ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Effect of leek (*Allium ampeloprasum* L.) extract on biotransformation enzymes and innate immunity of catfish (*Clarias gariepinus*) exposed to Benzo[a]Pyrene.

Wafaa T Abbas[a] and Elham Awad[a]*.

[a] Hydrobiology Department, Veterinary Research Division, National Research Center, Dokki, Giza, Egypt.
[a]* Department of Hydrobiology, National Research Center, El-Tahrir Street, Dokki, Giza, Egypt.

ABSTRACT

Aquaculture is subjected to various toxicants which affect the health status ending with dramatically lost production. The study investigated the prospective effect of Leek extract (*Allium ampeloprasum* L.) on detoxification and innate immuno response of catfish (*Clarias gariepinus*) exposed to benzo[a]pyrene (BaP). Fish were divided into 4 groups before being injected with 0 (control), 5, 50, 500 mg/kg of leek extract (dissolved in Dimethyl sulfoxid). After 2 weeks, fish were exposed to 1mg/L of BaP for 24 h and sampling was done before and after exposure. Biotransformation enzymes (cytochrome P4501A1 (CYP4501A1), Ethoxyresorufin-O-Deethylase (EROD) and Glutathione-S-transferase (GSH) activities), BaP metabolites in bile and innate immune parameters (antiproteases, myeloperoxidase, total protein, albumin and globulin) were examined. Results demonstrated significant induction in EROD and GSH activities in all fish groups injected leek extract, while after exposure to BaP, their induction recorded the highest values in the low dose group (5mg/Kg). Moreover, CYP450 content showed no significant differences before and after exposure to BaP. No metabolite residues were detected in fish bile in both control and treated groups after BaP exposure using GC-MASS analysis. Furthermore, all doses of leeks showed enhancement in all examined innate immune parameters. However after exposure to BaP, only 5 mg/kg dose demonstrated ability to resist the toxic effect of BaP and enhanced immune parameters. To conclude, the injection of catfish with low dose of leek extract may contribute in protecting fish from harmful effect of BaP through enhancement biotransformation and immune systems.

Keywords: *Allium ampeloprasum* L., *Clarias gariepinus,* immunity

Corresponding author

ISSN: 0975-8585

INTRODUCTION

Fish represent one of the most valuable and cheapest animal protein sources, they are subjected to many environmental stresses, water pollution is the most effective one that lead to great losses and decrease in fish production. Benzo[a]pyrene (BaP) is an organic environmental contaminant that is widely spread in both aquatic and terrestrial ecosystems. It belongs to the polycyclic aromatic hydrocarbons (PAHs) and finds its way to the environment through incomplete combustion of any organic substance such as plants, dead animals, garbage, oil and coal (Hardin et al., 1992 ; Mudzinski, 1993).

Metabolism and detoxification of xenobiotics are performed in two metabolic phases. BaP is firstly metabolized into quinine metabolites through some oxidation-reduction reactions that mediated by phase I biotransformation enzyme, cytochrome P450. The redox reactions create reactive oxygen species (ROS), such as superoxide, hydrogen peroxide and hydroxyl group (Kiruthiga et al., 2007). The over release of ROS create oxidative stress where bind and damage DNA, RNA and other proteins, finally causing carcinogenic and mutagenic alterations (Oldham & Bowen, 1998). Phase II involves the conjugation of the phase I products with larger molecules, such as glutathione, glucuronic acid or sulphate to produce more hydrophilic metabolites that could be excreted and eliminated from the fish body (Široká & Drastichova, 2004).

The main idea of the immune system is that it is a group of mechanisms that protects the animal from invading pathogens. Therefore any suppression of the immune system could lead to a dramatic malfunction of the cells and resulted in death. On the other hand, the bacterial infection was responsible for suppressing biotransformation activities in the fish tissue. Carp (*Cyprinus carpio*) infected with *Listeria monocytogenes* bacteria, showed reduction in the liver, spleen, and head kidney enzyme activities such as cytochrome P450 levels and ethoxyresorufin O-deethylase (EROD) activity (Chambras, 1999). Toxicants including metals, pesticides and polycyclic aromatic hydrocarbons may induce immunosuppression. Moreover, these compounds may cause inadequate responses towards host tissues (Burnett, 2005).

Several variations in the immune response of fish have been reported by many authors toward exposure to BaP (Abbas et al., 2016 ; Holladay et al., 1998 ; Smith, 1999). For example, Japanese medaka (*Oryzias latipes*) injected intraperitoneal with BaP (2–200 ug/g BW) which showed suppression of respiratory burst, lymphocyte proliferation and humoral immunity in addition to weak resistance against infection with *Yersinia ruckeri* (Carlson et al., 2002a). Similarly, Japanese medaka exposed to BaP showed suppression in leukocyte superoxide production at head kidney cells (Carlson et al., 2004). Moreover, exposure to BaP (20 and 200 mg/g BW) resulted in suppression of antibody-forming cell and superoxide production accompanied by an increase in cytochrome P4501A1(CYP1A) expression/activity in Japanese medaka (Carlson et al., 2002b). It must be noted that stimulating the immune system relies upon the route and time of administration as well as the dosage (Sakai, 1999).

Recently, the world turns to use natural products to get rid of toxicants and avoid the non degradable substances which cause stress in the environment. Plants and their derivatives are considered as the most important natural products, since it being easy and cheap to obtain. It is well known that plants possess antioxidant compounds which act as protective agents that inactivate reactive oxygen species and therefore delay or prevent oxidative damage and resist diseases (Hudec et al., 2007).

