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RESEARCH ARTICLE

Oxidative Stress-Induced Toxicity and DNA Stability in Some Agri-field Based Livestock/Insect by Widely Used Pesticides

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Abstract: ***Aim and Objective*****:** Humans continuously use pesticides in the field to control the pest population and weeds for considerable agricultural productivity. Side-by species like grazing-animals, insects and other species are adversely affected by or become resistant to pesticides. Insects, birds and cattle are highly abundant dwellers of the agriculture-field and represent three distinct phyla having versatile physiological features. Besides higher agricultural-productivity, protection to several species will maintain ecological/environmental balance. Studies on the effect of widely used pesticides on their DNA-stability and important enzymatic-activities are insufficient.

Materials and Methods**:** Antioxidant-activity (Superoxide-dismutase; SOD/Catalase- by gel-zymogram-assay) and DNA-stability (fragmentation-assay) in hepatic/gut tissues were studied after *in-vitro* exposure of Chlorpyrifos, Fenvalerate, Nimbecidine or Azadirachtin to goat/cow/poultry-hen/insect.

Results**:** In general, all pesticides were found to impair enzymatic-activities. However, lower organisms were affected more than higher vertebrates by azadirachtin-treatment. DNA fragmentation was found more in insects/poultry-birds than that of the cattle in hepatic/gut tissues. Inversely, toxicity/antioxidant marker-enzymes were more responsive in insect gut-tissues. However, mitochondrialtoxicity revealed variable effects on different species. It has been noticed that chlorpyrifos is the most toxic pesticide, followed by Fenvalerate/Nimbecidine (Azadirachtin, AZT). Nevertheless, AZT revealed its higher DNA-destabilizing effects on the field-insects as compared to the other animals.

Conclusion**:** Field-insects are highly integrated into the ecosystem and the local bio-geo-chemical cycle, which may be impaired. Pesticides may have toxic effects on higher vertebrates and may sustain in the soil after being metabolized into their different derivatives. Some of the sensitive biochemical parameters of this organism may be used as a biomarker for pesticide toxicity.

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1. INTRODUCTION

The liver is the largest organ in the vertebrate body and it is the major site of xenobiotics metabolism. Hepatic injury is a common pathological outcome, which exists in many liver diseases. Environmental toxicants have adverse effects on a large community of vertebrates; mammals and non-mammals. Chemical fertilizers and pesticides are used for plant growth promotion; high-yield and protecting crops, which may have adverse effects on agri-field consumers and humans also. Cows*(Bos taurus)*, goats (*Capra aegagrus hircus*) and poultry birds such as hen (*Gallus gallus domesticus*) have been recognized as the most effective livestock for promoting health and economy, worldwide. These animals are predominantly common in the agriculture field and consume those products.

Pesticides have adverse effects on animal and human health [1]. Chemical pesticides are classified as organophosphates, organochlorines, carbamates, *etc* [2]. These substances mostly remain un-metabolized compounds in the ecosystem. The toxicity of pesticides is categorized in relation to their ability to produce lipid peroxidation, to impair antioxidant

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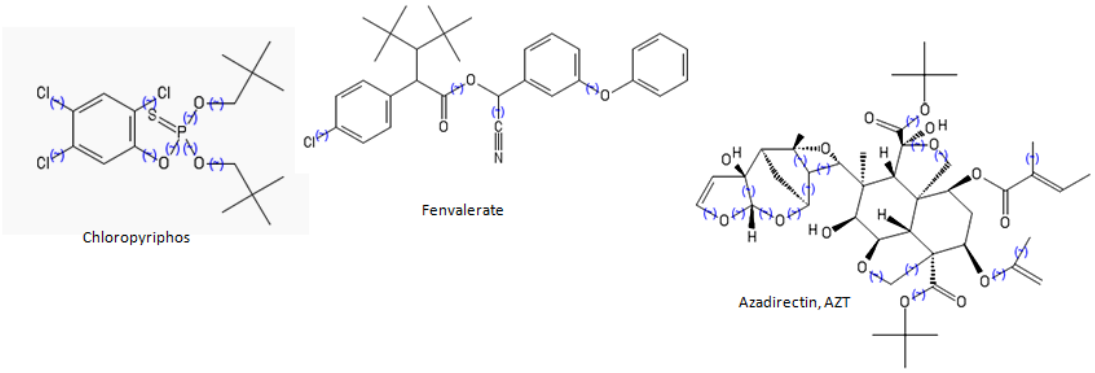

Fig. (1). Chemical structure of Chlorpyrifos, Fenvalerate (TATAfen) and Nimbecidine (Azadiractin, AZT).

status, mitochondrial/DNA stability. Stimulation of free-radical production, induction of lipid-peroxidation and disruption of the antioxidant components by pesticides may develop toxicity in the living system. Reactive oxygen species (ROS) are the main causative agents for oxidative stress, which cause several diseases in human, like Alzheimer's, diabetes, cancer *etc*. [3,4]. In many cases, ROS production is an integral part of the pathophysiological mechanism that helps in damaging major macromolecular and cytoskeletal structures. Degradation in the DNA structure and the mitochondrial membrane instability drastically damage the cellular structure. Necrotic and/or apoptotic death of the cells is the result of the toxicity [4]. Moreover, the mechanism of toxicity and its impact depend on the complexity of the exposed organisms. The complexity of the organism depends on its systematic position in the phylogenetic tree. There is an impact of evolution on the adaptive nature of the organism.

In the current study, four different living systems were selected from distinct phylogenetic positions. All are highly abundant agri-field dwellers and consumers. Therefore, the toxicity generated by pesticides on their metabolic system/organ will be due to their differences in physiological characteristics and systematic positions in animal kingdom. Sustained exposure of pesticides may affect animal health making them more sensitive to environmental-factors and especially the effects on agricultural field-insect are more detrimental. The purpose of this investigation is to provide a brief analysis of the toxicity effects of several pesticides on some common cattle, birds and insects and their comparative analysis. Biochemical/molecular parameters are evaluated here, which may represent their biomarker potentials. Further investigations may be designed to establish some toxicity indicator species.

2. MATERIALS AND METHODS

2.1. Pesticides used in this Study and their Chemistry (Fig. 1)

The chemical structures were drawn by the NCBI software PubChem Sketcher V2.4 utilizing the web address https://pubchem.ncbi.nlm.nih.gov/edit3/index.html

Chlorpyrifos: *O*,*O*-Diethyl *O*-3,5,6-trichloropyridin-2-yl phosphorothioate($C_9H_{11}Cl_3NO_3PS$),molar mass;350.57g·mol^{-1}, density; 1.398 gcm^{-3} (43.5°C), solubility in water; 2 mg/L, chemical nature; combustible, reacts strongly with amines, strong acids, caustics (Fig. **1**).

