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Effect of Black Mustard (*Brassica nigra*) on the Interaction between Immune and Biotransformation Systems of Nile Tilapia (*Oreochromis niloticus*) Exposed to Benzo-a-Pyrene

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ABSTRACT

Aquatic pollutants are responsible for any alterations in fish health. Our study was designed to examine the efficacy of dietary supplement of black mustard seed, *Brassica nigra* (30% crude and 1% alcoholic extract) on enhancement of the immunity and detoxification response of Nile tilapia, *Oreochromis niloticus* after exposure to a hydrocarbon pollutant; Benzo-a-Pyrene (BaP). Fish were divided into 3 groups before being fed for 28 days with crude black mustard seeds, 1% alcoholic extract and un-supplemented commercial diet as the control. At the end of experiment, fish were exposed to 1 mg L⁻¹ of BaP for 24 h. Humoral immune parameters (lysozyme activity, antiprotease activity and total protein), ethoxyresorufin-o-deethylase (EROD) and hepatic cytochrome P450 1A1 content (CYP1A1) were investigated. Results recorded high significant difference (p<0.05) of antiprotease and total protein values in group fed with 1% extract followed by crude seeds as compared with control. The highest significant lysozyme activity (p<0.05) was recorded also in group fed with 1% extract as compared with other groups. On the other hand the extract group revealed a significant decrease in EROD (p<0.05) and CYP1A1 content compared to other groups. Which lowering the possible toxic effect of bio-transformed metabolic intermediate compounds. While the EROD activity and CYP1A1 content in group fed with the crude seeds showed increase over the control one. Therefore, the results suggest using of black mustard extract as a dietary supplement to increase the immune function and enhance the detoxification process of pollutants and hence, reduce the harmful effect of aquatic pollutants on Nile tilapia health.

Key words: *Brassica nigra*, benzo-a-pyrene, cytochrome P4501A1, immunity, *Oreochromis niloticus*

INTRODUCTION

Now-a-days, aquaculture represents one of the most growing sectors of agricultural business which expands all over the world. Unfortunately, aquaculture is faced by some challenges; water pollution is the most dangerous one which coming mostly from urban industrial wastewater, agriculture and domestic sewage. In particular, river Nile can be considered as a model of polluted aquatic ecosystem because it receives untreated wastes from various drain outlets coming mainly from different sources of chemical contamination such as pesticides and petroleum hydrocarbons (Osman *et al.*, 2012; Osman and Kloas, 2010).

Polycyclic Aromatic Hydrocarbons (PAHs) are widely distributed in different water ecosystems (both marine and freshwater systems) and bio-accumulate in several aquatic species (Couch and

Harshbarger, 1985; Weeks *et al.*, 1990). Furthermore, it represent one of the most significant classes of organic pollution due to their carcinogenic and mutagenic potential (Barra *et al.*, 2001). Benzo-a-Pyrene (BaP) is a Polycyclic Aromatic Hydrocarbon (PAHs) which widespread in world environment due to their occurrence in petroleum, coal, soot, air pollutants and cutting oils (Hardin *et al.*, 1992; Mudzinski, 1993). It releases to the environment through the incomplete combustion of gasoline, garbage or any organic material and finds its way to the aquatic environment through runoffs, oil spill, industrial effluent and atmospheric disposition (Lee *et al.*, 1977).

Immuno-toxicity of BaP has been studied in mammalian systems (Ward *et al.*, 1985; White *et al.*, 1994). Furthermore, BaP also induces immune alterations in fish (Faisal and Huggett, 1993; Holladay *et al.*, 1998; Smith *et al.*, 1999). Previous study used single intraperitoneal (IP) injection of BaP and demonstrated suppression of innate, humoral and cell-mediated immunity of Japanese medaka (*Oryzias latipes*) as well as host resistance against infection with the bacterial pathogen *Yersinia ruckeri* (Carlson *et al.*, 2002a). Specifically, BaP dose-dependently suppressed T-lymphocyte dependent humoral immunity in fish 48 h following exposure to 2-200 µg BaP/g body weight. Moreover, BaP had been shown to induce immuno-suppression and cytochrome P4501A1 (CYP1A1) mediated catalysis in liver of Japanese medaka (Carlson *et al.*, 2002a, b). Furthermore, BaP had been shown to induce Cytochrome P4501A1 (CYP1A1) enzyme and EROD activity in liver of *Oreochromis niloticus* and *Clarias gariepinus* after 48 h exposure to 1 mg L⁻¹ (Hasaanain *et al.*, 2007). It is worth mentioning that CYP plays an important role the metabolism of numerous pollutants and represents a vital biomarker for PAHs presence in water bodies. The biotransformation of BaP may produce some reactive products which can bind to DNA and produce genetic alterations and mutations (Hasaanain *et al.*, 2007).