Leek, *Allium ampeloprasum* L. (family Amaryllidaceae) is one of the daily edible green vegetables for Egyptians; it is cheap and widely cultivated. It is mainly native to the Mediterranean region and cultivated later in other world regions of the world due its medicinal properties (Dey & Khaled, 2013). Leek is known to be rich with many active compounds like kaempferol, steroidal saponins, carotenoids, and chlorophyll(Nemeth & Piskula, 2007) . Therefore many medicinal properties including anti-hepatotoxic, antifungal (Morita et al., 1988), anti-inflammatory (Adão et al., 2011), anti-helmintic, antihypertensive activities (Guarrera & Savo, 2013) have been reported in leeks.

The current study was performed to study the effect of different doses of alcoholic leek extract, *Allium ampeloprasum* L. on the biotransformation enzymes and innate immunity parameters of catfish, *Clarias gariepinus* before and after being exposed to Benzo[a]Pyrene (BaP), in order to investigate the prospective effect of exposure to polycyclic aromatic hydrocarbons compounds.

ISSN: 0975-8585

MATERIAL AND METHODS

PLANT EXTRACT:

Leaves of leek (*Allium ampeloprasum*) were obtained from local market, Cairo, Egypt and allowed to dry in fresh air. 1 kg of dried leaves was extracted using 95% alcohol by percolation until it was exhausted and filtered off. Then the collected filtrates were evaporated under reduced pressure and low temperature using a rotator evaporator. The obtained residue was kept at 8˚C until use.

FISH, EXPERIMENTAL DESIGN AND SAMPLING:

Around 240 catfish, *Clarias gariepinus* (average weight 80 ± 5 g) were obtained from commercial fish farms at Kafr El-Shaik, Egypt. Fish were allowed to acclimatize in aerated free-flowing freshwater for 2 weeks. Fish were randomly distributed into 4 groups, each group containing 60 fish (20 X three replicates). The first 3 groups were injected with 0.2 ml of 5, 50 and 500 mg/kg of fish of leek extract dissolved in Dimethyl sulfoxid (Sigma). The fourth group was injected with 0.2 ml of Dimethyl sulfoxid only (control). During acclimatization and experiment time, fish were fed twice daily with commercial diets. The first sampling was done after 2 weeks of injection. Then, the fish, in four groups were exposed to 1mg/L of Benzo[a]pyrene (dissolved in 0.5 ml of Dimethyl sulfoxid) and the second sampling was done after 24 h of exposure. At each sampling blood was collected from the caudal vein of fish after being anesthetized with 3- amino benzoic acid ethyl ester (Sigma-Aldrich) using a syringe of 3 ml capacity. Then, blood samples were transferred to Vacuettes without heparin and allowed 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 collected and embedded directly in liquid nitrogen tanks, and then transported in -80^{o}C. Also gall bladders were collected in -20^{o}C for analysis by GC-MASS.

PREPARATION OF S-9 HEPATIC FRACTION:

After thawing liver samples on ice, about 0.5 g of each liver sample was homogenized by a high speed glass-Teflon homogenizer in 2ml of homogenization KCl-HEPES buffer, pH 7.5 (0.15 M KCl and 0.02 M HEPES). The homogenate was then centrifuged at 9000 g for 30 min at 4°C. The supernatant (S9 fraction) was collected, divided into four aliquots and stored in - 80 ^{o}C till enzyme assays (Parente et al., 2004). Protein content of S-9 fraction was determined spectrophotometrically at 595 nm by using Bio-Rad protein kit, using bovine serum albumin (Sigma-Aldrich) as a protein standard.

BIOTRANSFORMATION ENZYMES:

CYTOCHROME P4501A1 CONTENT (CYP4501A1):

Cytochrome P4501A1 was immuno-detected by a semi-quantitative Enzyme Linked Immuno-Sorbent Assay (ELISA) as developed by Goksøyr (1991). A 96-well plate was coated by 100 µl of S9 hepatic fraction (30 µg protein/ml in 50 mM Na-bicarbonate buffer, pH 9.5) overnight at 4^{o}C. The wells were blocked with 200 µl of 0.1% bovine serum albumin in Na-bicarbonate buffer for 1h at room temperature. 100 µl of anti CYP450 1A1 (Sigma-Aldrich) were added to the wells and incubated for 2h at 37^{o}C. 100 µl of 1:1000 Horseradish peroxidase-conjugated goat Anti-Rabbit IgG (Sigma-Aldrich), in Tween-20 phosphate buffer saline was added and incubated for 1h at 37 °C. Washing with Tween-20 phosphate buffer saline, pH 7.5 for three times must be done after each step. Finally, 100 µl of the o- phenylenediamine dihydrochloride (OPD substrate tablets, 60 mg) in 150 mM Na-phosphate + 50 mM Na-citrate buffer, pH 5.7 + 0.012% H_2O_2 was added for 30 min. and the reaction was stopped by adding 100 µl of 1N NaOH to each well and the optical density values were obtained by the ELISA reader at 492 nm.