Fenvalerate: (*RS*)-*alpha*-Cyano-3-phenoxybenzyl(RS)-2-(4-chlorophenyl)-3-methylbutyrate ($C_{25}H_{22}ClNO_3$), molar mass; 350.57 g·mol^{-1}, density; 1.175 gcm^{-3}, solubility in water; 2 µg/L, chemical nature; fenvalerate is most toxic to bees and fish. It is found in some emulsifiable concentrates, ULV, soluble powders, slow-release drug formulations, insecticidal fogs and in granules (Fig. **1**).

Nimbecidine: Azadirachtin AZT; Dimethyl (2a*R*,3*S*,4*S*,*R*, *S*,7a*S*,8*S*,10*R*,10a*S*,10b*R*)-10-(acetyloxy)-3,5-dihydroxy-4-[(1*S*,2*S*,6*S*,8*S*,9*R*,11*S*)-2-hydroxy-11-methyl-5,7,10-trioxatetra cyclo[6.3.1.0^{2,6}.0^{9,11}]dodec-3-en-9-yl]-4-methyl-8-{[(2*E*)-2-methylbut-2-enoyl]oxy}octahydro-1*H*-furo[3',4':4,4a]naphth o[1,8-*bc*]furan-5,10a(8*H*)-dicarboxylate($C_{35}H_{44}O_{16}$); molar mass; 720.72 g·mol^{-1}, chemical nature; this compound is found in the seeds (0.2 to 0.8 percent by weight) of the neem tree, *Azadirachta indica* (Fig. **1**).

2.2. Animal Tissue

Fresh liver tissue of adult specimens from common healthy livestock's goat (*Capra aegagrus hircus*) and cow (*Bos taurus* and *Bos indicas*), poultry bird as hen (*Gallus gallus domesticus*) were collected from the local Govt. registered market. Those were kept in plastic containers in the cold room at the temperature of about 4°C in darkness and experiments were immediately performed with the permission of the Institutional Ethical Review Board.

2.3. *In vitro* Treatment of Animal Tissues

Fresh liver tissues from different livestock's were incubated (for three hrs at room temperature) with different dilutions (w/v) of Chlorpyrifos such as 0.20%, 0.27%, 0.40%, 0.80%, 4.00%, or Fenvalerate 0.08%, 0.10%, 0.13%, 0.20%, 0.40% or Nimbecidine (Azadirachtin) 100 ppm, 133 ppm, 200 ppm, 400 ppm. 2000 ppm.The control marked dishes were treated with the vehicle. The biochemical studies were carried out with the experimental tissues after the incubation period.

2.4. *In vivo* Treatment of Insect Tissues

To determine LC_{50}, serial concentrations of AZT (Azadirachtin$C_{35}H_{44}O_{16}$, 1 ppm, 5 ppm, 10 ppm, 15 ppm, 20 ppm) were applied uniformly and separately to 25 grams of food plants placed in water containing conical flask in plastic

jars (11×11×26) cm covered with mosquito net fixed by rubber bands at room temperature. Then, same-aged male and female and male (1:1) insects (Orthoptera: Acridoidea) from newly hatched adults were placed in each jar and covered. Four replicates were used for each concentration (including control) and all jars were kept at 26±1ºC and 70±5% relative humidity. Alive insects from each treatment were dissected and fat-bodies free guts were taken separately from female and male insects and stored in -20ºC until use.

2.5. Preparation of Tissue Homogenate

Immediately after the treatment period, livers of decapitated livestock and gut tissues from insects were taken, weighed, cut into pieces and homogenized in 0.1 M chilled phosphate buffer, pH 7.4. The volume of the buffer was adjusted to obtain a 20% (w/v) homogenate. The homogenization was performed with the use of the Teflon homogenizer of the Potter-Elvehjem type. Next, the homogenates were centrifuged at 10,000xg for 30 min at 4°C in order to obtain supernatants, which were used to measure the enzyme activities.

2.6. Assay of Total Protein Contents

The total protein content of different tissues was estimated by the standard method of Lowry *et al.* 1951 [5]. Protein concentration was calculated and expressed as µg/mg wet tissue.

2.7. Determination of Antioxidant Enzymes

For the antioxidant status of SOD, CAT activity was determined using gel zymography.

2.7.1. Assay of Catalase Activities by Gel Zymography

The non-denaturing (8%) acrylamide gel was washed with distilled water for 10 minutes containing 25 µg proteins. The gel was shaken in 100 ml distilled water with 100 µl. H_2O_2 for 10 minutes and then it was washed with distilled water for 5 minutes. The control similar gel was stained with 30 ml, 2% Ferric Chloride ($FeCl_3$) and 30 ml 2% Potassium Ferricyanide [$K_3Fe(CN)_6$]. It was poured onto the gel at the same time. When the gel becomes yellowish green then the stain solution was removed. Finally, the gel was washed with distilled water and enzyme-activity bands appeared on the gel [6].

2.7.2. Assay of Super Oxide Dismutase Activities by Gel Zymography

SOD activity was performed according to the method described by Christine and Joseph, 2010 [7]. Supernatant from tissue homogenate was electrophoresed in riboflavin gel at 4ºC. To visualize SOD activity, gels were first incubated in 2.4 mM nitro blue tetrazolium (NBT) in deionized water for 15 min and then in 0.028 mM riboflavin/280 mM N,N,N′,N′-tetramethyl-ethylenediamine (TEMED) in 50 mM potassium phosphate buffer (pH 7.8) for 15 min in the darkroom. After washing, the gel was illuminated under fluorescent light to get a distinct SOD activity band.

All the zymogram gel bands were analyzed by ImajeJ software and the data was used for statistical analysis.

2.8. DNA Fragmentation Analysis

Liver tissues and gut tissues were treated with 500 µl of lysis buffer (50 mM Tris pH 8.0, 20 mM EDTA, 10 mM NaCl, 1% SDS) and centrifuged at 12,000×g for 30 min. The supernatant was extracted with a 1:1 mixture of phenol: chloroform and then precipitated in two equivalence of cold ethanol and one-tenth equivalence of sodium acetate. After spinning down and decantation, the precipitate was re-suspended in TE buffer and 5 µl of loading buffer. The 0.8% agarose gel was run and the band density was evaluated by the gel documentation system and the percentage of DNA stability was expressed as bar diagram [8]. A suitable DNA ladder (EZ Load 500 bp Molecular Ruler #1708354, Life Sciences, Bio-Rad) was also run to assume the damage pattern.

2.9. Mitochondrial Membrane Stability Assay by Fluorescence Microscopy

Mitochondria from the drug added liver tissues of cow, goat and poultry bird-hen were isolated and incubated at 37°C in a medium composed of 5 mM K_2HPO_4, and 5mM $MgCl_2$, 135 mM KCl, 20 mM MOPS, at pH 7.0. Incubations also contained Rhodamine123 (R123) [9]. R123 and all other chemicals were from Sigma Chemical Co. (St. Louis, MO). Fluorescent measurements of mitochondria and extracts were made using a fluorescence microscope (Nikon, Eclipse LV100 POL), with the VisComet (Impulse Bildanalyse) software.

