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Full Length Research Paper

Protective role of dietary corn silk supplements against *A. hydrophila* infection

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Corn silks are threads found in the maize plant (*Zea mays*) and traditionally used to treat urological infections and disorders. Corn silk is also known to possess nutrients and volatile compounds. However, this material is often disregarded and unused. This study therefore investigated the potential use of corn silk in aquaculture through its protective capacity in matured Nile tilapia (*Oreochromis niloticus* (L.) by measuring some nonspecific immune parameters (phagocytosis, production of reactive oxygen species, and plasma lysozyme level) in experimental *Aeromonas hydrophila*-challenged fish. The anti-oxidative property of corn silk was also investigated using paracetamol-induced hepatic toxicity in order to measure oxidative stress (malondialdehyde or MDA). Based on the results, phagocytosis was significantly higher in *A. hydrophila* infected fish fed with corn silk-coated feeds than in fish from the negative (PBS-injected) and positive control (*Aeromonas hydrophila* infected) treatments. Lysozyme level was also higher in corn silk-fed fish, but it was not significantly different from the positive control fish (*A. hydrophila* infected fish). Reactive oxygen species (ROS) was higher in corn silk-fed fish than the positive control fish but it was not statistically significant. MDA levels were significantly higher in paracetamol-treated fish than paracetamol-corn silk treated group. The results showed the potential immunostimulatory and antioxidant role of corn silk in Nile tilapia, but further studies are required to fully understand its mechanism of action and its full use in aquaculture.

Key words: Corn silk, lipid peroxidation, *Aeromonas hydrophila*, *Oreochromis niloticus,* immunostimulation.

INTRODUCTION

Presently, aquaculture is a fast growing food production industry that contributes nearly 50% of the annual fisheries production (FAO, 2012). In the Philippines, tilapia is the second most cultured fish species after milkfish. However, annual production of Nile tilapia is usually affected by episodes of high mortality mostly due to bacterial infections that could be attributed to very intensive culture practices and sometimes aggravated by seasonal effects of low environmental temperatures. One of the opportunistic bacteria that infect cultured Nile tilapia

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is *Aeromonas hydrophila* which causes septicemic infections in the fish (Cipriano, 2001). These pathogens are ubiquitous and are thus found in a variety of aquatic environments. To control these bacterial infections, antibiotics are mainly used.

However, antibiotic use may lead to the emergence or development of antibiotic-resistant bacteria, thus safer and more effective alternatives should be used (Pachanawan et al., 2008). Studies on the use of immunostimulants derived from natural bioactive products are presently gaining importance as an option instead of using chemotherapeutants and antibiotics. Immunostimulants enhance the non-specific immune responses (innate immunity) as well as the specific immune response mechanisms (adaptive or acquired immunity) of a certain organism (Anderson, 1992). Other immunostimulants like chitin, chitosan, and levamisole have been reported to enhance the non-immune responses of common carp (*Cyprinus carpio*) which led to a higher percentage survival of the fish (Gopalakannan and Arul, 2006).

Corn silk (*Zea mays*) is traditionally used in the treatment of cystitis, edema, gout, kidney stones, nephritis, and urological disorders (Ebrahimzadeh et al., 2008). It is also used as an anti-diabetic agent since it reportedly counteracts hyperglycaemia (Guo et al., 2009). It has phenolic compounds such as anthocyanins, vanillic acid, and protocatechuic acid which are reportedly responsible for its antioxidant capacity (Ebrahimzadeh et al., 2008). Corn silk extract has also shown great potential when it comes to prevention of diseases involving overproduction of radicals (Liu et al., 2011). Administration of natural products may be done via bathing, injection, or oral administration, the latter of which is the least stressful of all the three routes (Harikrishnan et al., 2011).

This study was therefore undertaken to investigate the protective role of dietary corn silk supplements against *A. hydrophila* infection and induced hepatic damage by measuring some nonspecific immune parameters and lipid peroxidation activity as indicator of oxidative stress.

