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EVALUATION OF NONYLPHENOL TOXICITY IN *ETROPLUS MACULATUS* (BLOCH, 1795): RESPONSE ON GILL ANTIOXIDANT DEFENSE SYSTEM

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ABSTRACT
The toxic effects of nonylphenol on adult freshwater cichlid fish, *Etroplus maculatus* at sublethal concentrations (one-fifth and one-tenth of LC_{50}) was evaluated for 24, 72 and 96 h in gill by determining the activities of antioxidant enzymes and lipid peroxidation. The body weight of the animal remained unchanged in both controls as well as in treatment groups. The weight of gill showed a significant decrease only at highest concentration after 96 h of nonylphenol exposure. The activities of antioxidant enzymes such as superoxide dismutase, catalase, glutathione reductase showed a time-dependent significant ($P<0.05$) decrease at both concentrations when compared to control groups. However, the levels of hydrogen peroxide generation and lipid peroxidation increased significantly ($P<0.05$) in time-dependent manner at low and high concentrations of nonylphenol treatment. After exposure to both the concentrations of nonylphenol the activity of one of the gill marker enzymes, alkaline phosphatase showed a significant reduction than that of control groups. From the present results, it was suggested that nonylphenol induces toxicity to fish and these effects altered the antioxidant defense system thereby induced oxidative stress in gill of *Etroplus maculatus*.

KEY WORDS: Alkaline phosphatase, Antioxidant enzymes, *Etroplus maculatus*, Gill, Lipid peroxidation, Nonylphenol, Oxidative stress

INTRODUCTION

Alkylphenol ethoxylates (APEs) are synthetic non-ionic surfactants found in variety of industrial and household appliances as detergents, cleaning products, lubricants, hair dyes and other hair care products, pesticides and even in spermicides (Talmage, 1994). The most common APEs are nonylphenol ethoxylates (NPEs) and are commercially used as surfactants over 50 years. NPEs are manufactured using nonylphenol with ethylene oxide under base condition that has been identified as most critical metabolite of APEs. Nonylphenol is produced in large volumes and about 60% of this production is released into the aquatic environment (Renner, 1997). Nonylphenol is highly toxic to aquatic organisms as its resistance to biodegradation and its ability to bioaccumulate. Nonylphenol has also been detected in human breast milk, blood and urine, and are associated with developmental and reproductive effects in fish (Gray and Metcalfe, 1997).

When animals ingest nonylphenol, its major absorption is from gastrointestinal tract which is probably rapid and extensive. The major metabolic pathways of nonylphenol include glucuronide and sulphate conjugation so that it is widely distributed throughout the body, with the highest concentration in fat. Recently there is an increasing concern over the widespread use of nonylphenol because of its toxicity to terrestrial, marine and aquatic organisms. However, only limited evidences are available for the significant effects of nonylphenol on human health. Nonylphenol has been shown to possess estrogenic activity and are shown to activate estrogen receptors in the multigeneration study in rats (Colerangle and Roy, 1996). In another study, when nonylphenol is administered to male Wistar rats orally for 45 days has been shown to decrease the weights of the testes and epididymides with reduction in epididymal sperm counts and also altered the activities of antioxidant enzymes in epididymal sperm of rats (Chitra et al., 2002). Nonylphenol has been also shown to induce oxidative stress in testis of rats while its toxic effects are reversed by the co-administration of natural antioxidant, vitamin E (Chitra and Mathur, 2004).

Several research data demonstrated the estrogenic effects of nonylphenol on mammals and fish. Various end points have been used to measure the estrogenic effects of nonylphenol in fish where increased vitellogenin production in male fish, morphological alterations of the gonads, and change in sex ratios are few among the endpoints extensively detected in fishes. Nonylphenol showed competitive displacement of estrogen from its receptor site in rainbow trout, *Oncorhynchus mykiss* (White et al., 1994). Exposure to nonylphenol has been shown to inhibit the testicular growth by significantly elevating the level of vitellogenin in male rainbow trout (Jobling et al., 1996). Arukwe et al. (1997) found

that nonylphenol exposure has been shown to cause an increase in the 6β-, 16α- and 17α-hydroxylase activities in salmon liver microsomes. In one of our studies it was found that nonylphenol at sublethal concentration has been shown to cause genetic damage in freshwater fish, *Oreochromis mossambicus* as confirmed by micronucleus test and Salmonella mutagenicity test (Balakrishnan et al., 2014).