2.10. Statistical Analysis

Statistical analysis with the raw data was performed in SPSS 17 to evaluate the level of significance and differences between the two groups (Student's t-test). One way ANOVA was performed to characterize the significance of differences between and within groups of cattle and insects or different drug-dose treated groups. Values in the bar diagrams are the mean ± SE from several independent experiments (3 to 5).

Levels of significances (P values) are presented as the SPSS software calculates it. $P<0.05$ or less than this value has been considered to be significant in the current experiment.

3. RESULTS AND DISCUSSION

In the current study, the catalase activity in goat liver decreased after in vitro incubation with pesticides. Besides, this inhibition was prominent in the case of chlorpyrifos (Fig. **2a**) and AZT (Fig. **2c**). Nevertheless, no significant alteration was noticed in response to fenvalerate treatment (Fig. **2b**). The activity of superoxide dismutase (SOD) in goat liver distinctly decreased after chlorpyrifos intoxication (Fig. **2f**). However, the statistical calculation did not present significant changes. However, in the case of gut tissues in the present experimental insect, both catalase and SOD activities increased after in vivo and *in vitro* AZT exposure, which is found to be highly significant (Fig. **2d** and **e**; Fig. **2i**

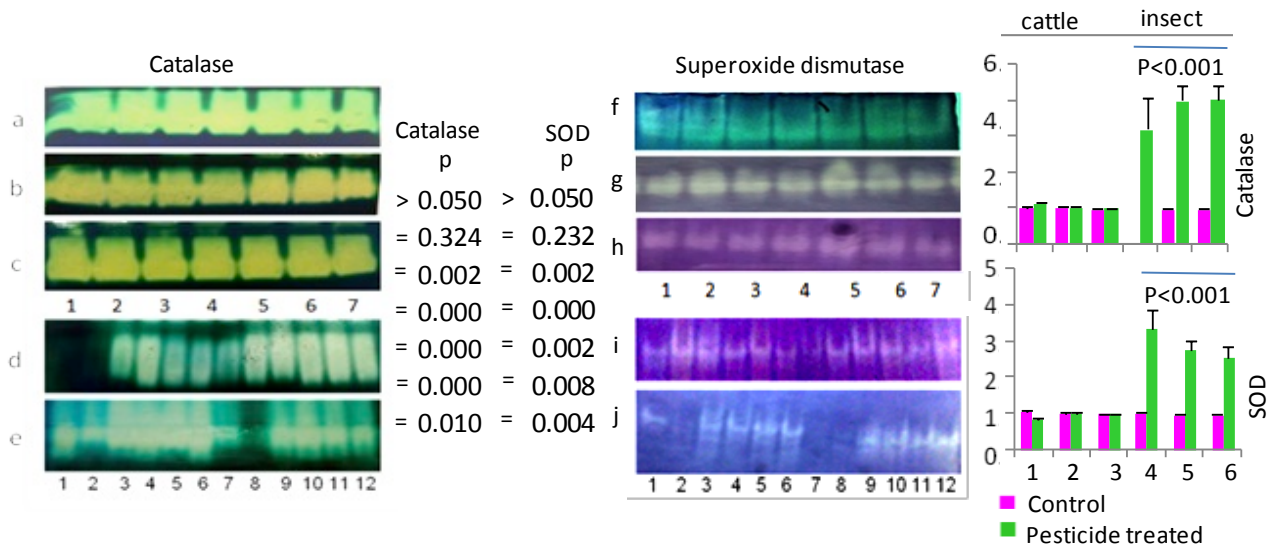

Fig. (2). Left panel: Goat (*Capra aegagrus hircus*) Liver (treated with Chlorpyrifos) catalase activity in gel zymogram. **-gel a-** Lane (1- 2 control, 3- 0.20 %, 4- 0.27 %, 5- 0.40 %, 6- 0.80 %. 7- 4.00 %) **gel b-** Catalase activity in Goat Liver (treated with Fenvalerate) : Lane (1- 2 control, 3- 0.08 %, 4- 0.10 %, 5- 0.13 %, 6- 0.20 %. 7- 0.40 %) **gel c-** Catalase activity in Goat Liver (treated with Nimbecidine) Lane (1- 2 control, 3- 100 ppm, 4- 133 ppm, 5- 200 ppm, 6- 400 ppm. 7- 2000 ppm) **gel d-** Catalase activity is shown in the gut of the insect in vivo treated with Nimbecidine. Lane distribution: Lane 1- 6 female gut (1- control, 2- 1 ppm, 3- 5 ppm, 4- 10 ppm, 5- 15 ppm, 6- 20 ppm.) and Lane 7- 12 male gut (7- control, 8- 1 ppm, 9- 5 ppm, 10- 10 ppm, 11- 15 ppm, 12- 20 ppm.) **gel e-** Catalase activity is shown in the gut of the insect in vitro treated with Nimbicidine. Lane distribution: Lane 1- 6 female gut (1- control, 2- 1 ppm, 3- 5 ppm, 4- 10 ppm, 5- 15 ppm, 6- 20 ppm.) and Lane 7- 12 male gut (7- control, 8- 1 ppm, 9- 5 ppm, 10- 10 ppm, 11- 15 ppm, 12- 20 ppm). Right panel: SOD activity is shown on a polyacrylamide gel- lane distribution in **gel f, g, h, i** and **j** are similar to gel a, b,c, d and e in the right panel respectively. Data in the last panel is the mean ± SE. Statistical data-P values of comparison between each drug-dose group of cattle and insects. Right most figure of bar diagram comparison of all drugs together in cattle versus insect. Statistical data shows that insects are more influenced and affected by pesticide exposure. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

and **j**). Moreover, the extent of increase of enzymatic activities with comparison to control is higher in female insect (lane 1-6) than that of male insects (lane 7-12). Small or negligible changes are noticed in Fig. (**2g)** and (**2h)**. This may suggest that these pesticides were less effective in the livestock and birds to generate radical toxicity, so the adaptive responses were less prominent. However, in the case of insects, the toxicity and the adaptive responses both were significant. Oxidative stress has been shown to play a crucial role in the cellular toxicity generated by a large number of pesticides [10]. Free radicals especially reactive oxygen species (ROS) are essential for different metabolic functions including the cell signaling and other physiological processes [11]. The role of AZT has been already reported as a potential inducer of mitochondrial superoxide anion radicals and H_2O_2 [12]. This has been clearly noticed in our present study.