ETHOXYRESORUFIN-O-DEETHYLASE (EROD) ACTIVITY:

Firstly, a mixture contained 0.1 M HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid) buffer, pH 7.8 + 1.28 M Magnisum Sulphate + BSA (Bovine Serum Albumin) + 0.5 mM freshly prepared NADPH (Nicotinamide Adenine Dinucleotide Phosphate; reduced form) was prepared in a glass centrifuge tube. Then S-9 fraction was added and the tubes were incubated in a water bath (25°C) for 10 min. The reaction was initiated by addition of 10 µL of ethoxyresorufin substrate (Sigma-Aldrich) to each tube, incubated in a water

bath (25°C) for another 10 min, and the reaction was terminated by addition of 2.5 mL of methanol. Protein was then precipitated by centrifugation at 9000 g for 10 min at 4°C. The supernatant was transferred to clean quartz cuvette and the fluorescence was measured by spectral-fluorimeter (JASCO EP777, Japan) at excitation/emission wavelengths of 530/585 nm. EROD activities were expressed as nmol resorufin/mg protein/min (Hodson et al., 1991)

GLUTATHIONE-S-TRANSFERASE (GST) ACTIVITY:

Glutathione-S-transferase activity was estimated through conjugation of 1mM CDNB (1-chloro-2,4-dinitrobenzene, Sigma-Aldric) in ethanol with 1mM GSH (reduced glutathione, Sigma-Aldrich) in a 0.1M phosphate buffer, pH 6.5. Then the absorbance of the formed GSH-CDNB conjugate was read kinetically at 340 nm. The specific activity of GST was calculated using a molar extinction coefficient of 9.6 $mM^{-1}cm^{-1}$, and it was expressed as µmole/min/mg protein (Habig et al., 1974).

BAP METABOLITES IN BILE:

The content of BaP metabolites in bile samples were determined by A Hewlett-Pakard (Palo Alto, CA, USA) HP 5890 series II plus gas chromatograph coupled with a 5972 mass selective detector, Ultra-2, 5% phenylmethylsilicon column. The gall bladders from each group were pooled and analyzed as a one sample. Briefly, a solution of potassium hydroxide in ethanol was added in a double volume to each sample, heated at 60^{o}C for two hours. After filtration, the extraction was achieved by n-hexane and the extract was stored in capped vials until GC-MASS analysis (Douglas et al., 1994).

INNATE IMMUNE PARAMETERS:

ANTIPROTEASE ACTIVITY:

The serum anti-trypsin activity was measured by the established methods of Ellis (1987) and Lange et al. (2001). Thus, 20 µl of standard trypsin solution (Sigma-Aldrich, 5 mg/ ml) was incubated with 20 µl of serum for 10 min at 22ºC. 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 and incubated for 1 h at 22ºC. The reaction was then ended with the addition of 500 µl of 10 % (v/v) trichloro acetic acid (TCA) and incubated for 30 min at 22ºC. The mixture was centrifuged at 6000 x g for 5 min and 100 µl of the supernatant was transferred to a flat-bottomed 96 well plates containing 100 µl of 1 N NaOH /well. The absorbance was read in the spectrophotometer at 410 nm, and the percentage inhibition of trypsin activity was calculated by comparing with a 100% control sample, in which the buffer replaced the serum. For a negative control, the buffer replaced both serum and trypsin.

MYELOPEROXIDASE CONTENT:

The total myeloperoxidase content present in serum was measured according to Quade and Roth (1997) . For this, 50 µl serum was diluted with 135 µl of Ca^{+2} and Mg^{+2} free HBSS (Sigma-Aldrich) in flat-bottomed 96-well microtitre plates. Then, 50 µl of 20 mM 3,3´,5,5´-tetramethylbenzidine hydrochloride (TMB, Sigma-Aldrich) and 5 mM H_2O_2 (Sigma-Aldrich) were added (both substrates of peroxidase). The colour-change reaction was stopped after 2 min by adding 50 µl of 4 M sulphuric acid (H_2SO_4). The absorbance was read at 450 nm in a fluorimeter. Standard samples without serum were also analysed.

TOTAL PROTEIN, ALBUMIN AND GLOBULIN:

Total protein albumin and globulin were measured by kits from Biodiagnostic (Egypt).

STATISTICAL ANALYSIS:

Data were analysed by a one-way analysis of variance (ANOVA). If the results of the ANOVA indicated significant differences among the mean values, differences were reported as statistically significant at $P<0.05$. All the statistical analyzes were performed using the statistical Minitab statistical software (Minitab, Coventry, UK).

ISSN: 0975-8585

RESULTS

BIOTRANSFORMATION ENZYMES:

CYTOCHROME P4501A1 CONTENT (CYP4501A1):

A slight increase in the basal hepatic CYP4501A1 content was observed in fish groups injected with doses of 5 and 500 mg/Kg of leek extract compared to the corresponding control before exposure to BaP, although the difference was not significant (Fig. 1). While after exposure to BaP, all leek doses showed no changes in CYP4501A1 content compared to their corresponding control. Moreover, there was a non-statistically significant increase in the hepatic CYP4501A1 content in the control group between before and after exposure to BaP.

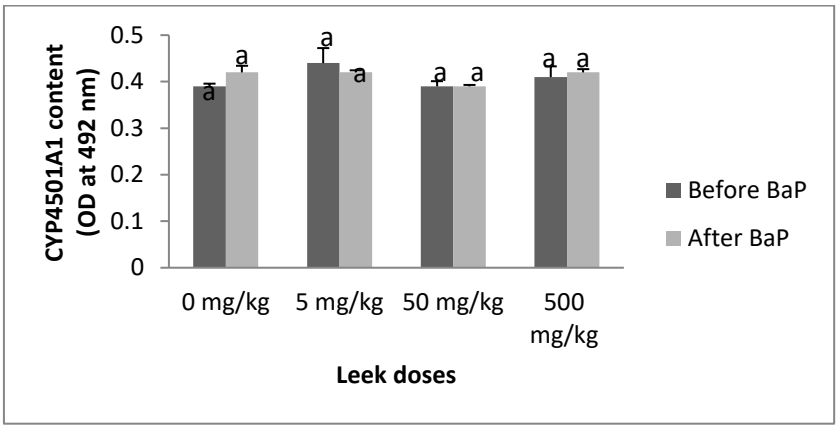

Fig. 1. Cytochrome P4501A1 content of catfish liver injected with 0 (control), 5, 50 and 500 mg/kg of leek extract before and after exposure to BaP. Letters were shown the significant differences (P<0.05) between treatment groups and the corresponding controls.

