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Carbaryl and dimethoate induced alterations of the antioxidant defense system in two freshwater pulmonate snails *Helisoma duryi* and *Lymnaea natalensis*

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ABSTRACT

Organophosphates and carbamates are extensively used to increase the quality and quantity of field crops. These pesticides may indirectly enter water bodies where they affect aquatic organisms. Once absorbed by aquatic organisms the pesticides are metabolised and normal metabolic processes may produce reactive oxygen species that have adverse effects on the aquatic organisms. The effects of exposure to carbaryl and dimethoate pesticides on antioxidant enzymes of two freshwater snail species Helisoma duryi and Lymanea natalensis were evaluated. Groups of snails were exposed to 25 ppb of carbaryl and/or dimethoate for 72 hours. After the exposure duration they were then analysed for their effects on the oxidative defense systems of the snails. Increased thiobarbituric acid reactive substances levels and activities of catalase, superoxide dismutase, glutathione peroxidase and glutathione S-transferase in both snail species were observed, probably as a means of combating oxidative stress due to pesticide poisoning. Increased lipid peroxidation, coupled with altered levels of oxygen free radical scavenging enzymes in snail homogenates are discussed in relation to oxidative stress.

Key words: Organophosphates; carbamates; biomarker; oxidative-stress; snails

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INTRODUCTION

Most organisms require oxygen in the catabolism of glucose which ultimately produces ATP and reduced forms of nicotinamide adenine dinucleotide and flavin adenine dinucleotide. During normal metabolism partial reduction of oxygen may occur, which results in the formation of reactive oxygen species [1]. Reactive oxygen species may be formed from the biotransformation of xenobiotics such as pesticides and industrial chemicals in biological systems [2]. Oxidative stress occurs when the production of ROS exceed the body's ability to eliminate these unstable oxygen species [1]. During detoxification processes of pesticides in living organisms free radicals may be generated which alter the organismal defense system leading to oxidative stress [3; 4]. Organophosphates and carbamates (CMs) exert their toxicity in both vertebrates and invertebrates by inhibiting acetylcholinesterase, an enzyme responsible for hydrolysing the neurotransmitter acetylcholine to products, acetate and choline which result in ending the transmission of a nervous impulse [5]. Organophosphorus and CM compounds prevent the breakdown of the neurotransmitter acetylcholine in the synaptic regions of cholinergic neurones leading to continued activation of the post synaptic membrane leading to convulsions that ultimately leads to respiratory failure and death [6]. Reactive oxygen species such as superoxide anions and hydroxyl free radicals are very reactive and they target lipids structures in cell membrane leading to lipid peroxidation and tissue damage. [7] reported oxidative stress in snails caused by oxidants during aestivation.

Most of the reports in literature discuss changes in levels and activities of enzymes that remove ROS in mussels that are used as indicators of exposure to environmental pollutants. [8] reported increased glutathione S-transferase

(GST) activity in *Monodonta lineate*, *Mytilus galloprovincialis* and *Nucella lapillus* exposed to petroleum hydrocarbons after an oil spill. Other studies have shown the adaptive responses of antioxidant enzymes in bivalve molluscs after exposure to metal pollutants. [9] reported increased activity of GST, catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) after exposure to sublethal levels of mercury.

The present study examined the oxidative effects of carbaryl and dimethoate, individually or as a mixture, in freshwater snail homogenates after *in-vivo* exposures.

The pesticides under investigation, an organophosphate (OP) dimethoate (O,O-dimethyl-S-N-methylcarbamoylmethyl-phoshorodithioate) and the CM carbaryl (1-naphthyl-N-methyl carbamate carbamate) are widely used in Zimbabwe to protect a broad range of crops against insects, mites and other pests. Despite the extensive use of carbaryl and dimethoate in crop protection and in households, information related to their effects on biochemical indicators in snails, which are used to assess the health status of these aquatic macro-invertebrates is not available in Zimbabwe. In order to understand level of carbaryl and dimethoate induced oxidative stress in aquatic snails, we investigated the effects of *in-vivo* exposure of the snails to the pesticides on the antioxidant enzyme activities of the snails.