Etroplus maculatus, an Indian cichlid fish, commonly known as orange chromid is an economically important food fish. In fact, in recent years it has been widely used as an experimental model in ecotoxicological studies as it has its ecological status within the food chain and suitable to maintain in laboratory. The present study was aimed to evaluate the toxic effects of nonylphenol on cichlid fish, *Etroplus maculatus* by assessing the antioxidant status on the gill tissues at varying concentrations and at varying durations.

MATERIALS AND METHODS

Maintenance of animal:

Cichlid fish, *Etroplus maculatus* weighing 7 ± 1 g and length 7 ± 1.5 cm were collected from a fish farm, KKF Nursery, Manjeri, Vaniyambalam. Fishes were transported with least disturbances to laboratory and were acclimatized prior to the experiment. They were sustained with proper aeration and light in 40 L capacity glass tanks, which was dechlorinated at regular intervals. Water in the tanks were maintained at standard temperature ranged from 28 ± 2°C, oxygen saturation between 70 and 100 %, pH at 6.5 to 7.5 which were monitored using a standardized procedures as per APHA (1998). The LC_{50} values in the respective time intervals were determined by probit analysis, with a confident limit of 5 % level (Finney, 1971), which was 0.89 mg/ L ie. 890 µg/ L (Asifa et al., 2016). One-fifth (178 µg/ L) and one-tenth (89 µg/ L) of LC_{50} was the selected sublethal concentrations in the present study.

Toxicity testing:

For subacute toxicity tests the concentration of nonylphenol in water was maintained below the median lethal concentration. Based on the LC_{50} value, two sublethal concentrations such as one-fifth and one-tenth of LC_{50}-96 h were chosen and exposed to *Etroplus maculatus* for 24, 72 and 96 h. The experiment was designed as follows:

A. Control groups:	Group I:	Fishes maintained in water alone (solvent-free control)
	Group II:	Positive control group (1% DMSO)
B. Treatment groups	Group III:	Nonylphenol at 178 µg/ L concentration (one-fifth of LC_{50})
	Group IIIa:	Nonylphenol at 178 µg/ L for 24 h
	Group IIIb:	Nonylphenol at 178 µg/ L for 72 h
	Group IIIc:	Nonylphenol at 178 µg/ L for 96 h
Group IV:		Nonylphenol at 89 µg/ L concentration (one-tenth of LC_{50})
	Group IVa:	Nonylphenol at 89 µg/ L for 24 h
	Group IVb:	Nonylphenol at 89 µg/ L for 72 h
	Group IVc:	Nonylphenol at 89 µg/ L for 96 h

In each group, 10 animals were maintained and fishes were monitored throughout the study for any sign of health issues. At the end of every treatment, fishes were collected very gently using dip net, one at a time with least disturbance as it proved that handling of fish also stresses the animal and alter several parameters. Anesthetizing agents were not used and fishes were decapitated and gills were collected from both control and treated groups.

Tissue processing:

Collected gills were cleaned from mucous and blood and stored at 4ºC until biochemical analysis were performed. A 1% (w/ v) homogenate of gill was prepared in ice-cold normal saline with the help of a motor-driven glass Teflon homogenizer on crushed ice for a minute. The homogenate was centrifuged at 8000 ***g*** for 15 min at 4°C to obtain the supernatant, which was then used for the biochemical analyses.

Biochemical analysis:

Activities of antioxidant enzymes, superoxide dismutase -EC 1.15.1.1 (Marklund and Marklund, 1974), catalase - EC. 1.11.1.6 (Claiborne, 1985), glutathione reductase - EC. 1.6.4.2 (Carlberg and Mannervik, 1985), levels of hydrogen peroxide generation (Pick and Keisari, 1981), lipid peroxidation (Ohkawa et al., 1979) and the total protein concentration in the tissue were estimated by the method of Lowry et al. (1951). The activity of alkaline phosphatase (EC.3.1.3.1) was assayed by the method of Bessey et al. (1946).