(*) = significant difference between each dose (before and after exposure to BaP) (p <0.05). Bars= mean ± S.E

ETHOXYRESORUFIN-O-DEETHYLASE (EROD) ACTIVITY:

The injection of leek extract showed significant increase ($p < 0.05$) in the basal level of EROD activity in 5 and 50 mg/Kg doses compared to corresponding control before exposure to BaP (Fig. 2). After exposure to BaP, only the dose of 5mg/Kg recorded significant increase in EROD activities ($p < 0.05$) compared to the corresponding control while decreased in the higher doses (50 and 500 mg/Kg). Interestingly, the exposure to BaP caused about 1.8 fold increases in EROD activities in both control and 5mg/Kg dose compared to before exposure ($p < 0.05$).

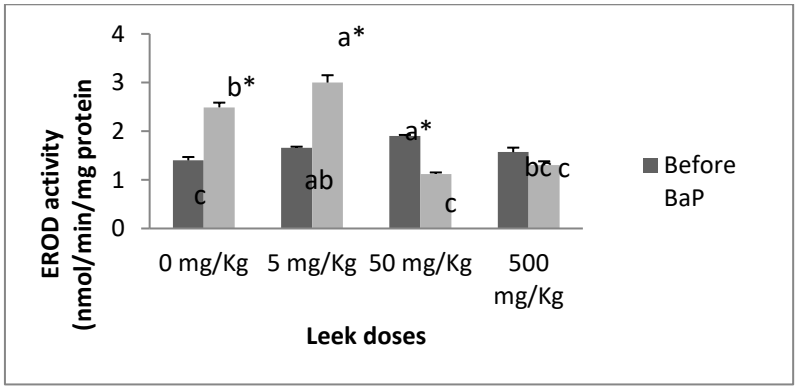

Fig. 2. EROD activity of catfish liver injected with 0 (control), 5, 50 and 500 mg/kg of leek extract before and after exposure to BaP. Letters were shown the significant differences (P<0.05) between treatment groups and the corresponding controls.

(*) = significant difference between each dose (before and after exposure to BaP) (p <0.05). Bars= mean ± S.E

ISSN: 0975-8585

GLUTATHIONE-S-TRANSFERASE (GST) ACTIVITY:

Before exposure to BaP, fish groups injected with all doses of leek extract showed significant increase in the basal level of GSH activity ($p < 0.05$) compared to the corresponding control (Fig 3). Moreover, the lowest dose (5 mg/Kg) recorded the highest value of GSH where the value was about two-fold increase compared to the corresponding control. After exposure to BaP, there was a significant increase in GSH activity in control group than before exposure ($p < 0.05$). Also the exposure to BaP led to insignificantly decreased in GSH activity in all doses of leek extract except in the dose of 5 mg/kg which recorded a higher activity than the corresponding control.

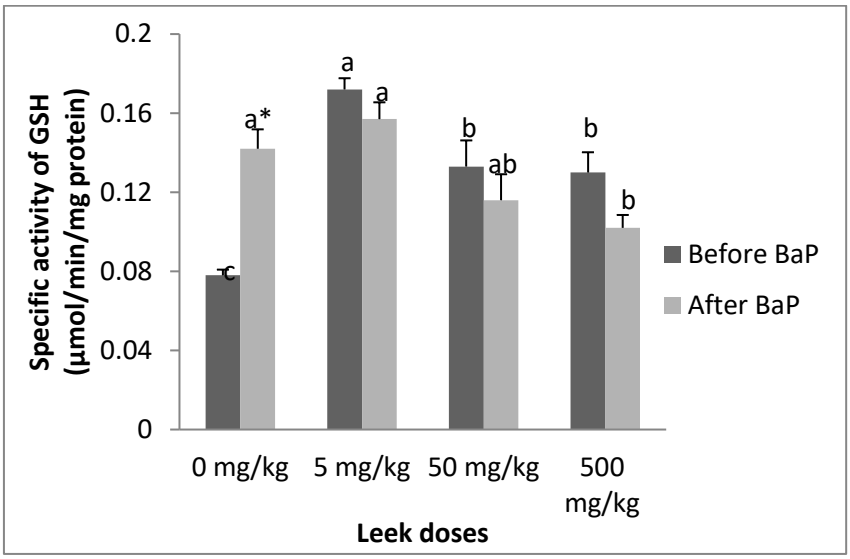

Fig. 3. GST activity of catfish liver injected with 0 (control), 5, 50 and 500 mg/kg of leek extract before and after exposure to BaP. Letters were shown the significant differences ($p < 0.05$) between treatment groups and the corresponding controls.

(*) = significant difference between each dose (before and after exposure to BaP) ($p < 0.05$). Bars= mean ± S.E

BAP METABOLITES IN BILE:

GC-MASS chromatograph of bile specimens revealed that BaP metabolites were not detected in both control and the treated groups after exposure to BaP.

INNATE IMMUNE PARAMETERS:

ANTIPROTEASE ACTIVITY:

Generally, high percentages of antiprotease activity were recorded in all fish injected with all doses of leek extract compared to corresponding controls before and after BaP exposure (Fig. 4), but without significant difference. Before BaP exposure, the higher doses (50 and 500 mg/kg) showed the highest antiprotease activity. But after BaP exposure, the highest activity was reported in 5 mg/kg dose. Moreover, the antiprotease activity was higher in control and 5 mg/kg doses after BaP exposure than before and vice versa in doses of 50 and 500 mg/kg.