The impairment in antioxidant defense mechanism is evident by the cellular damage caused by pesticides [13]. This has been reflected in the mitochondrial membrane degeneration studies (Fig. **4**). This degeneration is responsible to develop a free radical cascade possibly at the cytosolic level. Plasma membrane and organelle membrane stability is a determinant of cytotoxicity. Earlier it has been suggested that altered membrane fluidity can be responsible for the decreased activity of pesticide in mouse [14] and in the experimental chicken model [15]. Therefore, the pesticide may impair the physiological and biochemical processes across the species and that may be influenced by cellular physiology. The catalase activity was impaired in the experimental organisms after exposure to different concentrations of pesticides *i.e.*, Chlorpyrifos, Fenvalerate, Nimbicidine. It might be due to the binding of the pesticide residue to catalase or by inhibiting this enzyme synthesis [16]. An appreciable level of decrease in SOD activity was noticed in the liver of cattle (Fig. **2f** and **2g**). Nevertheless, a significant dose-dependent increase of SOD and catalase activity was noticed after both in vitro and in vivo AZT exposure to the insect of both sexes (bar diagram in the right panel of Fig. **2**). This suggests that antioxidant enzymes are more responsive (P<0.001) in environmental stress in the lower organisms and it may be selected as a biomarker in a variety of oxidative stress. A significant decrease in catalase activity was observed in the brain, liver and kidney tissues of *Channa punctatus* exposed to a different pesticide, triazophos [17]. Tripathi and Singh (2013) [18] also noticed that the catalase activity reduced in the brain, gill, liver and skeletal muscles of α-methrin treated *Channa punctatus*. The degree of effects of Chlorpyrifos, Fenvalerate and Nimbicidine on catalase and other protein profile was variable in different livestock-tissues because it may depend on the location and metabolic involvement of the tissues [19]. Tissue-specific antioxidative responses were noticed to take place during stress exposure.

In the present study, we have established that pesticides influence antioxidative enzymes in the liver of higher organisms and gut of the insects and, therefore, liver injury

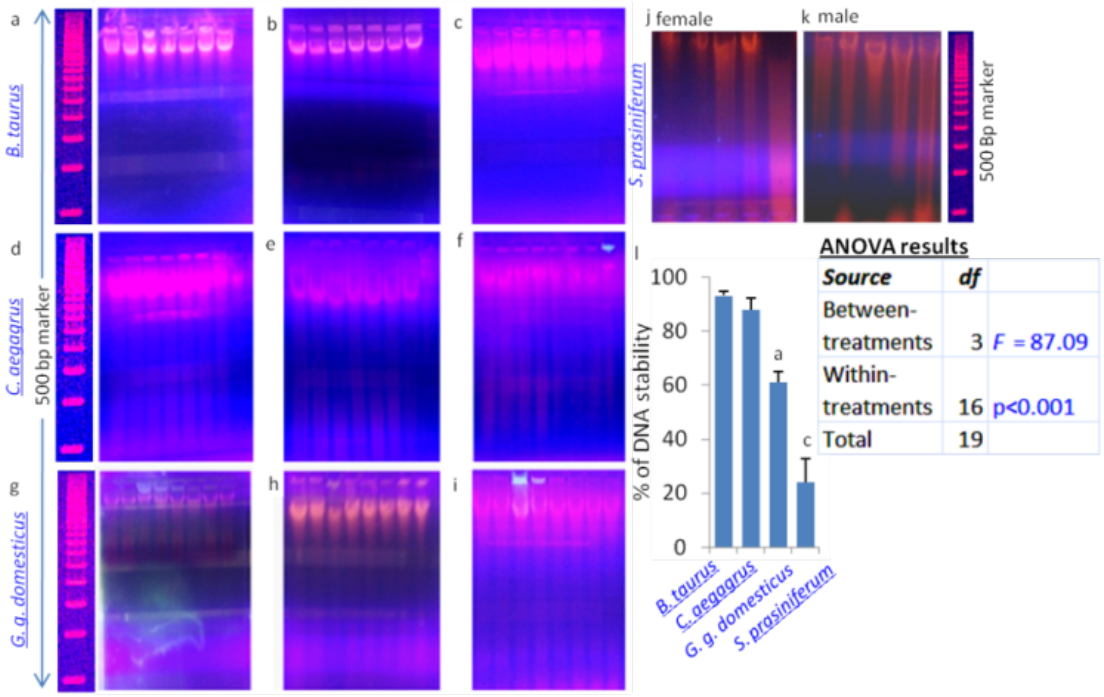

Fig. (3). (a, b, c) DNA fragmentation result is shown in the liver of Cow *(Bos taurus*) *in vitro* treated with Chlorpyrifos (a). Lane (1- 2 control, 3- 0.20 %, 4- 0.27 %, 5- 0.40 %, 6- 0.80 %. 7- 4.00 %); Flavaralate (b). Lane 1- 7 liver tissue (1- 2 control, 3- 0.08 %, 4- 0.10 %, 5- 0.13 %, 6- 0.20 %. 7- 0.40 %); Nimbecidine (AZT) (c). Lane (1- 2 control, 3- 100 ppm, 4- 133 ppm, 5- 200 ppm, 6- 400 ppm. 7- 2000 ppm). **(d, e, f)** DNA fragmentation result is shown in the liver of goat (*Capra aegagrus hircus*) *in vitro* treated with Chlorpyrifos (d). Lane (1- 2 control, 3- 0.20 %, 4- 0.27 %, 5- 0.40 %, 6- 0.80 %. 7- 4.00 %). Fenvalerate (e). Lane: (1- 2 control, 3- 0.08 %, 4- 0.10 %, 5- 0.13 %, 6- 0.20 %. 7- 0.40 %). Nimbecidine (AZT) (f). Lane (1- 2 control, 3- 100 ppm, 4- 133 ppm, 5- 200 ppm, 6- 400 ppm. 7- 2000 ppm). **(g, h, i)** DNA fragmentation result is shown in the liver of poultry bird, hen (*Gallus gallus domesticus*) in vitro treated with Chlorpyrifos (g). Lane (1- 2 control, 3- 0.20 %, 4- 0.27 %, 5- 0.40 %, 6- 0.80 %, 7- 4.00 %). Fenvalerate (h). Lane (1- 2 control, 3- 0.08 %, 4- 0.10 %, 5- 0.13 %, 6- 0.20 %. 7- 0.40 %). Nimbecidine (i). Lane (1- 2 control, 3- 100 ppm, 4- 133 ppm, 5- 200 ppm, 6- 400 ppm. 7- 2000 ppm). DNA fragmentation result is shown in in vivo gut treated with Nimbecidine (AZT) **(j)** female Lane (1- control, 2- 1 ppm, 3- 5 ppm, 4- 10 ppm, 5- 15 ppm) **(k)** male gut (1- control, 2- 1 ppm, 3- 5 ppm, 4- 10 ppm, 5- 15 ppm). Bar diagram results (5 independent experiments) and Students t-test values suggest that insects are the most affected and then the birds' group in terms of their DNA stability. Level of significances are shown; **a.** P<0.05 and **c.** P<0.001. ANOVA result suggests that between and within groups, the differences in the DNA stability are significantly different at P<0.001(F = 87.09). *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