Statistical analysis:
Statistical analysis were performed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range test using statistical package SPSS 17.0. Differences were considered to be significant at $p<0.05$ against control group. Data are presented as mean ± SD for ten animals per group. All biochemical estimations were carried out in duplicate.

RESULTS
Nonylphenol treatment did not alter the body weight of fish in controls and in treatment groups at both concentrations (Figure 1). The weight of gill showed a significant decrease only at highest concentration after 96 h of nonylphenol exposure (Figure 2). The activities of antioxidant enzymes such as superoxide dismutase, catalase, glutathione reductase showed a time-dependent significant ($P<0.05$) decrease at both concentrations when compared to control groups (Figures 3-5). However, the levels of hydrogen peroxide generation and lipid peroxidation increased significantly ($P<0.05$) in time-dependent manner at low and high concentrations of nonylphenol treatment (Figures 6 and 7). After exposure to nonylphenol at both the concentrations the activity of one of the gill marker enzymes, alkaline phosphatase showed a significant reduction than that of control groups (Figure 8).

DISCUSSION
In accordance to the previous studies on nonylphenol in *Etroplus maculatus* from our laboratory, the 96 h LC_{50} value was 890 µg/ L (Asifa et al., 2016). However, the median lethal concentration of nonylphenol for 96 h varies widely from 135 µg/ L to 1400 µg/ L depending upon several laboratory factors and the species of fish (Staples et al., 1998; Midhila and Chitra, 2015). There are several factors that contribute to the risk of nonylphenol which includes routes, duration and concentration of exposure. In the present study *Etroplus maculatus* was used to monitor the toxic effects of nonylphenol at laboratory condition. Nonylphenol dissolved in 1% DMSO was maintained as a positive control throughout the experiment and it was observed that DMSO-treated fish did not showed any marked changes in all parameters tested in the study and the data obtained are similar to the solvent-free control group. Acute exposure to toxicants is widely used in ecotoxicological studies that provide preliminary information about the toxic compound on the organism and its environment. In the present study nonylphenol at sublethal concentrations ie., one-fifth (20% of 96 h-LC_{50}) and one-tenth (10% of 96 h LC_{50}) was exposed for 96 h so as to evaluate the acute toxic effects of the compound on the antioxidant defense system in gill of fish.

The present observations clearly demonstrate the toxic effects of nonylphenol in gill of fish as evidenced by significant reduction in the activities of antioxidant enzymes at both sub lethal concentrations. Gill was chosen as primary target organ of nonylphenol toxicity as it is the principal organ that was in direct contact with water through which the toxicants are exposed (Gunkel, 1981). Antioxidants are molecules that are involved in the prevention of cellular damage caused due to highly reactive free radicals. Free radicals are unstable atoms or molecules that oxidize other molecules, and are produced as a part of normal metabolism. These oxidized products are more unstable and can react with other molecules leading to oxidative stress. In general, there are several antioxidant enzymes within the cell that scavenge free radicals such as superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase.

Nonylphenol used in the present study decreased the activities of antioxidant enzymes in time-dependent manner at both sublethal concentrations in fish gill. Superoxide dismutase and catalase are the vital first-line defensive enzymes against the oxidative stress, the inhibition of these enzymes may lead to the accumulation of hydrogen peroxide. The present results illustrate that the antioxidant defense enzyme system was affected due to nonylphenol exposure at both sublethal concentrations. The levels of hydrogen peroxide and lipid peroxidation was increased after exposure to nonylphenol in time-dependent manner. It can be used as an index for measuring damage in tissue due to reactive oxygen species and this could be due to the toxicity of nonylphenol. Therefore, the present study clearly demonstrates that nonylphenol possess toxic effect on gill of fish as confirmed by its effect on oxidative metabolism.