ISSN: 0975-8585

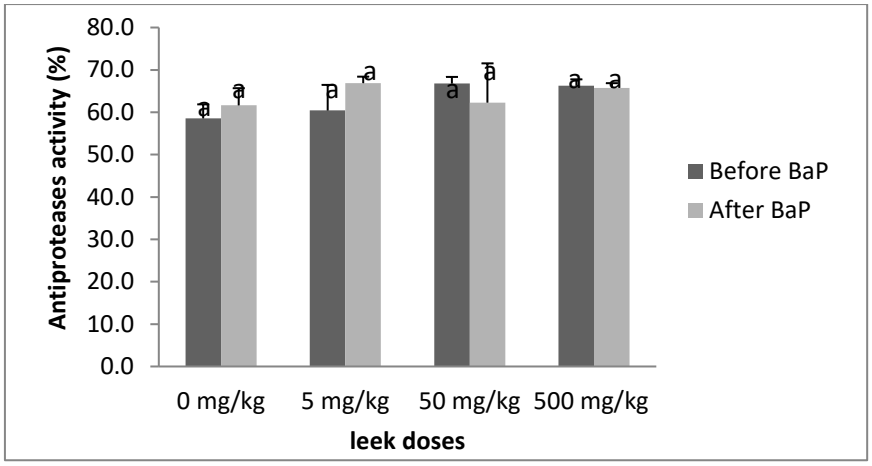

Fig. 4. Serum antiprotease activity of catfish injected with 0 (control), 5, 50 and 500 mg/kg of leek extract before and after exposure to BaP. Letters were shown the significant differences (p < 0.05) between treatment groups and the corresponding controls.

(*) = significant difference between each dose (before and after exposure to BaP) (p <0.05). Bars= mean ± S.E

MYELOPEROXIDASE CONTENT:

Similarly, all doses of leek extract showed higher myeloperoxidase values compared to the corresponding controls before and after BaP exposure (without significant difference), (Fig. 5). Moreover, the dose of 50 mg/kg recorded the highest myeloperoxidase values before BaP exposure however, after BaP exposure both 5 and 50 mg/kg doses were recorded the highest values. Interestingly, all groups injected with doses of leek extract revealed a higher myeloperoxidase content after BaP exposure than before exposure.

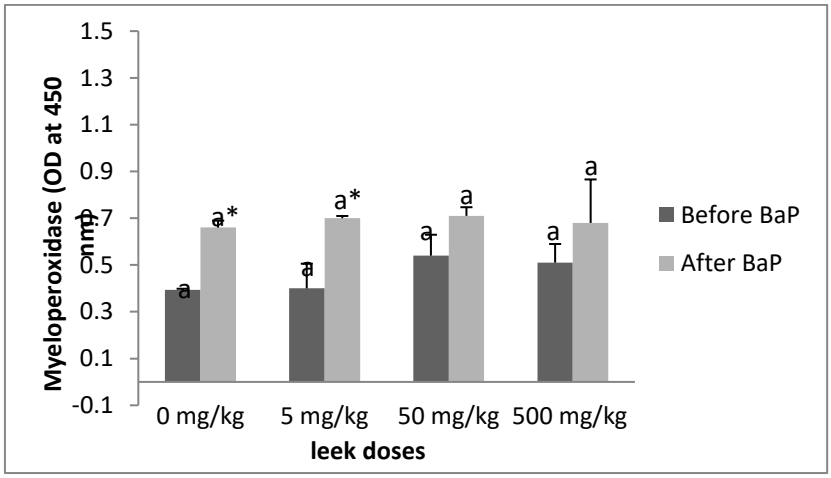

Fig. 5. Serum myeloperoxidase activity of catfish injected with 0 (control), 5, 50 and 500 mg/kg of leek extract before and after exposure to BaP. Letters were shown the significant differences (p < 0.05) between treatment groups and the corresponding controls.

(*) = significant difference between each dose (before and after exposure to BaP) (p <0.05). Bars= mean ± S.E

TOTAL PROTEIN, ALBUMIN AND GLOBULIN:

Before BaP exposure, a higher total protein and globulin values were recorded in treatment groups as compared to the corresponding control, especially the dose of 50 mg/kg which recorded the highest values (Fig. 6 & 7). By contrast, only the dose of 5 mg/kg showed an increase in total protein and globulin compared to the corresponding control after BaP exposure. On the other hand, total protein and globulin were elevated after BaP exposure in the dose of 5 mg/kg and the control than before exposure. It's necessary to emphasize the absence of any significant difference between the treatment groups and corresponding controls.

ISSN: 0975-8585

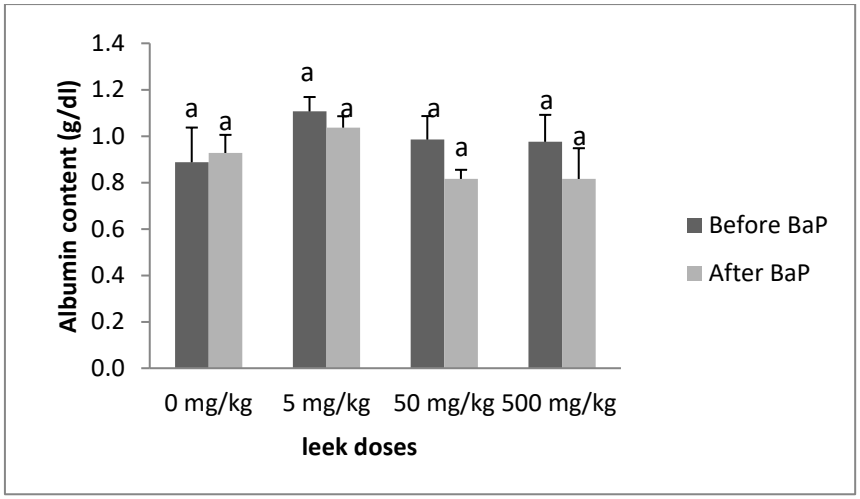

Fig. 6. Serum total protein of catfish injected with 0 (control), 5, 50 and 500 mg/kg of leek extract before and after exposure to BaP. Letters were shown the significant differences (p < 0.05) between treatment groups and the corresponding controls.