MATERIALS AND METHODS

Fish and experimental design

Mixed sex *Oreochromis niloticus* obtained from the Southeast Asia Fisheries Development Center, Freshwater Fisheries Station, Binangonan, Rizal and Philippines were used. Prior to the experiments, the fish were acclimatised in rectangular tanks with recirculating water system. They were fed daily at a feeding rate of 3% of the total body weight with commercial grow-out tilapia pellets. Regular water change was done during the pre-experimental period. For the experiments, three groups of tilapia, 20 fish each (58.9 ± 1.76 g, 180 fish total), were designated: a) negative control, which consisted of healthy tilapia fed with commercial tilapia pelleted feeds; b) positive control, which consisted of tilapia infected with *A. hydrophila* and fed with commercial tilapia feed pellets; and c) corn silk treated group, which consisted of tilapia infected with *A. hydrophila* and fed with the corn silk-coated tilapia feed pellets.

In order to determine the antioxidant property of corn silk, another experimental set-up consisted of four groups of tilapia (42.5 ± 0.98 g; 80 fish) that were designated as: a) negative control, which consisted of tilapia fed with commercial pelleted tilapia feeds; b) positive control, which consisted of tilapia fed with feeds coated with paracetamol; c) paracetamol and silymarin treated group, which consisted of tilapia fed with a mixture of paracetamol and silymarin-coated fish food; and d) paracetamol and corn silk treated group, which consisted of tilapia fed with a mixture of paracetamol and corn silk extract-coated fish food.

Fish food preparation

The powdered corn silk (1.750 g) produced by Britmix Wellness, Inc. was coated onto 500 g of commercial fish feed pellets. The powdered corn silk was mixed into a 22% gelatin solution and this mixture was coated onto the fish feed pellets. The coated feeds were air dried for 48 h and both the coated and uncoated feed pellets were stored in the refrigerator (4°C) to prevent bacterial and fungal contamination. The corn silk-coated fish food pellets were used for the fish in the first experiment. For the second experiment, four types of fish diets were used: a) for the negative control group, unsupplemented commercial fish food, b) for the positive control group, powdered paracetamol (1000 mg) was coated using gelatin onto 500 g of fish feed pellets, c) for the paracetamol-silymarin reference group, 1000 mg of paracetamol and 450 mg of silymarin (Steinbach Products, Inc.; Mandaluyong, Philippines) were coated onto 500 g fish feed pellets, and d) for the paracetamol-corn silk group, 1000 mg of paracetamol and 1000 mg of corn silk were coated onto 500 g fish feed pellets. The fish were fed at 3% body weight daily during the experimental period.

Preparation and Injection of bacteria

A. hydrophila culture was obtained from the National Institute of Molecular Biology and Biotechnology (BIOTECH) of the University of the Philippines - Los Baños. The bacterial innoculant was prepared through serial dilution and spread plate technique. Final bacterial concentration was adjusted to 6.2×10^5 CFU ml^{-1}. A 0.1 ml of the bacterial suspension was injected intramuscularly near the lateral line of the pectoral region of the fish from the positive control group and corn silk treated group of the first experimental set-up. Phosphate buffer saline (PBS) pH 7.2 with the same volume was injected into the negative control tilapia group. Bacterial infection was done after a 30 day feeding period.

Sample collection

For the immune response experiments, the fish in each treatment tanks were sacrificed at day 7 post-infection. Each fish was immobilized with a blow in the head. Blood was extracted from the midventral caudal peduncle. Plasma was obtained from the extracted blood by centrifuging at 10,000 rpm for 5 min at 4°C. Head kidney were dissected out and immersed in Petri dishes with cold supplemented fish physiological saline (FPS). For the antioxidant experiments, fish were sacrificed from each of the four tanks at day 16 of the experimental period. The livers were removed and immersed in PBS to be used in lipid peroxidation assay.

Lysozyme assay

Dilutions of hen egg white lysozyme (HEWL) was used as standard and prepared in phosphate citrate buffer (pH 5.8). *Micrococcus lysodeikticus* solution (75% w/v) buffered to pH 5.6 in phosphate citrate was mixed to HEWL standard dilution or tilapia plasma in a microtiter plate 7:1 ratio. Absorbance at 450 nm was read after 15 min using the ELISA plate reader. Using the standard curve of Vmax rates, plasma lysozyme concentrations were determined. Protein concentrations of the plasma samples were also determined.