Alkaline phosphatase, a hydrophilic lysosomal enzyme, which splits phosphorous esters at alkaline pH are released for the hydrolysis of antigenic particles and considered as a stress marker enzyme. It is found associated with cell membranes and is highly susceptible to free radical attack (Bajin-Katic et al., 2006). The present study showed that nonylphenol exposure significantly decreased the activity of alkaline phosphatase in the gill tissue at both sublethal concentrations in time-dependant manner when compared with the controls. The decreased alkaline phosphatase activity could be due to cellular oxidative damage in gill tissue induced by nonylphenol treatment. Such observation

has been observed in the gill and liver tissues of *Etroplus maculatus* when exposed to sublethal concentration of chlordecone (Asifa *et al.*, 2014). The results of the present investigation conclude that nonylphenol exposure at sublethal concentrations prove toxic to exposed fish as validated by imbalance in antioxidant defense system in gill tissue.

Figure 1

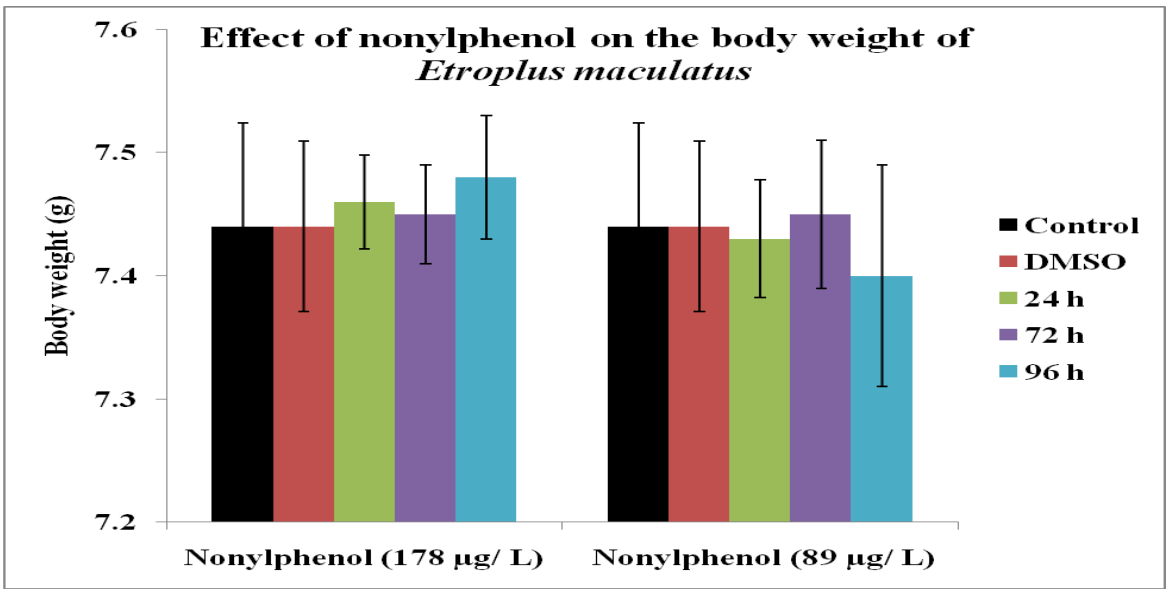

Figure 2

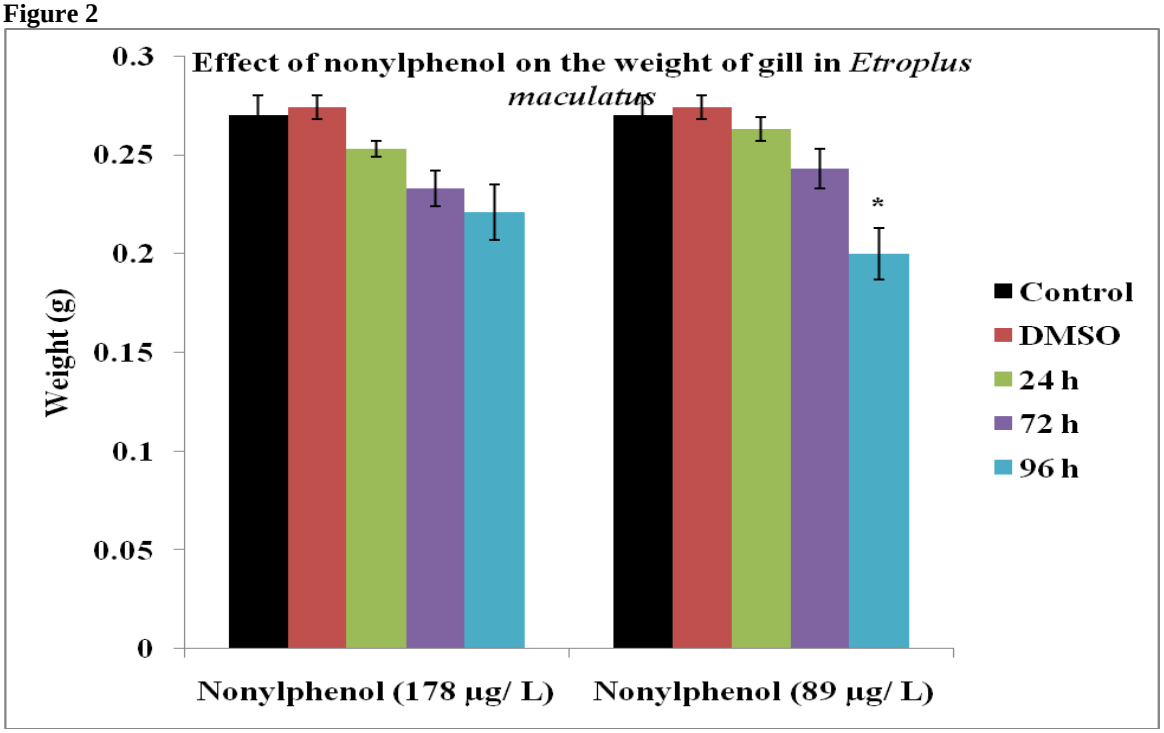

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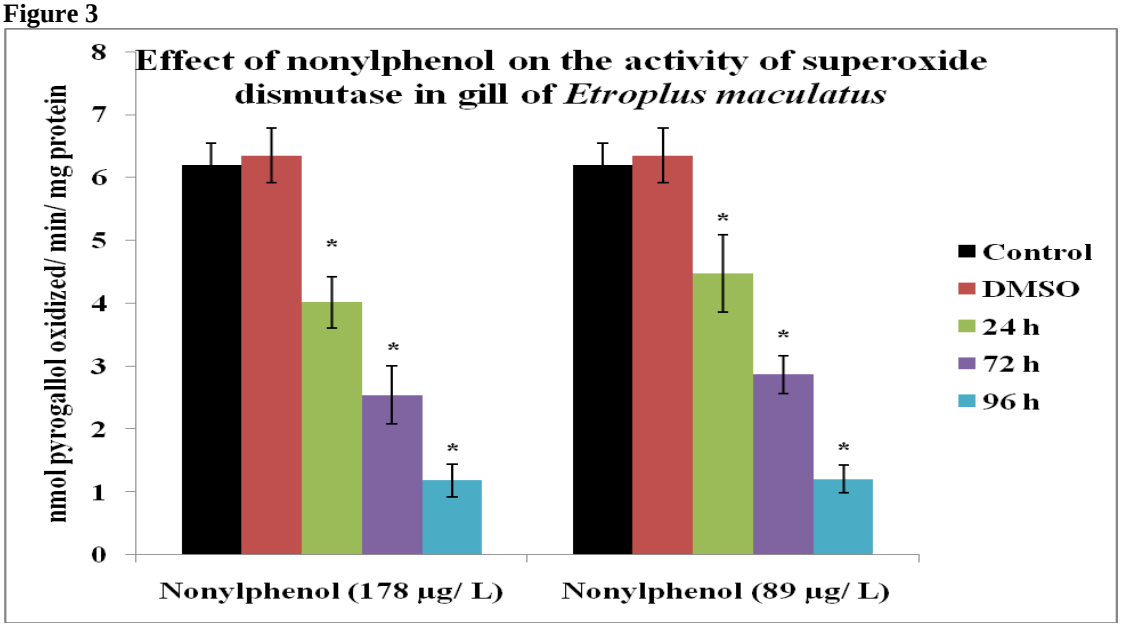
Figure 3