Generally, water pollution generates many stress factors which affect both immune and biotransformation systems in fish and increase the susceptibility to infectious diseases. Using antibiotics and other chemotherapeutics to control diseases may led to the development of strain resistant pathogens, environmental pollution and accumulation of residues in fish (Alexander *et al.*, 2010; Awad *et al.*, 2013). Therefore, there is a great direction toward using medical plants and its active ingredients to enhance the immune and biotransformation systems of farmed fish and elevate their resistance toward infectious diseases (Awad, 2010). The protection of aquaculture organisms against pollution effects by designing special diets able to modulate the enzymes involved in the phase-I and phase-II detoxification mechanism is of going interest. Family Brassicaceae has been studied due to the pharmacologic properties of its main metabolites; the glucosinolates. Glucosinolates have been shown to induce the activity of the phase II detoxification enzymes and inhibit phase I (activating) enzymes, exerting an anticancer effect (Johnson, 2002; Cartea and Velasco, 2008; Jeffery and Araya, 2009). They have also been shown to provide protection from oxidative stress through elimination of reactive oxygen species (Jeffery and Araya, 2009; Traka and Mithen, 2009). Sinigrin, a glucosinolate compound in *Brassica nigra* L. (black mustard) is hydrolyzed to allylisothiocyanate by myrosinase enzyme present in the plant tissues. Allylisothiocyanate are able to modulate phase I enzymes, particularly cytochrome P450 (Valgimigli and Iori, 2009). Interestingly, the immunological effect of black mustard has not examined before neither on human nor animal.

So the present work was carried on to study the possible effect of diets supplemented with crude seeds and alcoholic extract of black mustard (*Brassica nigra*) to enhance the immune and detoxification systems in fish and therefore modulate or/and resist the harmful effect of pollutant on fish health.

MATERIALS AND METHODS

Plant extract: *Brassica nigra* seeds were obtained from the Experimental Station of Medicinal plants, Department of Pharmacognosy, Faculty of Pharmacy, Cairo University. The seeds were firstly crushed (crude), before macerated in cold methanol. The filtrate is evaporated under reduced pressure resulting in solid residue kept for the experiment.

Fish, experimental design and sampling: Nile Tilapia (*Oreochromis niloticus*) of average weight 18 ± 0.2 g were obtained from commercial fish farms in Kanter alkairya and acclimatized in aerated free-flowing freshwater. During acclimatization, fish were fed twice daily with a commercial diet (27% protein ration) at rate of 3% of the fish body weight. Water in the aquaria was changed twice a week to avoid metabolite accumulations. Fish were divided into 3 groups (two replicates for each) before being fed for 28 days with, 1% extract of black mustard seeds (group 1), 30% of crashed crude seeds (group 2) and with un-supplemented commercial diet as the control (group 3). The experiment was conducted on August-September, 2014. At the end of experiment, fish were exposed to 1 mg L^{-1} of Benzo-a-pyrene (dissolved in 0.5 mL of Dimethyl sulfoxide (DMSO), Sigma) for 24 h. Blood was collected from fish (anesthetized by MS-222, Sigma-Aldrich, Basingstoke, U.K.) by venipuncture using 3 mL syringe before transferred to clean dry tubes and left to clot for 2 h at 4°C before centrifuging at 3000 rpm for 25 min at 4°C . Serum was collected and stored at -20°C until use.