MATERIALS AND METHODS

2.1 Chemicals

All enzymes, substrates and chemicals were bought from Sigma Chemical Company or Aldrich Chemical Company, Germany. The pesticides carbaryl and dimethoate were kind donations from Agricura (Pvt), Ltd, Zimbabwe. All other laboratory reagents, used in this study, were of analytical (ANALAR) grade.

2.2 Snail breeding and exposure

Snails used in the study were bred outdoors following the method of [10]. Before the exposure studies they were brought to the laboratory where they were acclimatised to laboratory conditions for 14 days. Juvenile snails exposed to 25 ppb of carbaryl or dimethoate, (1:1) mixture of carabryl and dimethoate for 72 hours. The exposures were performed in quadruplicate concentrations.

2.3 Preparation of homogenates

Fifteen whole snails from each experimental group were pooled and homogenized in ice-cold homogenization buffer (0.1 M potassium phosphate pH 7.4). The homogenates were centrifuged at 10,000 *X g* for 10 minutes and the resultant supernatant (S-10) fraction stored at -80^{o} C until analyzed. The remaining five snails from each group were kept at -80^{o} C for the determination of TBARS levels in tissue samples. Protein concentration was determined in the snail samples and bovine serum albumin used as standard [11].

2.4 Biochemical assays

2.4.1 Lipid peroxidation

Lipid peroxidation (LPO) was measured in snail tissue and was expressed as thiobarbituric acid reactive substances [12].

2.4.2 Superoxide dismutase

Superoxide dismutase activity was measured in in S-10 fractions following the formation of formazan the reaction of superoxide free radicals with 2-(4-indophenyl)-3(4-nitro-phenyl)-5-phenyl tetrazolium chloride [13]

2.4.3 Catalase

Catalase activity was measured in S-10 fractions by following the disappearance of the substrate hydrogen peroxide [14].

2.4.4 Glutathione S-transferase

Glutathione *S*-transferase activity was measured in S-10 fractions using 1-chloro-2-4-dintrobenzene as a substrate [15].

2.4.5 Glutathione peroxidase.

Glutathione peroxidase activity was determined in S-10 fractions using an indirect couple method that involve oxidation of glutathione (GSH) by H_2O_2 in the presence of glutathione reductase and reduced form of nicotinamide adenine dinucleotide phosphate. NADPH [16].

2.5.6 NAD(P)H quinone oxidoreductase

NAD(P)H quinone oxidoreductase activity was measured in S-10 fractions by following the disappearance of dichlorophenoindophenol [17].

2.6. Statistical analysis

The Dunnet and one-way analysis of variance in the Tukey's multiple comparison tests were used to show statistical differences between control and treated groups at *p<0.05 or **p<0.001.

RESULTS

3.1. Thiobarbituric acid reactive substances (TBARS) level

Snails exposed to pesticides showed significantly higher levels of TBARS when compared to control snails (Fig 3.1). In both snail species the amount of TBARS production was highest in snails exposed to the pesticide mixture (p<0.01).

