11.48

2. **Change in physiology of CAT activity:** Similar to SOD the CAT enzyme activity also got increased in second group of flies. And its activity is further increased in increase concentration of MTX from 5ppm to 25ppm in the culture media in a concentration dependent manner compared to that of control flies "**Graph 3**" & "Table 1". When 0.5 g of plant sample is added along with MTX of different concentration there is a considerable decrease in the activity of CAT in third group of flies "**Graph 4**" & "Table 1". The activity is further reduced in fourth group of flies compared to that of second group "Graph 4".

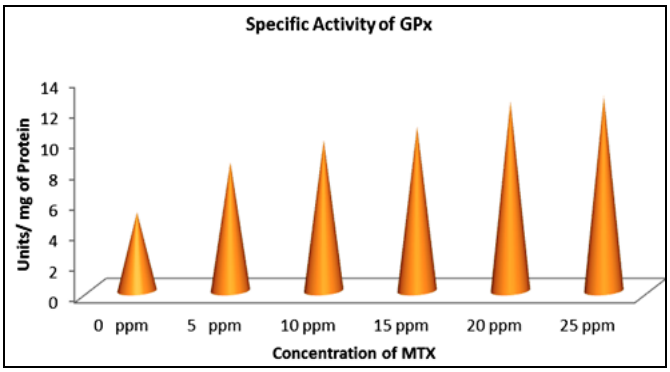


GRAPH 3: ACTIVITY OF CAT INCREASES GRADUALLY IN FLIES (SECOND GROUP) REARED ON MEDIA CONTAINING INCREASING CONCENTRATION OF MTX

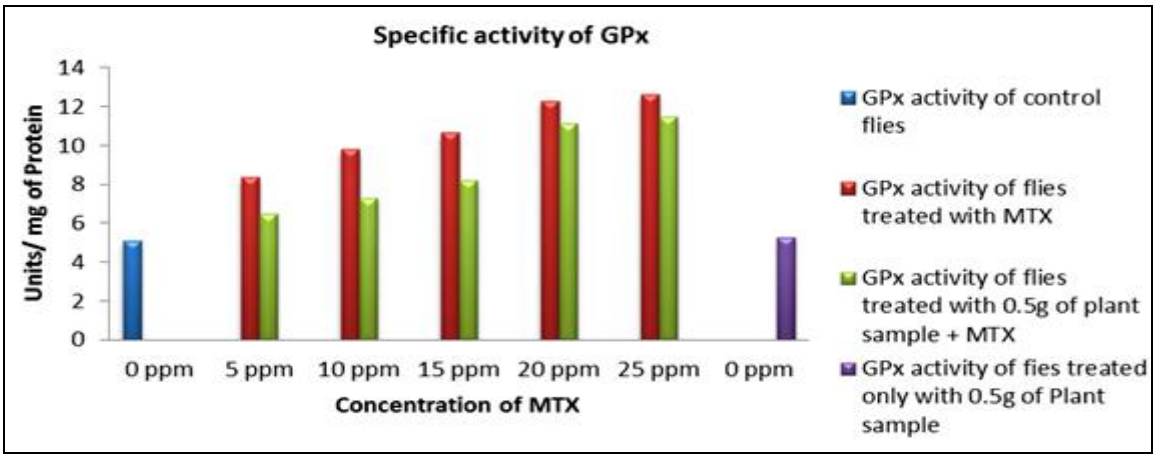


GRAPH 4: ACTIVITY OF CAT INCREASES GRADUALLY IN FLIES REARED ON MEDIA CONTAINING INCREASING CONCENTRATION OF MTX (SECOND GROUP) AND REDUCTION OF ACTIVITY IN THE PRESENCE OF 0.5G OF PLANT SAMPLE (THIRD GROUP)

3. **Change in physiology of GPx activity:** Along with SOD and CAT the GPx enzyme activity got increased in second group of flies. The activity is further increased successively as the concentration of MTX is increased from 5ppm to 25ppm in culture media in a concentration dependent manner when compared to that of control flies "**Graph 5**" & "Table 1". Decrease in the activity of GPx can be observed in the third group of flies by the addition of 0.5 g of plant sample "**Graph 6**" & "Table 1". The activity is further reduced in fourth group of flies compared to that of second group "Graph 6".