(*) = significant difference between each dose (before and after exposure to BaP) (p <0.05). Bars= mean ± S.E

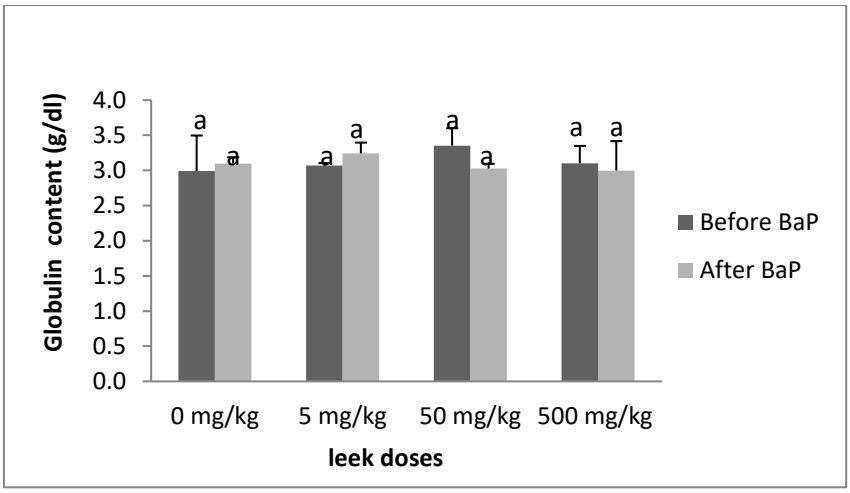

Fig. 7. Serum globulin of catfish injected with 0 (control), 5, 50 and 500 mg/kg of leek extract before and after exposure to BaP. Letters were shown the significant differences (p < 0.05) between treatment groups and the corresponding controls.

(*) = significant difference between each dose (before and after exposure to BaP) (p <0.05). Bars= mean ± S.E

All groups injected with doses of leek extract showed higher albumin values than the corresponding control before BaP exposure. Interestingly, the dose of 5mg/kg showed a higher albumin values before and after exposure to BaP compared to the corresponding controls (Fig. 8). There was no significant difference between treatment groups and corresponding controls. Generally, albumin values were higher before BaP exposure than after (except in control group).

ISSN: 0975-8585

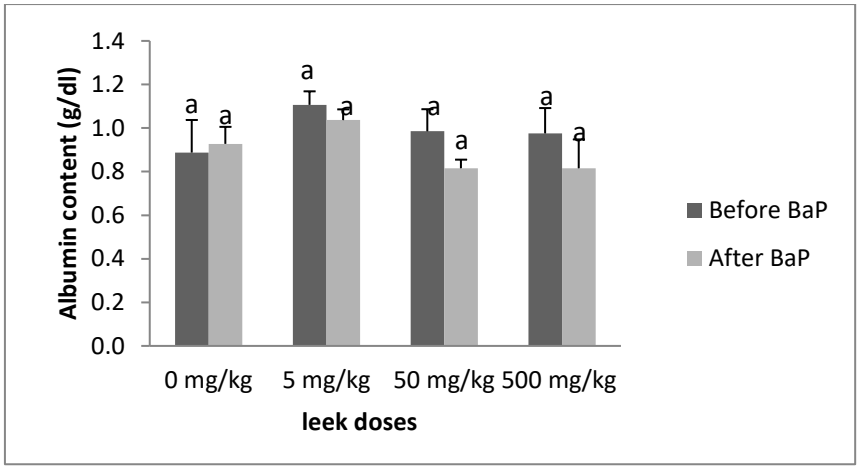

Fig. 8. Serum albumin of catfish injected with 0 (control), 5, 50 and 500 mg/kg of leek extract before and after exposure to BaP. Letters were shown the significant differences ($p < 0.05$) between treatment groups and the corresponding controls.

(*) = significant difference between each dose (before and after exposure to BaP) ($p < 0.05$). Bars= mean ± S.E

DISCUSSION

In fact, the ability of fish to resist the harmful toxicant effects in fish tissue as well as the invading pathogens is associated mainly with immune and biotransformation systems. The biotransformation system is the principal protective mechanism of living organisms against polycyclic aromatic hydrocarbons (PAH) which converts them to inactive hydrophilic metabolites (Tuvikene, 1995). At the same time, changes in the immune system of fish could represents a good biomarker for pollution (Risjani et al., 2014) since pollution induces abnormal functioning of immune cells and affect both innate and adaptive immune response (Fatima et al., 2000). In affirmation to this, some studies revealed a complementary relationship between biotransformation and immune systems (Reynaud et al., 2008). For example, Abbas et al. (2016) demonstrated that using black mustard extract in tilapia diet led to a simultaneous enhancement in the immune response as well as in the detoxification process of pollutants.