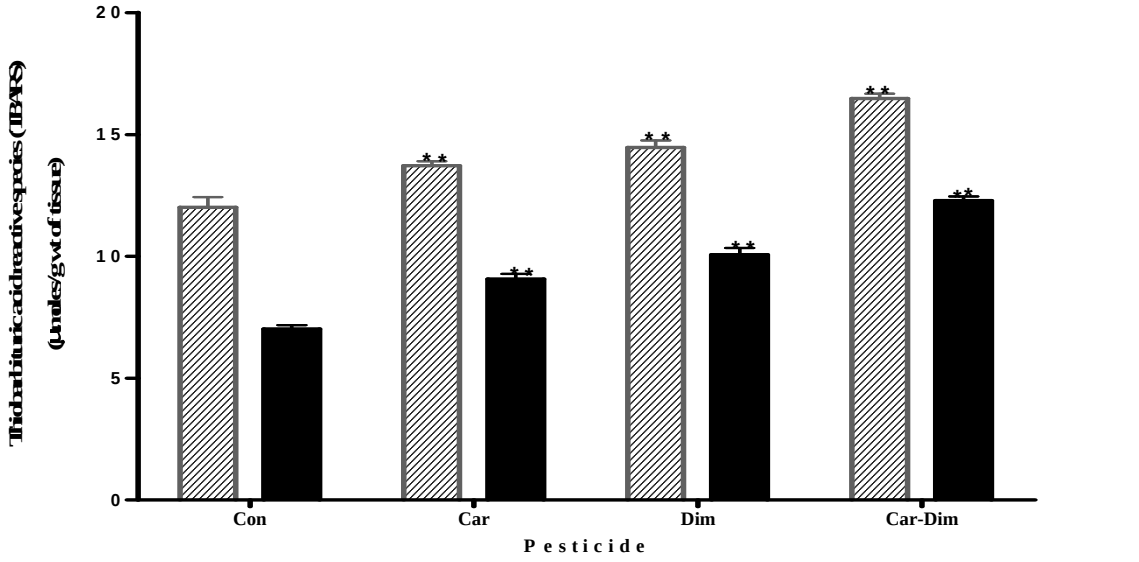
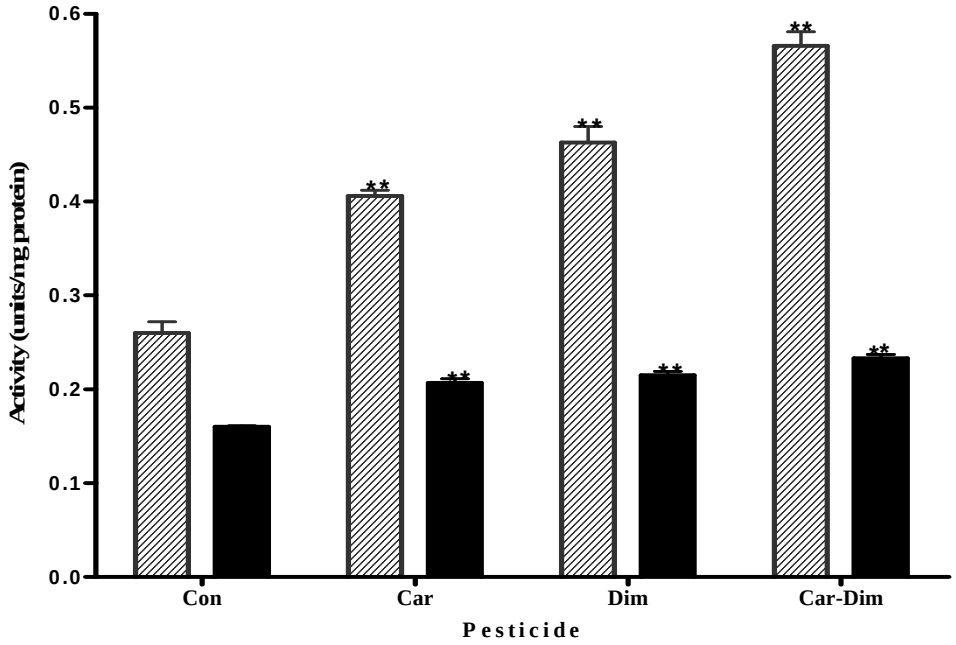


Fig 3.1 Effect of carbaryl, dimethoate or mixture of carbaryl and dimethoate on formation of thiobarbituric acid reactive substances in two aquatic snails *H. duryi* and *L. natalensis*. Con = control, Car = carbaryl, Dim = dimethoate and Car-Dim = carbaryl and dimethoate mixture. Values are expressed as mean ± S.D. Significantly different from control (***P<0.01*****)**

3.2. Superoxide dismutase activity

Increases in SOD activity of exposed snails of up to 74% in *H. duryi* and up to and 34% in *L. natalensis* depending on the pesticide exposed were observed (Fig 3.2). The mixtures increased SOD by as much as 110% in H. duryi and 47% in *L natalensis* when compared to the controls.