Macrophage phagocytic activity assay

The head kidney samples were homogenized using a screen mesh and suspended in 3 ml supplemented Leibovitz-15 medium (L-15). The cells were centrifuged at 400 rpm for 5 min in room temperature. The pelleted cells were washed twice with supplement L-15. After washing, the cells were suspended in FPS and the cell count and viability was determined by staining the cells with trypan blue (1:9), utilizing the trypan blue exclusion method. The cell concentration was adjusted to 10^6 viable cells ml^{-1}. Opsonized yeast cells were used as feeds to phagocytes, approximately with 10^8 yeasts ml^{-1} in PBS, prepared and mixed with one (1.0) ml of 0.8% Congo red dye. The yeast suspension was then autoclaved and washed with an equal volume of PBS until the excess Congo red dye was removed.

The suspension was centrifuged at 400 rpm for 5 min. The cells obtained were suspended again in PBS. The prepared yeast cells were added to the cell suspension from each sample at 2:1 ratio. An aliquot sample of 20 µl was smeared onto a glass slide after one hr of incubation at room temperature. The smears were air dried and fixed with 95% ethanol. After 24 h, the films were stained with 1% eosin for one min, rinsed with distilled water, and dipped in Giemsa stain for two minutes. These were air dried for 24 h. Cover slips were mounted onto the glass slides using Entellan. The smears were observed under the oil immersion objective. Percentage of active phagocytes was recorded from a hundred phagocytes that were counted in representative areas of the slide. Data were expressed as mean percentages of active phagocytes.

Reactive oxygen species (ROS) production assay

The assay for the assessment of superoxide anion produced outside the mitochondria was done according to the protocol of Zelikoff (1996). Head kidney macrophages were obtained from the previous preparation and adjusted to a cell concentration of 4 x 10^6 cells ml^{-1}. Four microcentrifuge tubes were labeled (1 to 4), and cells (125 µl of 10^6 cells. ml^{-1}) were added to each of these four tubes containing 250 µl ferricytochrome solution (final concentration = 2 mg ml^{-1}) and 62.5 µl of bovine superoxide dismutase was added to the second and fourth tubes.

Ten microliters of phorbol 12-myristate 13-acetate was added to the third and fourth tubes at a final concentration of 2 µg ml^{-1}. Fish physiological saline solution (FPS) was added to each tube to bring up the total volume to 0.5 ml. An additional tube containing all the reagents but without the cells served as the blank. Each tube was vortexed for approximately 30 s and 200 µl aliquot placed into the wells of a 96-well microtiter plate. The absorbance was measured at 492nm for up to an hour. Time points used for measurement include: 0, 15, 30, 45, and 60 min. The plates were incubated at room temperature, in a humid environment, between readings. The rate of superoxide anion radical production was determined from measurements taken over time, while OD readings at a single time point were used to make comparisons between different exposure groups. Change in absorbance was calculated by subtracting the mean of the “blank” wells and the wells containing SOD from the absorbance measured in the non-SOD-containing wells. By multiplying the change in absorbance by 15.87 the nmol concentration of SOD-inhibitable superoxide anion radical was computed. Data are expressed as nmol O2/2x105 cells/unit time.

Lipid peroxidation assay

The thiobarbituric acid reactive substance assay (TBARS assay) was used to measure lipid peroxidation in hepatic tissues obtained from tilapia. It measure malondialdehyde (MDA), one of the compounds formed by lipids after oxidative processes. Briefly, using a ground glass homogenizer, one gram of liver tissue was homogenized in 2 ml of PBS. An aliquot of 0.5 mL of the homogenate was transferred in clean test tubes and 2.5 ml of trichloroacetic acid (TCA) and 1 ml of thiobarbituric acid (TBA) was added to all test tubes and then mixed through vortexing.

The samples were then subjected to a hot water bath for 30 min. The samples were allowed to cool and then 4 ml of butanol was added to each of the test tubes. The samples were vortexed and the organic layer was removed and placed in centrifuge tubes. The organic layer was centrifuged at 3000 rpm for 10 min at room temperature. The absorbance was read at 532 nm using tetramethoxypropane was used as the standard. Data were expressed in µmol MDA. mg $protein^1$.