Liver samples were thawed on ice and then approximately 0.5 g of each liver was homogenized by a high speed glass-Teflon homogenizer in KCl-HEPES buffer; 0.15 M KCl, 0.02 M HEPES, pH 7.5. The homogenate was centrifuged at 9000 g for 30 min at 4°C . The supernatant (S9 fraction) was collected and stored in liquid nitrogen till CYP and EROD assays (Parente *et al.*, 2004).

Immune parameters

Antiproteases activity: The serum anti-trypsin activity was measured by established methods (Ellis, 1987; Lange *et al.*, 2001). Thus, 20 μL of standard trypsin solution (Sigma-Aldrich, 5 mg mL^{-1}) was incubated with 20 μL of serum for 10 min at 22°C . Subsequently, 200 μL of 0.1 M PBS (PH 7.2) and 250 μL of 2% azocasein solution (20 mg mL^{-1} PBS) were added before incubation for 1 h at 22°C . The reaction was stopped with the addition of 500 μL of 10 % (v/v) trichloro acetic acid (TCA) and incubated for 30 min at 2°C . The mixture was centrifuged at 6000x g for 5 min and 100 μL of the supernatant was transferred to a 96 microwell flat bottom plate containing 100 μL of 1 M NaOH/well. The absorbance was read in the spectrophotometer at 410 nm. Positive control (100%) was prepared by replace the serum with buffer. For a negative control, buffer replaced both serum and trypsin. The percentage inhibition of trypsin activity was calculated by comparing with a positive control sample.

Total protein: Serum protein and liver homogenates protein S9 were measured according to Lowry (Lowry *et al.*, 1951).

Lysozyme activity: The turbidimetric assay for lysozyme was carried out according to (Parry *et al.*, 1965). Thus, 40 μL of serum was added to 2 mL of a suspension of *Micrococcus lysodeikticus* (Sigma-Aldrich, 0.2 mg mL^{-1}) in a 0.05 M sodium phosphate buffer (pH 6.2). The reaction was carried out at 25°C and absorbance was measured at 530 nm after 0.5 and 4.5 min on a spectrophotometer. A unit of lysozyme activity was defined as the sample amount causing a decrease in absorbance of 0.001 min^{-1} .

Cytochrome P450 measurements

Determination of EROD activity: Cytochrome P450 activity (CYP1A1) was estimated as ethoxyresorufin-o-deethylase (EROD) activity, it was determined according to Hodson *et al.* (1991). In a glass centrifuge tube, the reaction mixture contained 0.1 M HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid) buffer, pH 7.8+1.28 M Magnesium Sulphate+ Bovine Serum Albumin (BSA)+0.5 mM freshly prepared Nicotinamide Adenine Dinucleotide Phosphate; reduced form (NADPH). Then S-9 preparation was added to each tube and the tubes were incubated for 10 min in a water bath at 25°C. The reaction was initiated by addition of 10 µL of Ethoxyresorufin substrate to each tube, incubated for 10 min in a water bath at 25°C and the reaction was terminated by addition of 2.5 mL of methanol to each reaction tube. Samples were then centrifuged at 9000 g for 10 min at 4°C to precipitate proteins. Each clear supernatant was transferred to clean fluorimeter cuvette and the fluorescence of the supernatant was measured by spectral-fluorimeter (JASCO EP777, Japan) at excitation/emission wavelengths of 530/585 nm. EROD activities were expressed as pmol resorufin/mg protein/min.

Immuno-detection of CYP content: Cytochrome CYP450 1A1 protein level was determined by a semi-quantitative Enzyme Linked Immuno-Sorbent Assay (ELISA) as developed by Goksoyr (1991) and the optical density values were obtained by the ELISA reader at 492 nm.

Statistical analysis: Data were analyzed by one-way analysis of variance (ANOVA). The differences among treatments were compared by Tukey's test using Minitab statistical software (Minitab, Coventry, UK). Data were considered significant at $p < 0.05$.