Superoxide dismutase activity in homogenates of two snail species *H. duryi* and *L. natalensis* exposed to carbaryl, dimethoate or mixture of carbaryl and dimethoate. Con = control, Car = carbaryl, Dim = dimethoate and Car-Dim = carbaryl and dimethoate mixture. Values are expressed as mean ± S.D. Significantly different from control ($P<0.01$)**

3.3. Catalase activity

Carbaryl and/or dimethoate caused an increase in CAT activity with carbaryl causing the lowest increases and the mixture causing the highest increases in enzyme activity in exposed snails (Fig 3.3). Catalase activity was higher in *H. duryi* than in *L. natalensis* after exposure to the same pesticide concentrations (Fig 3.3).

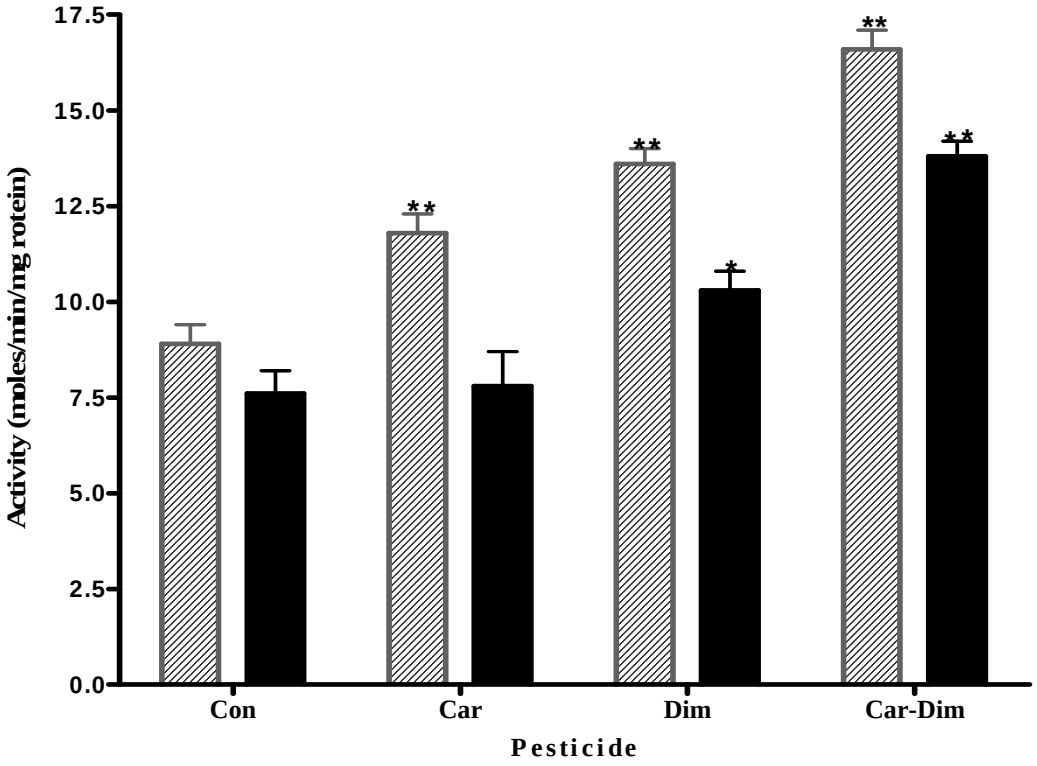


Fig 3.3 Catalase activity in *H. duryi* and *L. natalensis*. exposed to carbaryl, dimethoate or carbaryl and dimethoate. Con = control, Car = carbaryl, Dim = dimethoate and Car-Dim = carbaryl and dimethoate mixture. Values are expressed as mean ± S.D. Significantly different from control (*$P<0.05$ and **$P<0.01$)