associated with this insecticide may be due to oxidative tissue damage. There is little evidence of change in the antioxidant system after intoxication with organophosphate to agri-field dweller organisms. Hai *et al.* (1997) [20] observed an increase of SOD and catalase activities in the liver of dichlorvos-treated carps. They also found elevated levels of malondialdehyde in the livers of dichlorvos (DDVP)-treated fish. Fish is the organism of the aquatic ecosystem and exposure *via* contamination in water might have an instant effect. The mechanism of intoxication might suggest the pattern of toxicity in our present experimental organism. Yang *et al.* (1996) [21] suggested that the organophosphates and carbamates, besides their inhibitory effect on acetylcholinesterase, initiate the accumulation of free radicals leading to lipid peroxidation on such organism. Mitochondrial stability and function have not been observed in those previous experiments. In the current investigation, we evaluated mitochondrial membrane potential in cattle and birds and considered that pesticides have strong destabilizing activity. Due to the very low yield of mitochondria, the signal was undetectable in the insect. Nevertheless, direct evidence of antioxidative responses (catalase, SOD) and DNA instability was noticed in the insect after AZT exposure.

Antioxidant protection is accomplished by many enzymatic and non-enzymatic factors, which maintain the physiological level of reactive oxygen metabolites [22]. The antioxidant defense system includes superoxide dismutase, catalase and glutathione peroxidase enzymes. We measured the activity of two antioxidative enzymes, SOD and catalase. The SOD plays an important role in the first line of the antioxidant defense system by catalyzing the dismutation of superoxide radicals to form hydrogen peroxide and molecular oxygen. Further, this hydrogen peroxide undergoes a free-radical cascade reacting with transition metals or lipid-peroxidation products [23].

In the present study, a significant DNA laddering was found in the experimental insect induced by AZT (Fig. **3j** and **3k**). A moderate DNA laddering was also noticed in the poultry bird (3g, 3h and 3i for three pesticides). Nevertheless, in the higher vertebrate, DNA was found to be more stable (Fig. **3g, 3h** to **3i**). The pesticide treatment induces DNA fragmentation which is one of the criteria of necrotic or apoptotic cell death. This finding would be related to the magnitude of the exposure to pesticides and was directly involved in DNA fragmentation. Therefore, the major changes observed in DNA fragmentation of the liver

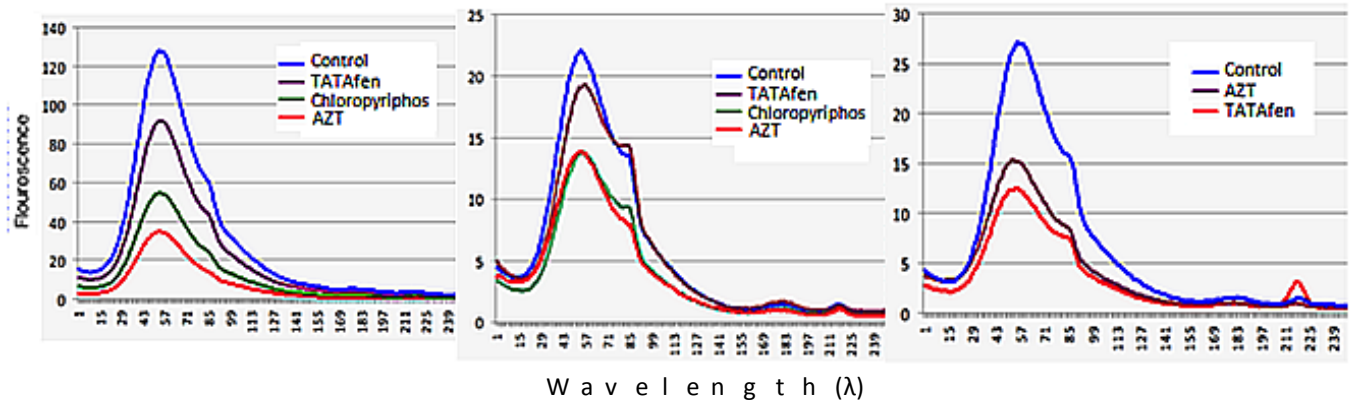

Fig. (4). Mitochondrial membrane potential/stability assays (Rhodamine method) by fluorescence microscopy. Depending on the sample availability, this experiment was conducted. Left panel: cow, middle panel: goat and right panel: poultry bird. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

may suggest a direct effect of the pesticides. The percent of DNA stability calculated from the densitometry analysis data (Fig. **3i**) suggests that cattle DNA is more protected than the poultry bird DNA damage ($P<0.05$) and insect DNA damage ($P<0.001$). Fungicide-induced DNA damages were caused by oxidative stress and might be responsible for the higher occurrence of apoptotic cell death [24]. Similar to our present study, the electrophoretic analysis confirmed the potential role of epoxiconazole in inducing DNA fragmented ladder that is regarded as the marker of apoptosis [25].

The present study was conducted to investigate the pesticides in alleviating oxidative stress in liver injury and DNA fragmentation. The liver is the site of biotransformation by which a toxic compound is transformed into a less harmful form to reduce toxicity [26]. However, during these processing hepatic cells may itself be damaged to some extent and develop chronic hepatotoxicity. Pesticides induce oxidative stress, which leads to the generation of free radicals, changes in antioxidants levels and lipid peroxidation [27] thus causing damage to proteins, lipids and DNA [28]. The report reveals that dimethoate induced DNA damage in the hemocytes of the insect *C. biguttulus* [29]. Some critical analyses have been shown earlier in *Drosophila* sp., but not in the present experimental grasshopper species. Dichlorvos (DDVP), an organophosphate pesticide was reported to induce DNA damage and also affect pre and post replication repair mechanisms in *Drosophila* sp. [30]. The normal error rectification processes were thus distorted and a certain degree of mutagenicity was generated at the level of biotransformation by the application of the pesticides.

DNA fragmentation observed in the present study has a very wider significance. It is the normal consequence of oxidative stress that was demonstrated through the inactivation of antioxidant enzymes (catalase and SOD) metabolic organs. This is also consistent with previous studies where DNA fragmentation was induced by pesticides in rat lymphocytes [31] and in rat brain by cypermethrin [32]. Not during the single generation period, even maternal pesticide (fenvalerate) exposure during pregnancy-period may impair growth and brain development in mouse offspring [33]. Hampering in the brain behavior is shown to trigger Parkinson-like symptoms through the initiation of autophagy and p38 MAPK/mTOR signaling pathway [34]. Moreover, interferences by fenvalerate in the expression of pro-apoptotic Bcl-2 family proteins increase the carcinogenicity potential of this pesticide [35]. The differences in the DNA stability of different organisms may be due to differences in DNA sequences between lineages, which noticed in our current study. These facts influence the stability, mutability and error-correction efficiency of the DNA. In lower organisms, the DNA was found to be less stable in response to xenobiotic/pesticide exposure (Fig. **3j** and **3k**). The fluorescent experiment by Rhodamine 123(R123) shows a notable decline in membrane potentials in any pesticides treated liver tissues compared to that of control groups of all species (Fig. **4**). R123 is one of the most dependable signaling molecules to estimate mitochondrial damage and apoptotic signaling. Fenvalerate (TATAfen) is noticed here to be the most damaging to mitochondrial structure (Fig. **4**). Mitochondrial membrane damage may generate a significant toxicity cascade and energy depletion in the cell and in the whole organism.