Protein content determination

Protein content was determined with the use of the BIO-Rad protein assay kit. The dye was prepared by diluting one part of the concentrated dye with four parts of deionized water. The filtrate was then collected. Dilutions of BSA were used as standard. 10 µl of each standard and sample solution were added to 96 well plates. 200 µl of the diluted dye was then added to all wells. The plate was incubated for 5 min at room temperature and absorbance was read at 595 nm with the use of an ELISA plate reader. Data were expressed as mg ml^{-1}.

Data analysis

All of the data were analysed for normality of distribution using Shapiro-Wilk test and homogeneity of variance prior to one way ANOVA test at $P<0.05$. Kruskal-Wallis test was used as non-parametric test for data with non-normal distribution. Comparison of the data was employed by using Least Significance Difference test for homogenous data sets and Games-Howell test for non-homogeneous data. MDA concentration values were analysed using Mann-Whitney U test.

RESULTS AND DISCUSSION

The efficiency of an immunostimulant is evaluated by testing its ability to protect the fish against pathogens, and also by measuring the immune response produced (Galindo-Villegas and Hosokawa, 2004). In the present study, the protective effects of dietary corn silk supplementation were determined in Nile tilapia. In order to determine the immune enhancing property of corn silk, fish were fed for 30 days with corn-silk coated feed pellets and then inoculated with *A. hydrophila*. Subsequently, phagocytic activity, plasma lysozyme levels and production of reactive oxygen species were measured as indicators of nonspecific immune response. Experiments were also undertaken in order wherein

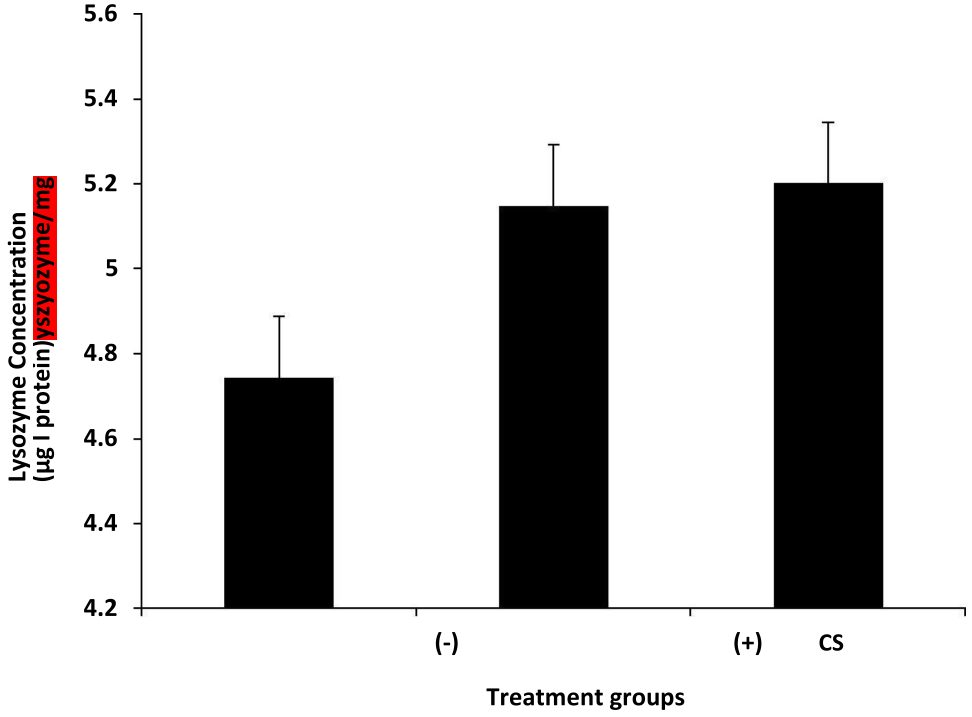

Figure 1. Plasma lysozyme levels from Nile tilapia (Oreochromis niloticus). Data are presented as mean ± SEM. (-), negative control, (+), positive control, CS, corn silk-treated group.

hepatic injury was induced through paracetamol-corn silk treatment to determine if corn silk could ameliorate oxidative stress.