This study demonstrated that the injection of catfish with different doses of leek extract (5, 50, 500 mg/Kg fish) effectively induced EROD activity, especially at the low dose (5 mg/Kg). This induction may be attributed to enrichment of leek with allyl sulfides which play an important role in changing and modifying some metabolic pathways associated with the tumours production (Nemeth & Piskula, 2007) and its components have the ability to block several carcinogenesis stages (Fleischauer et al., 2000). While CYP450 content showed no significant difference compared to the corresponding controls (before and after BaP exposure). Similarly, Wolkers et al. (1996) observed that EROD induction does not necessarily accompanied by increase in CYP content in Arctic charr (Salvelinus alpinus). Our study also revealed significant increase in EROD activity after exposure to BaP in the control group, which was mainly due to the toxic effect of BaP. Generally, induction of CYP450 1A1 can be explained as a result of binding xenobiotic to a cellular receptor (Aryl hydrocarbon receptor) in such a way that it triggers the expression of the gene coding CYP450 1A1 (Okey et al., 1994). EROD activity is also catalysed by the cytochrome P450 1A1 gene due to PAH pollution (Stegeman, 1989). BaP induce EROD activity in many fish species at different duration times, in gizzard shad, Dorosoma cepedianum (Levine & Oris, 1997), European eel, Anguilla anguilla (Gorbi & Regoli, 2004) and in both Nile tilapia, Oreochromis niloticus and catfish, Clarias gariepinus (Hassanain et al., 2007). The significant increase in EROD activity in fish injected with the dose of 5mg/Kg after exposure to BaP indicated the promised protective effect of this dose to reduce the toxic effect of BaP. Similarly, protective effect against the toxicity induced by carbon tetrachloride (CCL4) has been observed in male white rats after administration doses of leek extract (Jaffat et al., 2014). On the other hand, the obvious decrease in EROD activities at high doses (50 and 500 mg/Kg) after exposure to BaP revealed the inability of leek to enhance metabolic reactions at those high doses. Although CYP450 are involved in the conversion of BaP to highly reactive carcinogenic intermediates, their induction is the first step in the elimination of such pollutants from fish. So increasing the CYP450 activity using some natural plant extracts represents a very helpful solution to overcome the pollutants side effects.

Moreover, the increase in CYP450 activity levels could be considered beneficial as it is followed by phase II system (Villa-Cruz et al., 2009).

GST is one of the most important phase II biotransformation antioxidant enzymes which are responsible for detoxification and elimination of xenobiotic. Some organosulfur compounds which derived from allium plants can induce GSH synthesis and also has a hepatoprotective function (Shaarawy et al., 2009). It can interact with the reactive oxygen species produced from the phase I biotransformation and reduce its carcinogenic effect (Pastor et al., 1997 ; Baumgardner et al., 2008). Furthermore, they act as a substrate for glutathione and restore its hepatic level and provide enhanced protection against toxins (Vendemiale et al., 2001). Therefore, there was induction in GSH activities in leek injected groups, especially the low dose, which recorded the highest values before and after BaP exposure. Furthermore, inductions in both EROD and GSH (especially at the lowest dose of leek extract) trigger the complete transformation of BaP and prevent its toxic effect upon fish which are compatible with previous studies that used plants or their extracts to enhance the detoxification system, such as broccoli (Villa-Cruz et al., 2009) and black mustard (Abbas et al., 2016).

Presence of PAH metabolite in fish bile provides information about the pollution state in the surrounding environment and the exposure of fish to PAH's (Vuorinen et al., 2006). Unfortunately, our study did not report any of the BaP metabolites in both control and treated fish groups. This may be attributed to the short exposure duration (only 24 h) and also to the route of administration (in the water at 1mg/l dose). Some previous studies reported the presence of BaP metabolites after three days of intraperitoneal injection of BaP in Oreochromis niloticus (Villa-Cruz et al., 2009) or in Fundulus heteroclitus which were exposed to BaP in water for two weeks (Zhu et al., 2008).

Fish plasma contains immune soluble molecules which play a vital role as inhibitors or lysins against the invading foreign microorganisms. Antiproteases are one of inhibitors molecules which inhibit the action of destructive proteases to prevent protein hydrolysis , and thus limit the bacteria from growing in fish (Laskowski Jr & Kato, 1980 ; Ellis, 1999). Several studies recorded enhancement in antiprotease activity of fish using either plant or their extracts as immunostimulant (Christybapita et al., 2007 ; Awad et al., 2013 ; Awad, 2010 ; Rao Y & Chakrabarti, 2005) . In agreement to previous studies, our results recorded a high antiprotease activity in fish injected all doses of leek extract, especially the highest doses (50 and 500 mg/kg) compared to their corresponding controls after 2 weeks (before BaP exposure). Similarly, the highest dose of soluble fraction of Tinospora cordifolia leaves (600 mg/kg) injected in Oreochromis mossambicus, showed the highest antiprotease activity after 8 days. In similar manner, tilapia fed with Eclipta alba leaf extract showed an increasing level of antiprotease activity after 2 weeks (Christybapita et al., 2007). Furthermore, the enhancement in innate immune response was recorded after 8 weeks in olive flounder Paralichthys olivaceus, injected with 50 and 100 mg/kg dose of leaf extracts of Punica granatum (Harikrishnan et al., 2010). On the other hand, all doses of leek extract showed a higher level in antiprotease activity even after exposure to BaP. Particularly, the dose of 5mg/kg revealed the highest percentage. Similarly, a dose of 1% of black mustard extract showed an elevation in antiprotease percentage of Nile tilapia after exposure to BaP (Abbas et al., 2016). Interestingly, 5mg/kg dose also recorded a higher level in antiprotease activity after exposure to BaP than before exposure. Such this observation could reflect the effectiveness of this dose to resist toxicant effect and elevate immune response at the same time.