The insects originated between the Cambrian period (Euarthropoda, 535 MY) and the Devonian period (385 MY). During this period and further, during the long tenure of speciation, insects have gone through less diversification (Fig. **5**). Therefore, the adaptive modification and DNA stabilization are less likely to happen here. In contrast, Aves originated 135 MY ago in the first part of the Cretaceous period and went through stages of more diversification (Fig. **5**). Comparatively, less DNA fragmentation is noticed in *G. g. domesticus*, the poultry hen (Fig. **3g**, **3h** and **3i**). The report reveals that in prokaryotes like bacteria, environmental stress and other 'mutants' have influenced the selection pressure. Nevertheless, those have lower DNA repair efficiency, hence a higher mutation rate [36]. In cattle, very less amount of DNA-fragmentation is noticed (Fig. **3a** to Fig. **3f**). Extensive diversification and adaptive modification are noticed in Mammals, which originated in the Cenozoic period, 65 MY. Moreover, these mammals diverged in rodents, primates, cattle and others in a very short (Cenozoic) time (Fig. **5**). Adaptation in higher eukaryote may be governed more by natural selection-pressure than mutation associated forced error on DNA sequences/stability. Even species can differ in rate of copy

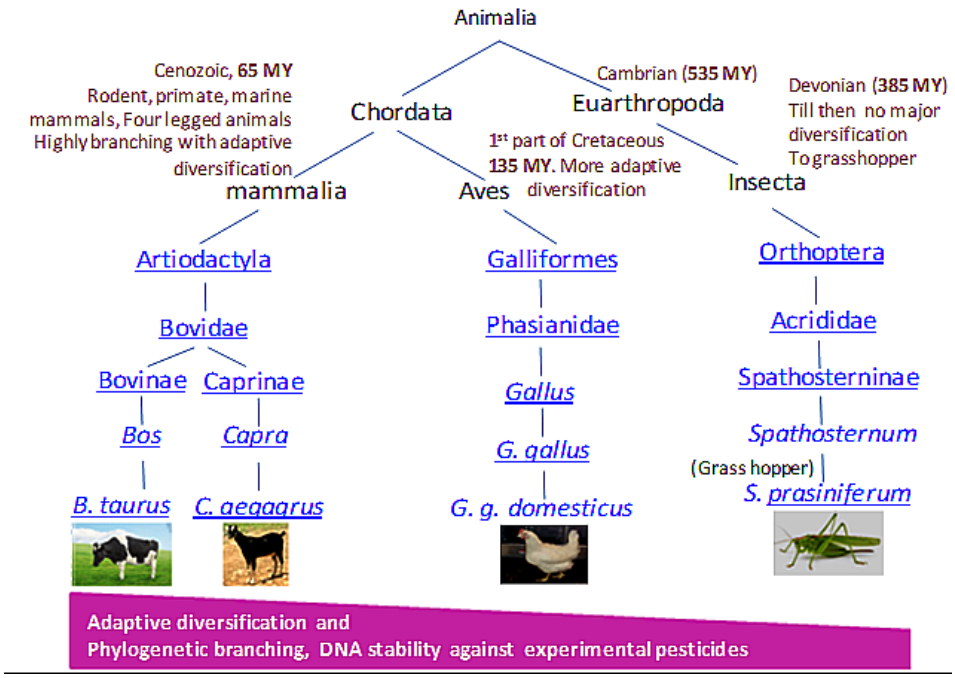

Fig. (5). Respective position and lineage diversification of the four living systems investigated in the current study; cow, goat, poultry-hen and an insect-grasshopper. DNA stability has been shown to be related to the relative phylogenetic position of the organism. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

error detection and repair. Number of DNA replications per generation can also vary with population structure and mating system [37]. Beside the toxicity and adaptation studies, and their comparative analysis in different living system, there may have some wider messages in our study. This work may resemble a reference model of environmental stress and its ecological/evolutionary impact dictated by the xenobiotic-interfered DNA stability. As for example, Daphnia hemoglobin (Hb) has been established as the widely recognized respiratory pigment. As a reliable biomarker, it can predict the water contamination with bisphenol A (BPA), benzo[a]pyrene (B[a]P), chlorpyrifos [38]. Azadirachtin has been shown to suppress the growth and development of *Bactrocera dorsalis* by releasing cathepsin [39] and induce apoptosis in *Bombyx mori* by releasing extra Ca^{2+} [40]. Lower organisms are more social and actively participate in the formation of a composite niche/ecosystem. Therefore, any kind of changes or deterioration in the ecosystem may affect their fecundity, reproductive ability, life span and mortality. The changes in their DNA structure/stability may modulate their phenotypic behavior and their interaction with neighboring organisms.

CONCLUSION

From the present study, we can infer that pesticide intoxication may induce oxidative stress more in the lower organisms. However, the effect of subchronic exposure of pesticides on livestock leading to oxidative stress requires further evaluation at the molecular level. After an acute exposure, the risk assessment should be performed to quantify the exposure of humans to that particular contamination. For a sustained or chronic exposure, the risk assessment is important at a community level with a focus on its long-term ecological and environmental impact.

LIST OF ABBREVIATIONS

AZT	=	azadirachtin
CAT	=	catalase
DDVP	=	dichlorvos
ppm	=	parts per million
ROS	=	reactive oxygen species
SOD	=	superoxide dismutase

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

For all animal experiments, proper permissions were obtained from the Institutional (Oriental Institute of Science and Technology) Review Board.

HUMAN AND ANIMAL RIGHTS

No humans were used for studies that are the basis of this research. All the animal experiment procedures followed were in accordance with the standards set forth in the eighth edition of "Guide for the Care and Use of Laboratory Animals" (grants.nih.gov/grants/olaw/guide-for-the-care-and-use-of-laboratory-animals_prepub.pdfpublished by the National Academy of Sciences, The National Academies Press, Washington, D.C.).

All ethical norms and maintain requisite regulatory affairs. The firm house is a Government accredited (CPCSEA-Committee for the Purpose of Control and Supervision of Experiments on Animals: Reg. no 1A2A/PO/BT/S/15/CPCSEA. <http://cpcsea.nic.in/Auth/index.aspx>) organization under the Dept. of Animal Husbandry and Dairy, Ministry of Agriculture and Farmer's Welfare, Govt. of India.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

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AVAILABILITY OF DATA AND MATERIALS

The dataset used and/or analyzed during the current study are available from the corresponding author [SM] on a reasonable request.