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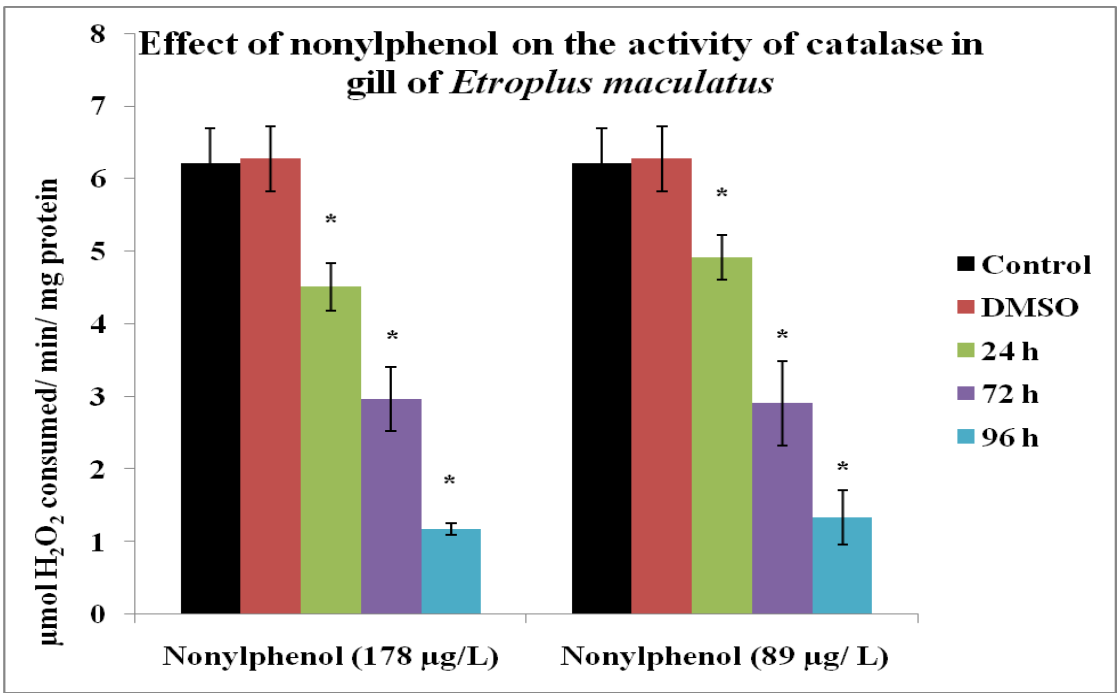