3.4. Glutathione S-transferase activity

The activity of GST was significantly increased in all snails exposed to the pesticides when compared to control snails except for carbaryl exposed *L. natalensis* where the increase was not statistically different in Dunnet test (Fig 3.4). The snails exposed to the pesticide mixture significantly increased ($p<0.01$) GST activity when compared with snails exposed to individual pesticides.

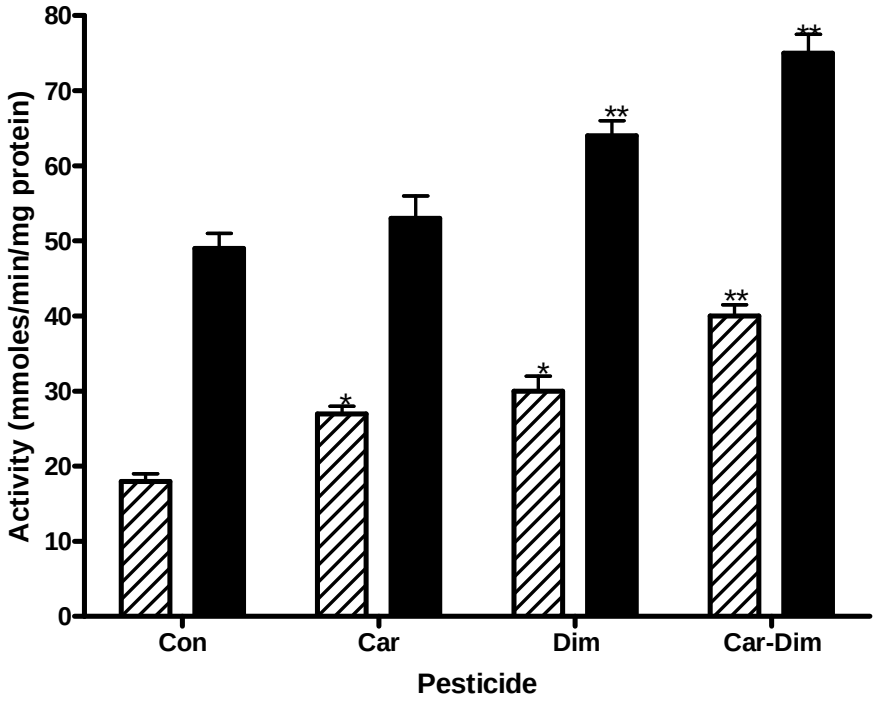
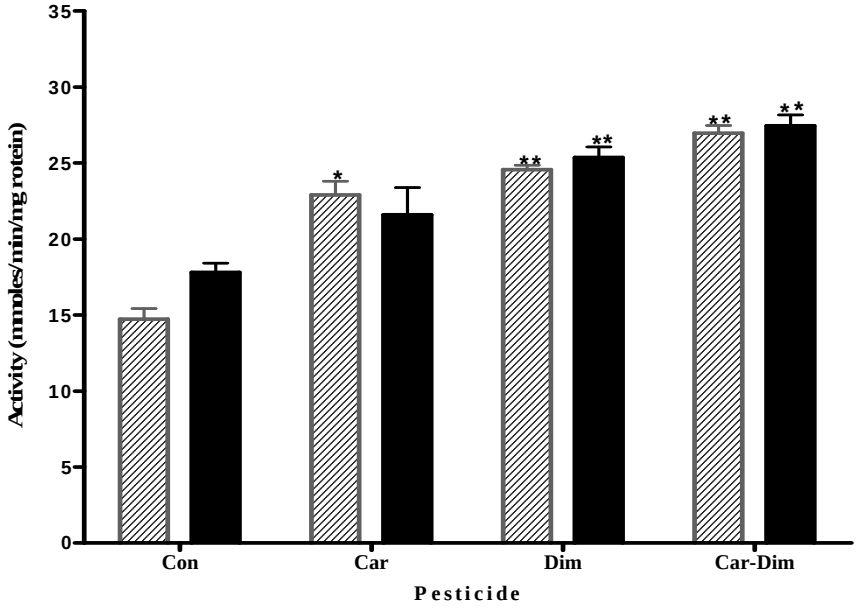