Corn silk is composed of stigmas and styles of the *Zea mays* (maize) plant, and it has been used in traditional Chinese medicine for the treatment of various ailments (Ren et al., 2009). It has been reported that corn silk is an excellent source of many bioactive compounds such as flavonoids, saponin, alkaloids, tannins, phytosterols, allantoin, vitamin E and K, etc. (Hu and Deng, 2011; Ren et al., 2013). It is possibly due to these bioactive components that research studies in corn silk were undertaken to identify its relevance to human health, which includes its reported immune enhancing effects. Similarly, fish health researchers have continually searched for bioactive compounds that could be used to enhance fish immunity and protection against various pathogens. Best known immune stimulants are glucans and lipopolysaccharides, and synthetic compounds, animal and plant extracts and vitamins that usually target the nonspecific immunity of cultured fish species (Ardo et al., 2008).

Based on the results, higher lysozyme level was exhibited by the group of fish which were fed with corn silk coated feed pellets and infected with *A. hydrophila* (Figure 1) but the increase was not significantly different from that of the positive control group. It is likely that the concentration of the powdered corn silk added may not have been potent enough to raise the lysozyme levels in tilapia significantly. Alternately, the duration of the feeding period prior to the bacterial challenge could have been longer in order to boost the immune system.

However, it is likely that this increase in lysozyme level could also aid in the destruction of *A. hydrophila*. It is also worthy to note that bioactive compounds from corn silk could also inhibit bacterial infection. In a study by Nessa et al. (2012), antimicrobial activities of corn silk extracts and bioactive compounds were compared with that of gentamycin, and they found out that the extracts and flavonoids were significantly more sensitive against a number of bacteria.

Phagocytosis is the most primitive immune response mechanism, and basically involves the ingestion of a pathogen by macrophages or neutrophils. Phagocytic activity has been significantly enhanced by the corn silk treatment in this study. The corn silk treated group has statistically significant phagocytic activity than the positive and negative control groups (Figure 2). These results likewise indicate a potential immunostimulatory effect of corn silk in *A. hydrophila* infected tilapia. This response has also been exhibited by some fish species when treated with bioactive extracts. Yin et al. (2009) reported increase in phagocytosis in carp when treated with *Ganoderma lucidum* and *Astragalus radix*. They attributed these mainly to the polysaccharides, monosaccharides, flavonoid and alkaloid contents of both herbs.

Production of ROS is considered as an important

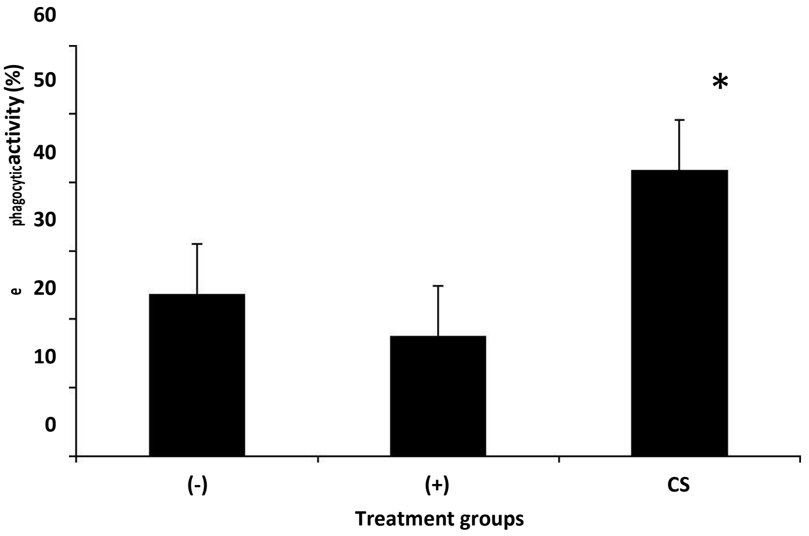

Figure 2. Percentage phagocytic activity of head kidney phagocytes. Data are presented as mean ± SEM. *- CS, corn silk treated group significantly different ($P < 0.05$) from the positive control.