GRAPH 5: ACTIVITY OF GPX INCREASES GRADUALLY IN FLIES (SECOND GROUP) REARED ON MEDIA CONTAINING INCREASING CONCENTRATION OF MTX

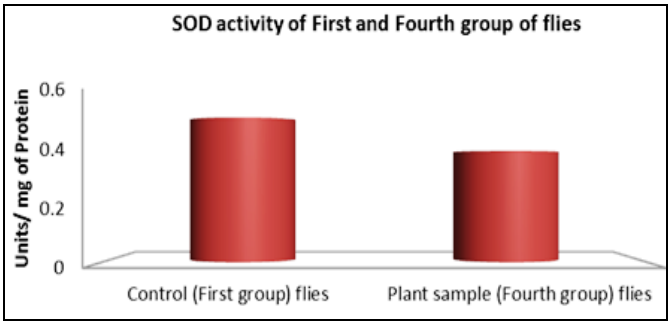


GRAPH 6: ACTIVITY OF GPX INCREASES GRADUALLY IN FLIES REARED ON MEDIA CONTAINING INCREASING CONCENTRATION OF MTX (SECOND GROUP) AND REDUCTION OF ACTIVITY IN THE PRESENCE OF 0.5G OF PLANT SAMPLE (THIRD GROUP)

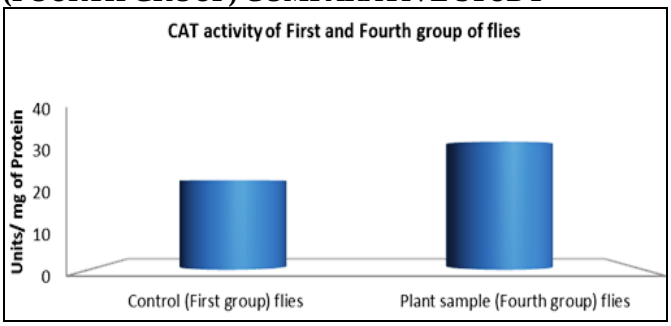
4. **Variation of enzyme activity in normal flies treated with plant sample alone:** Comparative study of the fruit flies grown in the media containing only 0.5gm of plant sample with that of control is carried out. The enzyme activity is different in the fourth group of flies reared on the media containing only 0.5 gm of the plant sample "**Table 2**".

   There is decreased SOD activity in the fourth group flies grown on medium containing only 0.5g of Vacha over the control flies "**Graph 7**", and the activity of CAT and GPx is found to be decreased in the same flies grown on medium containing only 0.5g of japatrae to that of control flies "**Graph 8, 9**" and "Table 2".

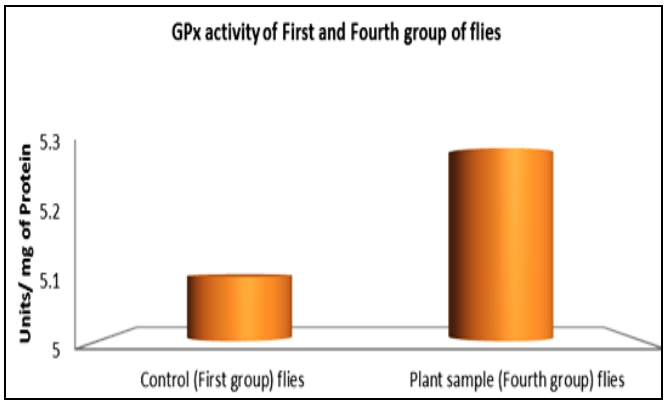
   From the above result, shows that SOD, CAT, and GPx activity is increased when MTX is added and the plant sample is found to reduce the stress.



GRAPH 7: SOD ACTIVITY IN FRUIT FLIES OF CONTROL (FIRST GROUP) AND PLANT SAMPLE (FOURTH GROUP) COMPARATIVE STUDY



GRAPH 8: CATALASE ACTIVITY IN FRUIT FLIES OF CONTROL (FIRST GROUP) AND PLANT SAMPLE (FOURTH GROUP) COMPARATIVE STUDY



GRAPH 9: GPX ACTIVITY IN FRUIT FLIES OF CONTROL (FIRST GROUP) AND PLANT SAMPLE (FOURTH GROUP) COMPARATIVE STUDY

TABLE 2: VARIATION OF ENZYME ACTIVITY IN NORMAL *D. MELANOGASTER* AND THE FLIES TREATED WITH PLANT SAMPLE

	SOD activity in units/mg of protein	CAT activity in units/mg of protein	GPx activity in units/mg of protein
Control (1st group)	0.52	23	5.1
Plant sample (4th group)	0.40	32.9	5.3

DISCUSSIONS: Stress is defined as a physical or psychological stimulus that can produce mental tension or physiological reactions that may lead to illness [25]. Origin of stress may vary but its effect is deleterious. It is a condition or circumstance which can disturb the normal physiological and psychological functioning of an individual. It is a well-known fact that stress of any nature produces a non-specific state in the organism i.e. the state of stress or "stress syndrome" which is characterized by adrenal hypertrophy, depletion of adrenal ascorbic acid and cortisol and a decrease in the size of lymphoid tissue [26, 27].

An imbalance between antioxidant defense and ROS results in oxidative stress, leading to cellular damage. 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 [1]. The results of the current study demonstrated increase in the activity of the stress related marker enzymes in stress induced flies.

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 [28].

Oxidative damage to proteins plays a crucial role in ageing because oxidized proteins lose catalytic function and are preferentially hydrolyzed. It is hypothesized that oxidative damage to specific proteins constitutes one of the mechanisms linking oxidative stress damage and age-associated losses in physiological functions [29].