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Figure 5

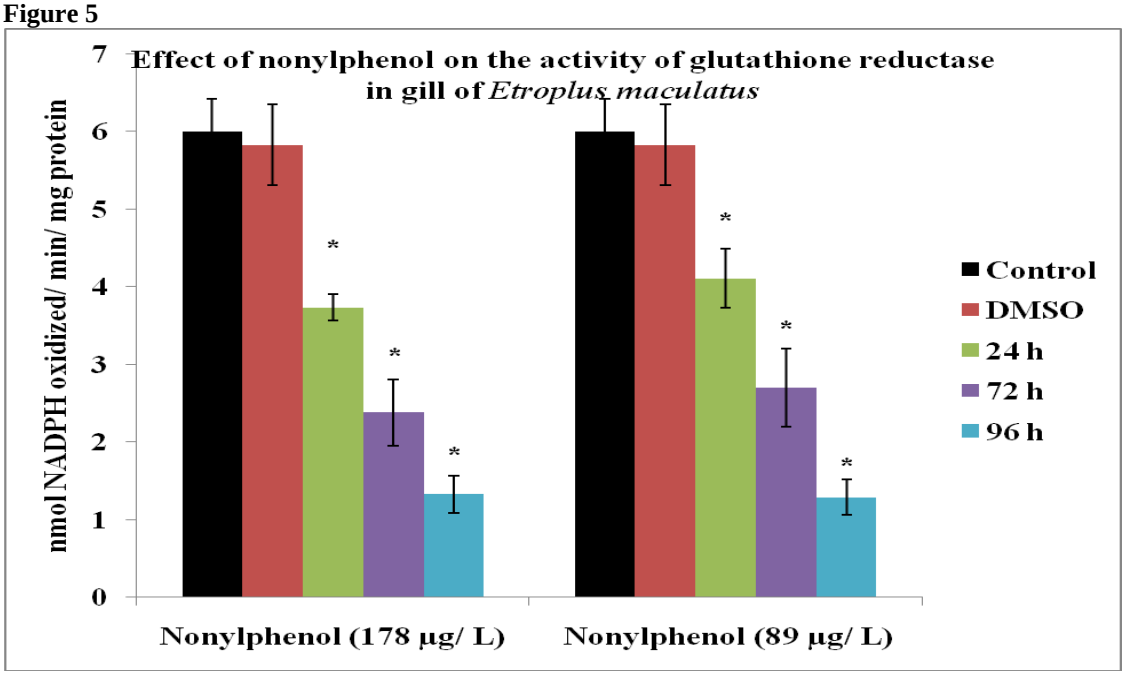
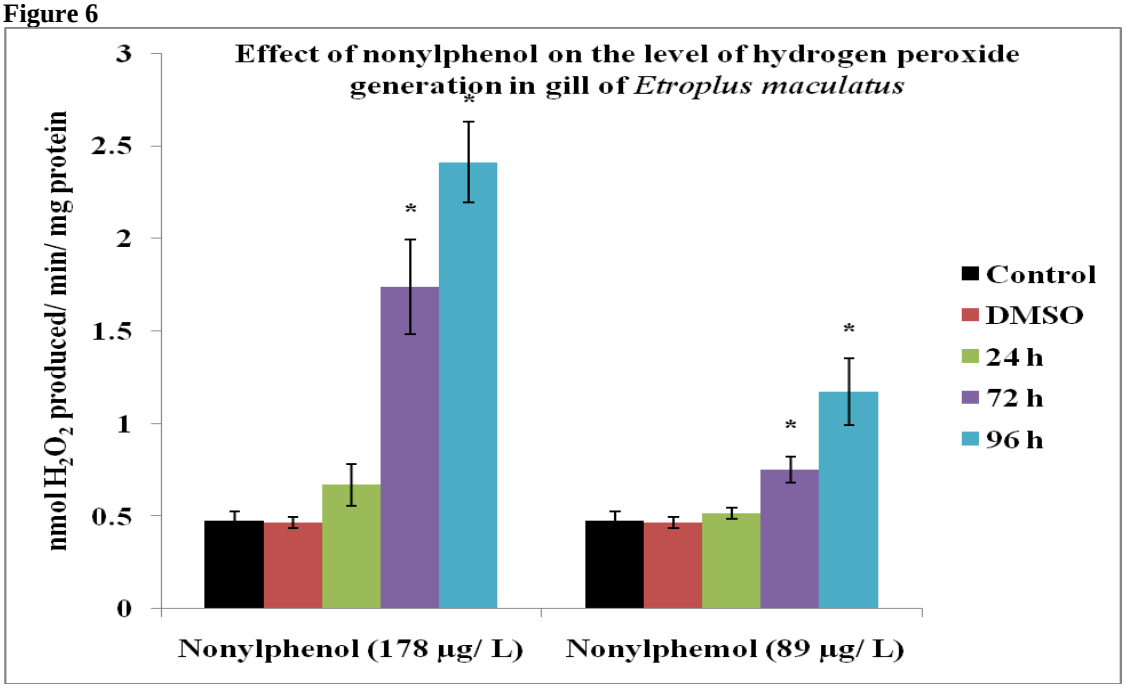