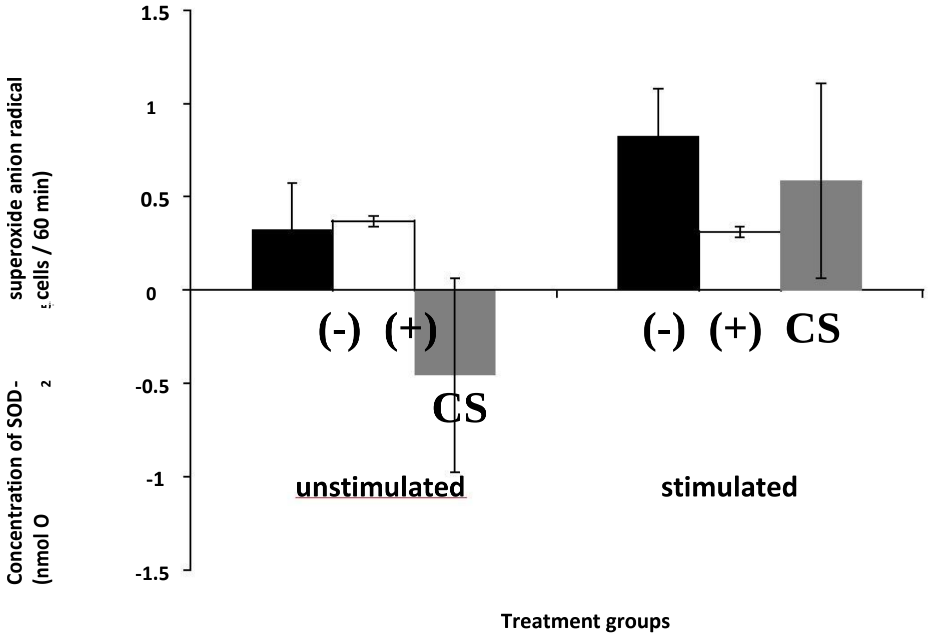

Figure 3. Concentration of SOD-inhibitable superoxide anion radical (nmol O_2/2 x 10^5 cells/60 min) of head kidney macrophages in Nile tilapia. Data are presented as mean ± SEM. (-) negative control, (+) positive control, CS- corn silk-treated group.

microbial killing mechanism (intracellular and extracellular) in vertebrates. Animals have inherent enzymes (antioxidants) to detoxify these anions to counteract the possible adverse effects in normal cells and tissues. Antioxidants include superoxide dismutase, catalase, glutathione S-transferase, glutathione peroxidase, vitamin E components such as α- tocopherol and γ-tocopherol. One study suggested that α-terpineol, citronellol, and eugenol, are some of the main compounds involved in the antioxidant action of corn silk (El-Ghorab et al., 2007). More recent studies relate flavonone glycosides as potent antioxidants (Hu and Deng, 2011; Ren et al., 2013).

This study confirmed the ROS scavenging capacity of corn silk especially in unstimulated head kidney cells (Figure 3) where ROS production was greatly inhibited.