Fig 3.4. Effect of carbaryl, dimethoate or mixture of carbaryl and dimethoate on glutathione S-transferase activity in *H. duryi* and *L. natalensis*. Con = control, Car = carbaryl, Dim = dimethoate and Car-Dim = carbaryl and dimethoate mixture. Values are expressed as mean ± S.D. Significantly different from control (P*<0.05 and ***P<0.01*)**

3.5. Glutathione peroxidase activity

The GPx activity of snails exposed to the pesticides showed significant increases when compared to control snails except in *L natalensis* exposed carbaryl where the increase was not statistically significant (Fig 3.5).



. Fig 3.5 Effect of carbaryl, dimethoate or carbaryl and dimethoate mixture on glutathione peroxidase activity in two snail species *H. duryi* and *L. natalensis*. Con = control, Car = carbaryl, Dim = dimethoate and Car-Dim = carbaryl and dimethoate mixture. Values are expressed as mean ± S.D. Significantly different from control (P*<0.05 and ***P<0.01*)**

3.6. NAD(P)H quinone oxidoreductase activity

The NAD(P)H quinone oxidoreductase activity was not significantly different between the controls and snails exposed to carbaryl and/or dimethoate in both snail species as shown in Fig 3.6.

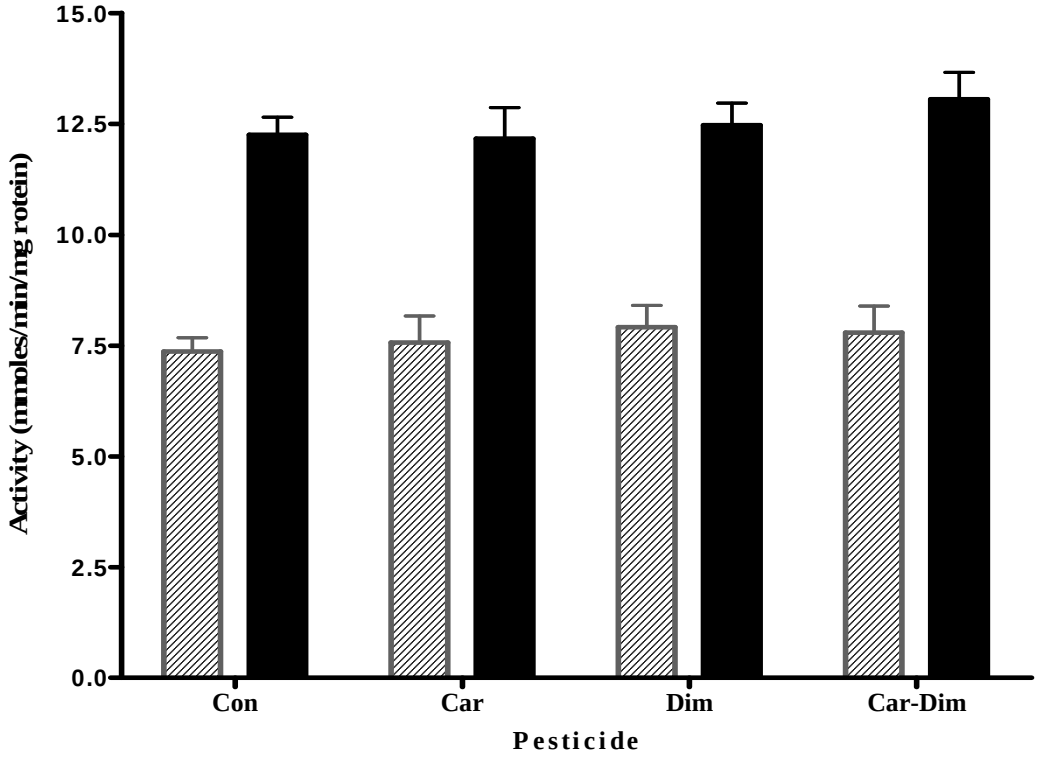


Fig 3.6 Effect of carbaryl , dimethoate or mixture of carbaryl and dimethoate on NAD(P)H quinone oxidoreductase activity in two snail species ▨ *H. duryi* an■ *L. natalensis.* Con = control, Car = carbaryl, Dim = dimethoate and Car-Dim = carbaryl and dimethoate mixture. Values are expressed as mean ± S.D

DISCUSSION

Pesticides are sometimes used improperly in large amounts which ultimately results in environmental pollution [18] Their toxicological effects may involve inducing stress of an oxidative nature in non-target species leading to generation of free radicals and alterations antioxidant enzymes.

Our results have shown that exposure to the pesticides carbaryl or dimethoate as well as their mixture increased the activities of SOD, CAT, GPx and GST and levels of TBARS of the two snail *species H. duryi* and *L. natalensis*. Catalase and SOD are considered as the first line of defense against the effects of ROS in all organisms and as such have evolved in different tissues in aerobic organisms [19].

4.1 Superoxide dismutase activity

Significant increase in SOD activity observed in the present study suggests that the toxicity of carbaryl and/or dimethoate possibly involve generation of ROS such as superoxide anion radicals. The snails counteract the effects of the superoxide free radicals by increasing levels of SOD, an enzyme that decomposes the free radical molecules. Our findings are comparable with studies of [2] who reported increased SOD activity in mussels exposed to urban pollutants. Comparing