CONFLICT OF INTEREST

None declared.

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REFERENCES

[1] al-Saleh, I.A. Pesticides: a review article. *J. Environ. Pathol. Toxicol. Oncol.,* **1994**, *13*(3), 151-161. PMID: 7722882

[2] Pajoumand, A.; Jalali, N.; Abdollahi, M.; Shadnia, S. Survival following severe aluminum phosphide poisoning. *J. Pharm. Pract. Res.,* **2002**, *32*, 297-299. http://dx.doi.org/10.1002/jppr2002324297

[3] Butterfield, D.A.; Lauderback, C.M. Lipid peroxidation and protein oxidation in Alzheimer's disease brain: potential causes and consequences involving amyloid beta-peptide-associated free radical oxidative stress. *Free Radic. Biol. Med.,* **2002**, *32*(11), 1050-1060. http://dx.doi.org/10.1016/S0891-5849(02)00794-3 PMID: 12031889

[4] Zarkovic, N. 4-hydroxynonenal as a bioactive marker of pathophysiological processes. *Mol. Aspects Med.,* **2003**, *24*(4-5), 281-291. http://dx.doi.org/10.1016/S0098-2997(03)00023-2 PMID: 12893006

[5] Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; Randall, R.J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.,* **1951**, *193*(1), 265-275. PMID: 14907713

[6] Zerbetto, E.; Vergani, L.; Dabbeni-Sala, F. Quantification of muscle mitochondrial oxidative phosphorylation enzymes via histochemical staining of blue native polyacrylamide gels. *Electrophoresis,* **1997**, *18*(11), 2059-2064. http://dx.doi.org/10.1002/elps.1150181131 PMID: 9420170

[7] Weydert, C.J.; Cullen, J.J. Measurement of superoxide dismutase, catalase and glutathione peroxidase in cultured cells and tissue. *Nat. Protoc.,* **2010**, *5*(1), 51-66. http://dx.doi.org/10.1038/nprot.2009.197 PMID: 20057381

[8] Compton, M.M. A biochemical hallmark of apoptosis: internucleosomal degradation of the genome. *Cancer Metastasis Rev.,* **1992**, *11*(2), 105-119. http://dx.doi.org/10.1007/BF00048058 PMID: 1327565

[9] Sakamuru, S.; Attene-Ramos, M.S.; Xia, M. Mitochondrial Membrane Potential Assay. *Methods Mol. Biol.,* **2016**, *1473*, 17-22. http://dx.doi.org/10.1007/978-1-4939-6346-1_2 PMID: 27518619

[10] Stevenson, D.E.; Kehrer, J.P.; Kolaja, K.L.; Walborg, E.F., Jr; Klaunig, J.E. Effect of dietary antioxidants on dieldrin-induced hepatotoxicity in mice. *Toxicol. Lett.,* **1995**, *75*(1-3), 177-183. http://dx.doi.org/10.1016/0378-4274(94)03178-A PMID: 7863524

[11] Li, R.; Jia, Z.; Trush, M.A. Defining ROS in Biology and Medicine. *React. Oxyg. Species (Apex),* **2016**, *1*(1), 9-21. http://dx.doi.org/10.20455/ros.2016.803 PMID: 29707643

[12] Huc, L.; Tekpli, X.; Holme, J.A.; Rissel, M.; Solhaug, A.; Gardyn, C.; Le Moigne, G.; Gorria, M.; Dimanche-Boitrel, M.T.; Lagadic-Gossmann, D. c-Jun NH2-terminal kinase-related Na+/H+ exchanger isoform 1 activation controls hexokinase II expression in benzo(a)pyrene-induced apoptosis. *Cancer Res.,* **2007**, *67*(4), 1696-1705. http://dx.doi.org/10.1158/0008-5472.CAN-06-2327 PMID: 17308111

[13] Lobo, V.; Patil, A.; Phatak, A.; Chandra, N. Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacogn. Rev.,* **2010**, *4*(8), 118-126. http://dx.doi.org/10.4103/0973-7847.70902 PMID: 22228951

[14] Maiti, P.K.; Kar, A.; Gupta, P.; Chaurasia, S.S. Loss of membrane integrity and inhibition of type-I iodothyronine 5′-monodeiodinase activity by fenvalerate in female mouse. *Biochem. Biophys. Res. Commun.,* **1995**, *214*(3), 905-909. http://dx.doi.org/10.1006/bbrc.1995.2372 PMID: 7575562

[15] Maiti, P.K.; Gupta, P.; Chaurasia, S.S.; Kar, A. Dimethoate induced lipid peroxidation and inhibition of type-I iodothyronine 5′-monodeiodinase activity in young cockerel. *Bull. Environ. Contam. Toxicol.,* **1996**, *57*(2), 335-340. http://dx.doi.org/10.1007/s001289900195 PMID: 8661918

[16] Tripathi, G.; Verma, P. Fenvalerate-induced changes in a catfish, Clarias batrachus: metabolic enzymes, RNA and protein. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.,* **2004**, *138*(1), 75-79. http://dx.doi.org/10.1016/j.cca.2004.05.005 PMID: 15313449

[17] Naveed, A.; Janaiah, C. Effect of Triazophos on protein metabolism in the fish. *Channapunctatus. Curr. Res. J. Biol. Sci.,* **2011**, *3*, 124-128.

[18] Tripathi, G.; Singh, H. Impact of alphamethrin on biochemical parameters of *Channa punctatus. J. Environ. Biol.,* **2013**, *34*(2), 227-230. PMID: 24620583

[19] Vioque-Fernández, A.; de Almeida, E.A.; López-Barea, J. Biochemical and proteomic effects in Procambarus clarkii after chlorpyrifos or carbaryl exposure under sublethal conditions. *Biomarkers,* **2009**, *14*(5), 299-310. http://dx.doi.org/10.1080/13547500902913211 PMID: 19476409

[20] Hai, D.Q.; Varga, S.I.; Matkovics, B. Organophosphate effects on antioxidant system of carp (Cyprinus carpio) and catfish (Ictalurus nebulosus). *Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol.,* **1997**, *117*(1), 83-88. http://dx.doi.org/10.1016/S0742-8413(96)00234-4 PMID: 9206589

[21] Yang, Z.P.; Morrow, J. WU, A.; Roberts, L.J.; Dettbarn, W.D. Diisopropyl phosphoro fluorodate-induced muscle hyperactivity associated with enhanced lipid peroxidation in vivo. *Biochem. Pharmacol.,* **1996**, *52*, 357-361. http://dx.doi.org/10.1016/0006-2952(96)00214-6 PMID: 8694861

[22] Ondreicka, R.; Beno, I.; Cerná, O.; Grancicová, E.; Staruchová, M.; Volkovová, K.; Bobek, P.; Tatara, M. [Relation between levels of vitamins C, E, A and beta-carotene and activity of antioxidant enzymes in the blood]. *Bratisl. Lek Listy,* **1998**, *99*(5), 250-254. PMID: 9673039

[23] Ho, Y.S.H.; Gargano, M.; Cao, J.; Bronson, R.T.; Heimler, I.; Hutz, R.J. Reduced fertility in female mice lacking copper-zinc superoxide dismutase. *J. Biol. Chem.,* **1998**, *273*(13), 7765-7769. http://dx.doi.org/10.1074/jbc.273.13.7765 PMID: 9516486

[24] Schwarzbacherová, V.; Wnuk, M.; Lewinska, A.; Potocki, L.; Zebrowski, J.; Koziorowski, M.; Holečková, B.; Šiviková, K.; Dianovský, J. Evaluation of cytotoxic and genotoxic activity of fungicide formulation Tango(®) Super in bovine lymphocytes. *Environ. Pollut.,* **2017**, *220*(Pt A), 255-263.