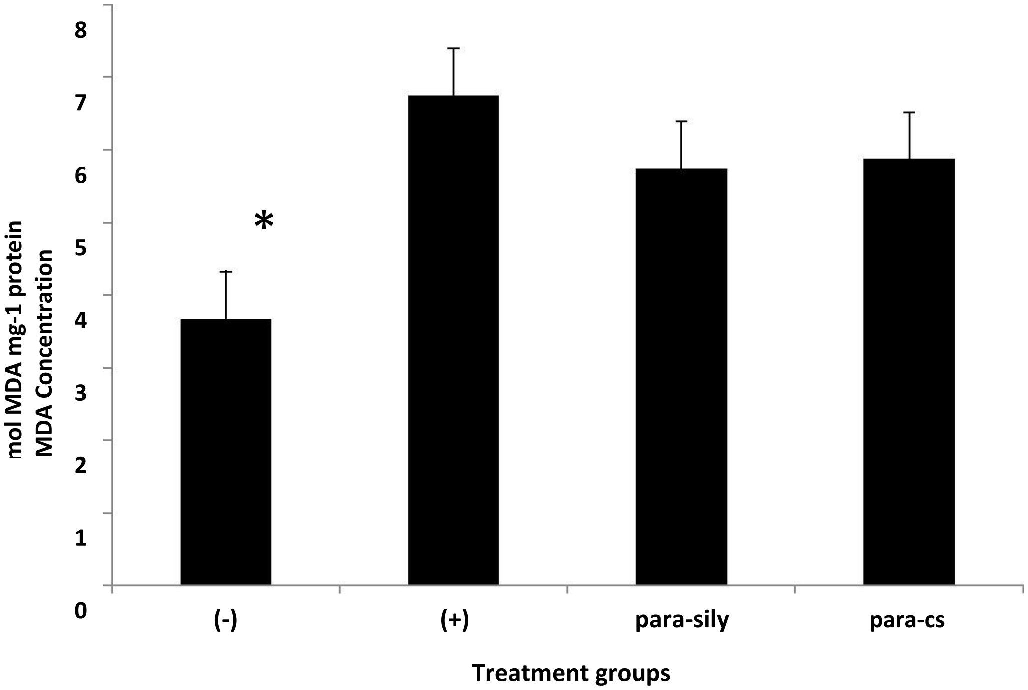

Figure 4. MDA concentrations in Nile tilapia at day 16 of paracetamol treatment. Data are presented as mean values ± SEM. ***** (-) group significantly different from the (+) and CS groups ($P < 0.05$). **(-)**, negative control fish, fed with unsupplemented diet; **(+)**, positive control fish, diet with paracetamol; para-sily, paracetamol and silymarin-treated group, diet with paracetamol and silymarin; para-cs, paracetamol and corn silk-treated group, diet with paracetamol and corn silk.

This effect was also shown by lower ROS levels obtained in PMA-stimulated cells from corn silk-treated fish. The scavenging activity and antioxidative capacity (anti-lipid peroxidation) have been recently confirmed also by Hu and Deng (2012) and Ren et al. (2013). It should be taken note of that ROS are actually produced by macrophages as microbial killing mechanisms. Kim et al. (2005) reported that corn silk activates murine macrophages to produce cytokines and enzymes that are important in regulating normal physiological functions inflammatory response in macrophages. The data from this study likewise showed the immunostimulatory role of corn silk in macrophages from tilapia head kidney. As for oxidative stress results, the negative control fish had significantly lower MDA levels than the positive control fish as shown in Figure 4. The paracetamol-corn silk treated group has a lower concentration of MDA compared to the paracetamol-silymarin treated group, but this was not statistically different. The results showed that corn silk had comparable antioxidant effects as silymarin, and these treatments were able to alleviate, although not significantly, the oxidative effects of paracetamol on the hepatic cells. Paracetamol hepatotoxicity is highly dependent on the cytochrome P450 enzyme system. As paracetamol is metabolised at the cytochrome P450 system, it is converted to the toxic metabolite N-acetyl-P-benzenequinoneimmine (NAPQI). NAPQI is a toxic free radical that binds to proteins and fatty acids and reacts with glutathione, a natural antioxidant, eventually depleting it. Most importantly, paracetamol leads to the formation of reactive oxygen and nitrogen species that induce oxidation of cellular membranes.

In the present study, silymarin was used as reference substance since it is known to have four different flavonoids that make it a good antioxidant. Its main flavonoid component is silbin with isosilibinin, silydianin, and silychristin. It has been proven to protect against carbon tetrachloride toxicity, acetaminophen (paracetamol), phalloidin, galactosamine, and thioactemide (Pradhan and Girish, 2006; Pradeep et al., 2007). Similarly, corn silk contains many isolates of flavonoids like myricetin, fisetin, quercetin, naringin and luteolin which have been shown to possess antioxidant and prooxidant properties of varying degrees.

However, some studies showed different flavonoid synergists as most effective in hepatoprotection. Moreover, it has been reported that corn silk effectively increases antioxidant enzyme levels such as sodium dismutase and glutathione peroxidase (Hu and Deng, 2011; Nurhanan et al., 2012). In a study by Liu et al. (2011), two flavones glycosides were isolated from the n-butanol extracts of corn silk that exhibited very high antioxidant and free-radical scavenging activities. The present study confirmed the potential of corn silk as a potent antioxidant in a cultured fish species. This could be relevant in feed formulation where high concentrations of lipids or fatty acids are used, and thus necessitates the addition of antioxidants. However, the appropriate dietary dosage in fish still need to be further investigated. Likewise, further immune-based trials are needed to maximize fully the protective property of corn silk against fish pathogens.

Conflict of Interest

The authors have not declared any conflict of interest.

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