enzyme activity in the two snail species, SOD activities observed in *H. duryi* were almost 2 fold those observed in *L. natalensis* for all pesticide groups and this can be attributed to species difference. Possibly the SOD gene expression in *H. duryi* is greater compared to *L. natalensis* implying possible difference in sensitivity to pollutants and detoxification mechanisms taking place in the two snail species.

4.2 Catalase activity

Catalase decomposes the hydrogen peroxide, the product of SOD activity, to water and molecular oxygen and the observed increased levels of CAT activity are possibly an adaptive mechanism in the snail system to minimize the oxidative effects of the pesticides. [20] also observed enhanced CAT activity in terrestrial snails exposed to carbaryl. In the present study in both snail species, a similar response trend was observed whereby, carbaryl caused the

minimum increases in CAT activity, followed by dimethoate with the highest activities observed in snails exposed to the pesticide mixture. When comparing the enzyme activities of the two snail species, higher CAT activities were observed in *H. duryi* than in *L. natalensis* again reinforcing the species difference response to exposure to the studied pesticides.

4.3 Glutathione S-transferase activity

The marked increase in GST activity in the snails exposed to carbaryl and/or dimethoate in comparison to controls observed in the present study, indicate an attempt by the snail system to render the pesticides harmless or soluble for excretion. The snails achieve this by conjugating the electrophiles that may be generated during metabolism of the pesticides. The increased GST levels support the assumption that the toxicity of carbaryl and/or dimethoate may involve generation of reactive oxygen species. Pesticide induced activation of glutathione S-transferase has been also reported in land snails. [21] reported activation of GST in the terrestrial snail Helix aspersa exposed to the pesticide, imidacloprid. The enhanced levels of GST observed in snails exposed to pesticides in the present study is suggestive of the defense system in the snails adjusting to the increased levels of free radicals generated during metabolism of the pesticides, carbaryl and/or dimethoate. The increases in GST activity were lowest in snails exposed to carbaryl and highest in snails exposed to the mixture of carbaryl and dimethoate in both snail species. However on comparing the activities of GST in the two species, significantly higher activities were observed in *L. natalensis* than *in H. duryi* for all pesticide exposures suggesting the difference in how the two species respond to identical exposure conditions probably due to the difference in their genetic makeup.