RESULTS

Immune parameters: The serum antiprotease activity (Fig. 1) and total protein (Fig. 2) values were significantly increased ($p < 0.05$) in specimens fed with extract (group 1) followed with crude (group 2) as compared to control (group 3) which showed the lowest values. Similarly, the highest significant serum lysozyme activity ($p < 0.05$) was recorded in extract (group 1) as compared with other groups (Fig. 3). On the contrary to other result, the lowest lysozyme value was recorded in crude.

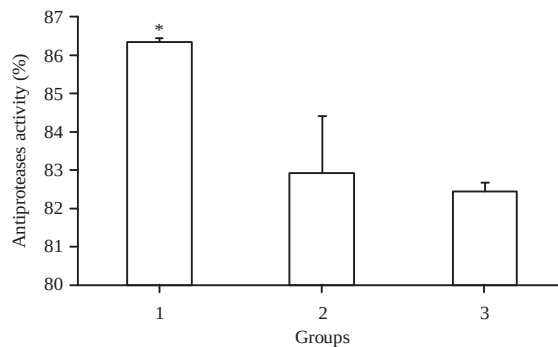


Fig. 1: Antiprotease activity of Nile tilapia fed diets supplemented with mustard extract (group 1), mustard crude (group 2) and un-supplemented diet as control (group 3), *Significant difference from control $p < 0.05$, Bars: Mean $\pm$ SE

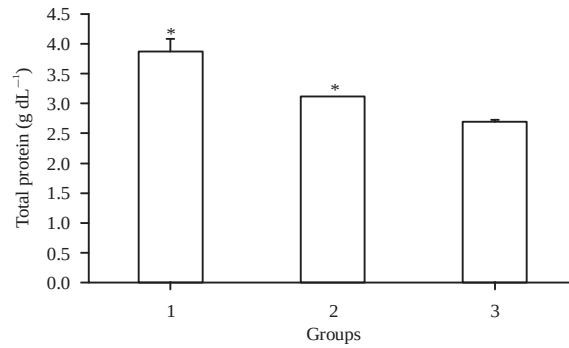


Fig. 2: Total protein activity of Nile tilapia fed diets supplemented with mustard extract (group 1), mustard crude (group 2) and un-supplemented diet as control (group 3), *Significant difference from control $p<0.05$, Bars: Mean $\pm$ SE

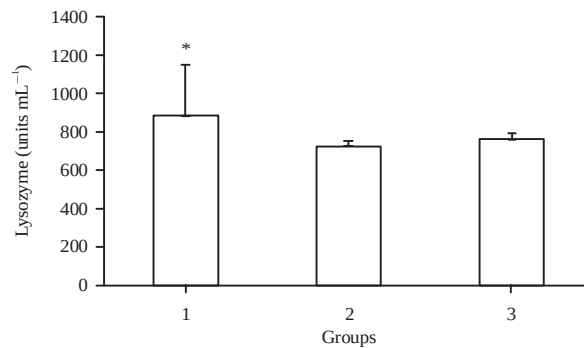


Fig. 3: Lysozyme activity of Nile tilapia fed diets supplemented with mustard extract (group 1), mustard crude (group 2) and un-supplemented diet as control (group 3), *Significant difference from control $p<0.05$, Bars: Mean $\pm$ SE

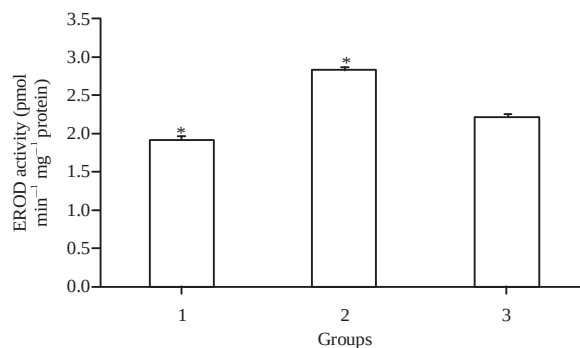


Fig. 4: Hepatic EROD activity of Nile tilapia fed diets supplemented with mustard extract (group 1), mustard crude (group 2) and un-supplemented diet as control (group 3), *Significant difference from control $p<0.05$, Bars: Mean $\pm$ SE