Figure 6



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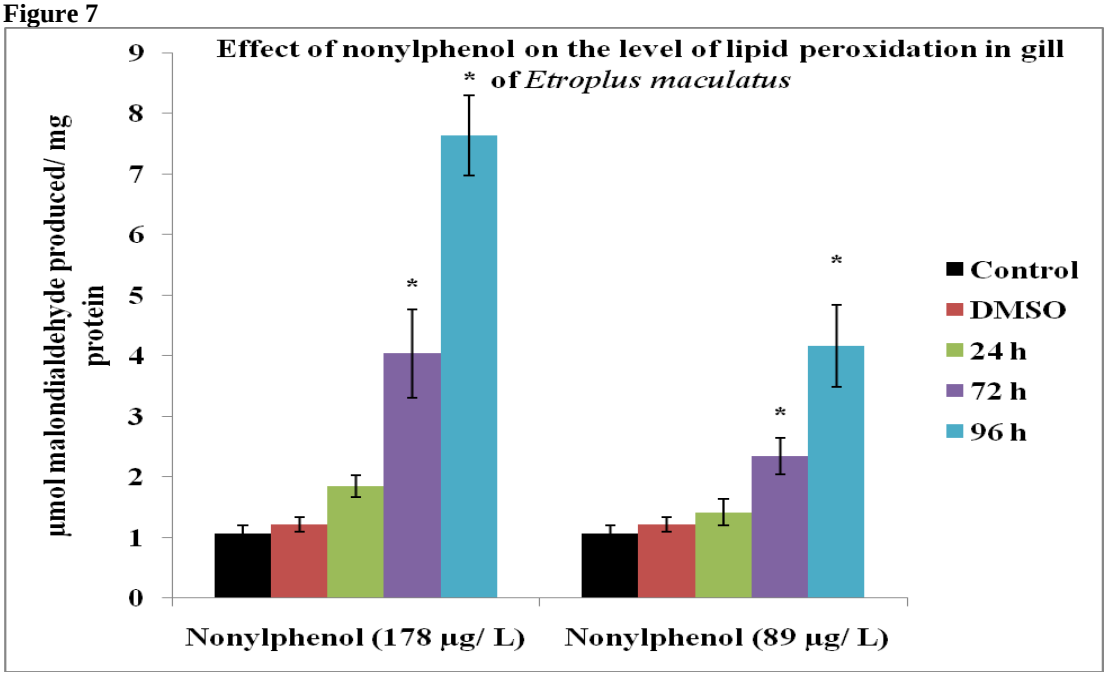
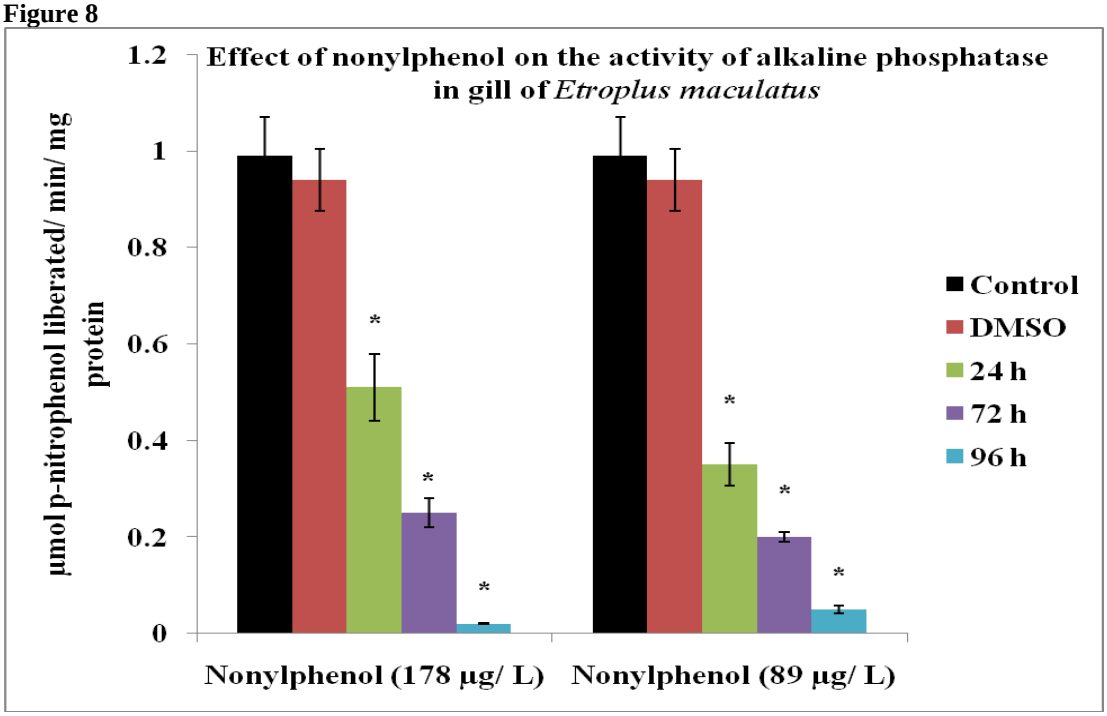


Figure 8



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