[25] Šiviková, K.; Holečková, B.; Schwarzbacherová, V.; Galdíková, M.; Dianovský, J. Potential chromosome damage, cell-cycle

kinetics/and apoptosis induced by epoxiconazole in bovine peripheral lymphocytes in vitro. *Chemosphere,* **2018**, *193*, 82-88. http://dx.doi.org/10.1016/j.chemosphere.2017.11.008 PMID: 29127838

[26] Hodgson, E. *A textbook of modern toxicology,* 3rd ed.; John Wiley and Sons: Inc New Jersey, **2004**, pp. 203-211. http://dx.doi.org/10.1002/0471646776

[27] Ender, Y.; Onder, C. Effects of dichlorvos on lipid peroxidation in mice on subacute and subchronic periods. *Pestic. Biochem. Physiol.,* **2006**, *86*, 106-109. http://dx.doi.org/10.1016/j.pestbp.2006.02.002

[28] Rahman, M.F.; Mahboob, M.; Danadevi, K.; Saleha Banu, B.; Grover, P. Assessment of genotoxic effects of chloropyriphos and acephate by the comet assay in mice leucocytes. *Mutat. Res.,* **2002**, *516*(1-2), 139-147. http://dx.doi.org/10.1016/S1383-5718(02)00033-5 PMID: 11943619

[29] Karpeta-Kaczmarek, J.; Kubok, M.; Dziewięcka, M.; Sawczyn, T.; Augustyniak, M. The level of DNA damage in adult grasshoppers Chorthippus biguttulus (Orthoptera, Acrididae) following dimethoate exposure is dependent on the insects' habitat. *Environ. Pollut.,* **2016**, *215*, 266-272. http://dx.doi.org/10.1016/j.envpol.2016.05.032 PMID: 27213568

[30] Mishra, M.; Sharma, A.; Shukla, A.K.; Kumar, R.; Dwivedi, U.N.; Kar Chowdhuri, D. Genotoxicity of dichlorvos in strains of Drosophila melanogaster defective in DNA repair. *Mutat. Res. Genet. Toxicol. Environ. Mutagen.,* **2014**, *766*(766), 35-41. http://dx.doi.org/10.1016/j.mrgentox.2014.02.004 PMID: 24614193

[31] Sharma, D.; Saxena, P.; Singh, V.; Sharma, R. Assessment of DNA degradation in lymphocytes of albino rat (*Rattusnorvegicus*) under lambda cyhalothrin stress. *World Appl. Sci. J.,* **2010**, *11*, 24-28.

[32] Hussien, H.M.; Abdou, H.M.; Yousef, M.I. Cypermethrin induced damage in genomic DNA and histopathological changes in brain and haematotoxicity in rats: The protective effect of sesame oil. *Brain Res. Bull.,* **2011**, *92,* 76-83. http://dx.doi.org/10.1016/j.brainresbull.2011.10.020 PMID: 22085743

[33] Liu, J.J.; Guo, C.; Wang, B.; Shi, M.X.; Yang, Y.; Yu, Z.; Meng, X.H.; Xu, D.X. Maternal fenvalerate exposure during pregnancy impairs growth and neurobehavioral development in mouse offspring. *PLoS One,* **2018**, *13*(10)e0205403 http://dx.doi.org/10.1371/journal.pone.0205403 PMID: 30321209

[34] Zhu, J.; Xia, R.; Liu, Z.; Shen, J.; Gong, X.; Hu, Y.; Chen, H.; Yu, Y.; Gao, W.; Wang, C.; Wang, S.L. Fenvalerate triggers Parkinson-like symptom during zebrafish development through initiation of autophagy and p38 MAPK/mTOR signaling pathway. *Chemosphere,* **2020**, *243*125336 http://dx.doi.org/10.1016/j.chemosphere.2019.125336 PMID: 31734597

[35] Cui, Z.G.; Jin, Y.J.; Sun, L.; Zakki, S.A.; Li, M.L.; Feng, Q.W.; Kondo, T.; Ogawa, R.; Inadera, H. Potential hazards of fenvalerate in massive pollution influence the apoptosis sensitivity. *J. Appl. Toxicol.,* **2018**, *38*(2), 240-247. http://dx.doi.org/10.1002/jat.3517 PMID: 28949029

[36] Denamur, E.; Matic, I. Evolution of mutation rates in bacteria. *Mol. Microbiol.,* **2006**, *60*(4), 820-827. http://dx.doi.org/10.1111/j.1365-2958.2006.05150.x PMID: 16677295

[37] Bromham, L. Why do species vary in their rate of molecular evolution? *Biol. Lett.,* **2009**, *5*(3), 401-404. http://dx.doi.org/10.1098/rsbl.2009.0136 PMID: 19364710

[38] Ha, M.H.; Choi, J. Effects of environmental contaminants on hemoglobin gene expression in Daphnia magna: a potential biomarker for freshwater quality monitoring. *Arch. Environ. Contam. Toxicol.,* **2009**, *57*(2), 330-337. http://dx.doi.org/10.1007/s00244-007-9079-0 PMID: 19471991

[39] Zhao, T.; Lai, D.; Zhou, Y.; Xu, H.; Zhang, Z.; Kuang, S.; Shao, X. Azadirachtin A inhibits the growth and development of Bactrocera dorsalis larvae by releasing cathepsin in the midgut. *Ecotoxicol. Environ. Saf.,* **2019**, *183*109512 http://dx.doi.org/10.1016/j.ecoenv.2019.109512 PMID: 31398584

[40] Zhang, J.; Liu, H.; Sun, Z.; Xie, J.; Zhong, G.; Yi, X. Azadirachtin induced apoptosis in the prothoracic gland in *Bombyx mori* and a pronounced Ca2+ release effect in Sf9 cells. *Int. J. Biol. Sci.,* **2017**, *13*(12), 1532-1539. http://dx.doi.org/10.7150/ijbs.22175 PMID: 29230101