Cytochrome P450 measurements: The hepatic EROD activity of *Oreochromis niloticus* fish fed on extract (group 1) was significantly lower ($p<0.05$) than that of group 3 which received the ordinary food (control). While fish fed on the crude *Brassica* seeds (group 2) showed a significant increase ($p<0.05$) in EROD activity over the control group (Fig. 4). Also there was a decrease in CYP

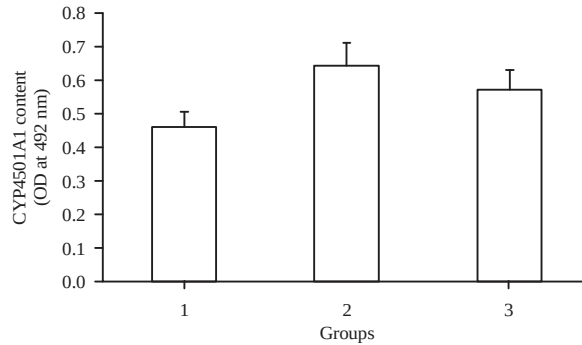


Fig. 5: Cytochrome P450 1A1 content of Nile tilapia fed diets supplemented with mustard extract (group 1), mustard crude (group 2) and un-supplemented diet as control (group 3), *Significant difference from control $p < 0.05$, Bars: Mean $\pm$ SE

content of Nile Tilapia feed on alcoholic extract (group 1) compared to the control (group 3) and the fish which received the crude seeds exhibited more CYP content than that of control group (Fig. 5).

DISCUSSION

Organic water contaminants, especially Polycyclic Aromatic Hydrocarbons (PAHs), represent a serious hazard to fish health, since they cause immuno-suppression and increase the susceptibility to many infectious diseases. Biotransformation and immune systems are both concerned with the capacity of the organism to resist many of the environmental stress factors like; viral infection, bacterial infection and exposure to xenobiotic. Some studies have cleared the complementary relationship between biotransformation and immune systems in many animals (Reynaud *et al.*, 2008).

Antiproteases play vital roles in the defense mechanism of various organisms by regulating and inhibiting the activities of potentially destructive proteases. Antiproteases inhibits the action of proteases either by binding to their active sites or by 'trapping' the protease to prevent protein hydrolysis (Laskowski and Kato, 1980) and thus, restrict the ability of bacteria to invade and to grow in fish (Ellis, 2001). Several studies showed remarkable enhancement in antiprotease activity after administration diets contains plants/ plant extracts meal (Vasudeva Rao and Chakrabarti, 2005; Christyapita *et al.*, 2007; Awad, 2010). In similar manner, feeding with Mustard extract (group 1) showed significant increasing in antiproteases than control. Also, mustard crude (group 2) showed increasing but without significant difference. Interestingly, the groups treated with either mustard extract or crude resisted the harmful effect of pollutant and enhanced the immune status of fish.

Serum proteins have diverse functions, including regulation of the water balance in fish (Wedemeyer and Yasutake, 1977) and protective agents through the role of acute phase proteins in limiting the dispersal of infectious agents (Larsen *et al.*, 2001; Gerwick *et al.*, 2002). Therefore, many researchers attributed the high concentrations of total protein in fish fed with immuno-stimulants to the enhancement of the non-specific immune response. For example, Ardo *et al.* (2008) recorded enhanced total protein in Nile tilapia fed with diets supplements with plant immuno-stimulant (0.1% yellow leader, *A. membranaceus* and Japanese honeysuckle, *Lonicera japonica*). Similarly, rainbow trout recorded enhanced total protein levels fed with 0.1 and 1% ginger, mistletoe and stinging nettle (Dugenci *et al.*, 2003). Fortunately, significantly increased in

serum total protein ($p < 0.05$) were observed in groups fed with either mustard extract (group 1) or crude (group 2) compared to control after exposure to benzo-a-pyrene. Such observation are strengthens the effective role of those immuno-stimulant to increase the immune status to resist the pollutants.