Cells have a variety of defense mechanisms to ameliorate the harmful effects of ROS. Superoxide dismutase (SOD) catalyzes the conversion of two superoxide anions into a molecule of hydrogen peroxide (H_2O_2) and oxygen (O_2) [14, 30]. In the peroxisomes of eukaryotic cells, the enzyme Catalase converts H_2O_2 to water and oxygen, and thus completes the detoxification initiated by SOD. GPx is a group of enzymes containing selenium, which uses Glutathione to catalyze the degradation of hydrogen peroxide, as well as organic peroxides (lipid peroxides) to alcohols [6]. In addition to this Cells can able to defend themselves using Guaiacol peroxidase, Ascorbate peroxidase against ROS damages.

The activity of SOD, CAT and GPx increases significantly in a concentration dependent manner after inducing stress by MTX "Graph 1, 3, 5" & "Table 1". 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 nucleosides may cause drug induced oxidative stress and much ROS is produced [9].

Similarly Increased activity levels in Arginase, GPx, CAT and SOD enzyme levels was observed on prolonged ammonia exposure in kidney tissues of fish due to oxidative stress [31]. Oxidative stress has been implicated as the main causal factor in aging and antioxidant defense is therefore considered critically important in longevity assurance [1]. In order to antagonize the elevated level of ROS, the level of the defensive enzymes such as CAT, SOD and GPx also got increased

under stressful condition to protect the organism from drug induced oxidative stress "Graph 1, 3, 5". 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) [32].

MTX has a greater toxic effect on rapidly dividing cells (such as malignant and myeloid cells, and gastrointestinal and oral mucosa), which replicate their DNA more frequently, and thus inhibits the growth and proliferation of these noncancerous cells, as well as causing several side effects. Facing a scarcity of dTMP, rapidly dividing cancerous cells undergo cell death via thymine less death [8]. Thus MTX causes drug induced oxidative stress. Oxidative stress is the steady state level of oxidative damage in a cell, tissues or organ caused by the ROS. Oxidative modification of nucleic acids by ROS is of remarkable biological importance, as it results in the transformation of nonmalignant cells into malignant ones [33]. Oxidative stress has been causally linked to many diseases, including cancer, atherosclerosis, ischemic injury, inflammation, and neurodegenerative diseases, such as Parkinson's disease and Alzheimer's disease [1]. There are a number of non-enzymatic small molecule antioxidants that play a role in detoxification. Glutathione may be the most important intra-cellular defense against the deleterious effects of ROS. Other small molecule antioxidants such as Ascorbic acid (vitamin C), Tocopherol (vit E), Uric acid, and β-carotene also play important roles as cellular antioxidants [1]. Similarly, polyphenol antioxidants assist in preventing ROS damages by scavenging [34].

In current herbal drug scenario, plant derived antioxidants are gaining importance because of their potential health benefits, no toxicity and side effects over synthetic antioxidants like butyl hydroxy anisole and butyl hydroxy toluene (BHA and BHT, respectively) [35].

As per our results, the plant powder (*Vacha*) used in this study is found to reduce the stress induced by MTX. This is 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 0.5g of plant sample "Graph 2, 4, 6 "and "Table 1".

Similarly *Convolvulus pluricaulis* [20], *Glycyrrhiza glabra* [36], and *Rauwolfia serpentine* [32] has reduced the level of antioxidant enzymes in stress induced fruit flies treated with respective plant sample in different concentrations.

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 is less to that of control "Graph 7". The hypothetical reason behind this is may be the plant molecules are reducing the level of ROS much lesser to that of normal level which intern reduced the antioxidant enzyme levels. Whereas the fruit flies reared in the medium containing *Convolvulus pluricaulis* [20] and *Glycyrrhiza glabra* [36], the activity of SOD in control and fourth group of flies were almost similar to that of control.

In the current study, specific activity of CAT and GPx has increased slightly in fourth group flies over the control flies "Graph 8, 9" & "Table 2" which may indicate the antioxidant and free radical scavenging property of *Vacha*. Slight increase in the level of defensive enzymes within the normal range aid in better scavenging of ROS and also it indicates the healthiness of fruit flies. But the specific activity of CAT has decreased in flies reared on medium containing *Glycyrrhiza glabra* [36] and there is a slight increase in CAT activity in the flies with *Convolvulus pluricaulis* [20] to that of control flies.

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 [37].

The stress induction in the Drosophila was confirmed by the increase 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 here may be used to combat stress related disorders. The anti- stress property (Reduction of elevated ROS) 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 decreased the level of ROS thereby reducing antioxidant enzyme activity. Thus, *Acorus calamus* (*Vacha*) tends to balance between ROS and a variety of enzyme system that can deactivate ROS, thereby it aids in improving and maintaining the health of *D. melanogaster* even under stressful conditions. This experiment have 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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