4.4 Glutathione peroxidase activity

The results of the present study showed similar trend in GPx activities in both snail species with the lowest GPx activity in snails exposed to carbaryl and the highest activities in snails exposed to the mixture of carbaryl and dimethoate. Although carbaryl and dimethoate have quite different chemical structures, they bring forth similar oxidative stress responses in the two snail species brought about by free radicals generated indirectly during the biotransformation of the two pesticides. Our results are supported by [20] who observed elevated GPx activity in snails exposed to carbaryl. The increased presence of GPx observed compliments catalase in breaking down the toxic hydrogen peroxide, a product of the dismutation of superoxide anion radicle, to water. Effects of pesticides like dimethoate differ in different organisms. [22] for instance reported diminished GPx activity in *Oreochromis niloticus* exposed to dimethoate contaminated river water indicating organismal response difference to pollutants.

4.5 NAD(P)H quinone oxidoreductase activity

The results of the present study showed no statistical differences in the NAD(P)H quinone oxidoreductase activities between pesticide-treated groups and the controls. Though a possibility exists for formation of quinone–like structures during metabolism of carbaryl because of the hydroxylated benzene rings in its structure, the results obtained with both snail species showed no evidence of altered NAD(P)H quinone oxidoreductase activity, suggesting that the metabolic pathway of carbaryl, does not involve production of quinone-like intermediates. The results obtained suggest that NAD(P)H quinone oxidoreductase in the two snail species is not a useful biomarker for oxidative stress caused by the two environmental pollutants, carbaryl and dimethoate.

4.6 TBARS level

Lipid peroxidation is one of the most frequently used indicators of the level of ROS induced damage in living organisms. Malondialdehyde, one of the end products of lipid peroxidation is used as a biomarker of radical damage and oxidative stress, and was expressed in the present study as thiobarbituric acid reactive substances. Increase in levels of thiobarbituric acid reactive substances observed was lowest in snails exposed to carbaryl, than snails exposed to dimethoate and the highest levels of thiobarbituric acid reactive substances were observed in snails exposed to the mixture of carbaryl and dimethoate in both snail species. While, the enhanced activities of SOD, CAT, GPx and GST, the antioxidant enzymatic defense system that scavenge toxic ROS reflect an adaptive mechanism within the snails, there was failure by the total antioxidant defense mechanism to protect the tissues from mechanical damage caused by the two pesticides, as evidenced by enhanced levels thiobarbituric acid reactive substances. This failure to protect the snails was more pronounced in *H. duryi* snails which generally caused higher levels of all antioxidants enzymes except for the levels of DT diaphorase and GST activities which were higher in *L. natalensis* when compared *H. duryi*.

CONCLUSION

Carbaryl and/or dimethoate pesticides induced oxidative stress in the two snail species as evidenced by increased lipid peroxidation products coupled with altered activity of antioxidant enzyme activities was observed. The two pesticides though structurally different appear to have similar biotransformation mechanisms which involve monooxyegenase system and production of free radicals as by-products that cause alterations of antioxidant enzyme levels. Antioxidant enzymes except for NAD(P)H quinone oxidoreductase in the *H. duryi* or *L. natalensis* were very

sensitive to exposure to carbaryl and/or dimethoate and these enzyme systems in both snail species therefore have a potential of being exploited as biomarkers of exposure to carbaryl/dimethoate or pesticides with similar chemical properties.

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