Lysozyme is considered as important parameter in the immune defense system of both invertebrates and vertebrates (Wang *et al.*, 2010). In fish, lysozyme is recognized to be an opsonin and activates the complement system and phagocytes (Grinde, 1989). Thus, it plays an important role in the host defense mechanisms against infectious diseases (Lundblad *et al.*, 1979; Lindsay, 1986; Lie *et al.*, 1989). The results of this study revealed a significant increase in lysozyme activity in mustard extract group than the other groups. Also, that could attributed to the efficiency of mustard extract as immuno-modulator to resist the dramatically effect of BaP. The harmful effects of PAHs on serum lysozyme activity have been demonstrated in fish. For example; Rainbow trout (*Oncorhynchus mykiss*) showed reduced in lysozyme level after injected with diesel-oil drilling mud (Tahir and Secombes, 1995). Similarly, decreasing in serum lysozyme activity was recorded in dab (*Limanda limanda*) exposed oil-contaminated sediments (Secombes *et al.*, 1997). On other hand, several studies showed remarkable efficiency by medical plants to enhance immune system in fish and elevate its status to resist infection diseases (Ardo *et al.*, 2008; Awad, 2010; Ndong and Fall, 2011; Awad *et al.*, 2013).

Biotransformation of xenobiotic involve two main phases, cytochrome P450 (CYP450) is the main enzyme responsible to phase I, in which the organic PAH pollutant transformed to more hydrophilic products like phenols, dihydrodiols, quinones and epoxides that easily conjugated and excreted from the organism (Livingstone, 1998). Some of the metabolic intermediates that formed during the phase I biotransformation can bind covalently to DNA and RNA that cause cytotoxicity, mutagenicity and even cancer. This carcinogenic activity of the PAHs can be increased by enhancement the metabolism through activation of detoxification pathways (Gelboin, 1980). Fortunately, in our study there is a significant decrease in EROD activity in fish fed on 1% mustard alcoholic extract (group 1) and this may represent a good protection of fish health after exposed to BaP. Moreover the decrease in CYP content in the same group which was accompanied by increase in different immunity measurements has confirmed the possible interaction between biotransformation and immunity systems. This was also in accordance with previous studies which stated the inhibition of phase I enzymes after exposure to glucosinolates; the main metabolite of *Brassica nigra* (Cartea and Velasco, 2008; Jeffery and Araya, 2009).

On the other hand, the significant increase in EROD activity and the insignificant one in CYP content in fish feed on crude mustard seeds (group 2) revealed its inefficiency to control the biotransformation system and the possibility of producing carcinogenic products. The mustard seeds contain many active compounds; alkaloid, sinapine, myrosin, sinigrin, inosite, albumins, gums and coloring matters (Upwar *et al.*, 2011). The extraction process plays an important role in determining the constituents of the extract and their concentrations. For example, the alcoholic extraction contains higher concentrations of hydrophobic alkamides and water extraction contains the more hydrophilic polysaccharides (Bauer, 1998). Accordingly, the extraction method and the resulting extracts may be completely different in their biological activity and action, in turn differ from the crude one. Some previous studies reported the efficiency of the alcoholic extracts than crude or water extracts (Bae *et al.*, 2012). Herein we report on the first study of protective and immuno-modulatory effects of the alcoholic extract of black mustard seeds on Nile tilapia (*Oreochromis niloticus*) exposed to Benzo-a-pyrene.

Therefore, diet supplementation with black mustard seeds extract resulted in protective effects against the toxic influence of Benzo-a-pyrene on some examined biochemical parameters in the liver tissue of fish. Our findings demonstrated that black mustard seeds may be helpful in reducing the adverse effects of benzo-a-pyrene by down regulate biotransformation CYP enzyme and its EROD activity. The effect is more pronounced with the methanol extract due to high glucosinolate content extracted with methanol than that extracted by water (Devi and Thangam, 2010). However, further investigations are necessary to elucidate the mechanism of black mustard seeds mediated protection against benzo-a-pyrene toxicity in aquatic animals.

CONCLUSION

In conclusion, addition of a medicinal plant, *Brassica nigra*, to *Oreochromis niloticus* food improves both the immune and biotransformation systems after exposure to a polycyclic aromatic hydrocarbon, BaP. This integration between the two systems could protect fish against pollution and adverse effects on fish health and productivity.

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