The first line toward the invading pathogen/or any foreign particles in fish are neutrophils and monocytes. They play essential role (as phagocytic cell) in engulfing and digesting the invading pathogen as well as stimulating other leukocytes and immune molecule trigger an inflammatory response (Rao et al., 2006). Myeloperoxidase is an enzyme that exists in cytoplasmic granules of neutrophils, which have a strong influence in killing microbes (Dalmo et al., 1997). Previous investigations reported the efficiency of using plant immunostimulant to enhance the myeloperoxidase content in fish (Awad & Austin, 2010 ; Awad et al., 2013 ; Awad et al., 2015). Our result revealed an increase in myeloperoxidase values in all fish injected with doses of leek extract before exposure to BaP, although the increase wasn't statistically significant. In agreement with this study, Oreochromis mossambicus injected with 6 and 600 mg/kg doses of soluble fraction of T. cordifolia leaves recorded significant increase in myeloperoxidase activity after 6 days. Similar, common tilapia showed an increase in myeloperoxidase value after fed with different doses of aqueous leaf extract of E. alba for one week (Christybapita et al., 2007). It's interesting that the myeloperoxidase content in fish isn't affected by the exposure to BaP, where all treatment groups recorded slightly higher values than their corresponding controls. Moreover, comparing the myeloperoxidase values of doses before and after exposure to BaP revealed an

increase in the values after exposure especially at 5 and 50 mg/kg dose. It could be attributed to the effectiveness of leek compounds to reduce or to resist the toxic effect of BaP, especially when applied once.

Fish serum proteins have important functions such as water balance regulation, repairing tissue damage and killing micro-organisms (Wedemeyer & Yasutake, 1977 ; Atanasova & Hadjinikolova, 2008). Protein consists of albumin and globulin. Globulins are active protein which plays a role in the immune defence system (Jha et al., 2007). Our study revealed an increase in total protein, globulin, and albumin values in all fish injected with doses of leek extract before exposure to BaP. Moreover, the dose of 50 mg/kg also recorded the highest total protein and globulin values. The increase in total protein and globulin was demonstrated in other fishes that used plant immunostimulant. For example, previous studies carried on rohu recorded higher values of total protein, globulin and albumin after fed diet mixed with different concentrations of mango kernel (1, 5 and 10%), and garlic (0.1, 0.5 and 1%) (Sahu et al., 2006 ; Sahu et al., 2007). It is worthy to mention that the dose of plant immunostimulant which provide the best immune response is varied according to fish species as well as which type or form (crude or extract) of plant being used. Thus, diet supplemented with 1% lupin showed higher protein and globulin values in rainbow trout than of 1% mango, while albumin didn't increase at all (Awad & Austin, 2010). After exposure to BaP, the total protein, globulin and albumin values were increased only in the fish group that received 5 mg/kg. Only few investigations are carried out regarding the efficiency of plants to resist toxicant effect and enhance the immunity. Among them, a study done by Abbas et al. (2016) which recorded an increase in total protein of Nile tilapia fed with 1% of black mustard extract and 30% of crude black mustard and exposed to BaP as opposed to the control. Furthermore, using mustard extract showed a higher value of protein than with using crude.

Generally, catfish (Clarias gariepinus) injected with doses of leek extract (5, 50 and 500 mg/kg) showed an increase in detoxification enzymes as well as innate immune parameters. However after exposure to BaP only 5 mg/kg dose showed ability to resist BaP effect and enhanced innate immune response. Further investigation is necessary to examine the effect of oral administration of leek extract with different time and dosage on general fish health status which lead to the best protection and then enhance the fish productivity.

ACKNOWLEDGEMENTS

This research has been supported and funded by the National Research Center of Egypt (P101 203).

REFERENCES

[1] Abbas WT, Awad E, Abdel-Rahman EH (2016) Effect of Black Mustard (*Brassica nigra*) on the Interaction between Immune and Biotransformation Systems of Nile Tilapia (*Oreochromis niloticus*) Exposed to Benzo-a-Pyrene. *Journal of Fisheries and Aquatic Science,* 11, 56.

[2] Adão CR, da Silva BP, Parente JP (2011) A new steroidal saponin from *Allium ampeloprasum* var. porrum with antiinflammatory and gastroprotective effects. *Phytochemistry Letters,* 4, 306-310.

[3] Atanasova R, Hadjinikolova L (2008) Cellural and humoral (non-specific) immunity of carp stocking material (*Cyprinus carpio* L.) before and after wintering. *Bulgarian Journal of Agricultural Science,* 14, 251-255.

[4] Awad E, Austin B (2010) Use of lupin, Lupinus perennis, mango, *Mangifera indica*, and *stinging nettle*, *Urtica dioica*, as feed additives to prevent *Aeromonas hydrophila* infection in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J Fish Dis,* 33, 413-420.

[5] Awad E, Austin D, Lyndon AR (2013) Effect of black cumin seed oil (*Nigella sativa*) and nettle extract (Quercetin) on enhancement of immunity in rainbow trout, *Oncorhynchus mykiss*(Walbaum). *Aquaculture,* 388, 193-197.

[6] Awad E, Cerezuela R, Esteban MÁ (2015) Effects of fenugreek (*Trigonella foenum graecum*) on gilthead seabream (*Sparus aurata* L.) immune status and growth performance. *Fish Shellfish Immunol,* 45, 454-464.

[7] Awad ES (2010) Studies on plant based dietary supplements for control of *Aeromonas hydrophila* infections in rainbow trout (*Oncorhynchus mykiss* Walbaum). Heriot-Watt University.

[8] Baumgardner JN, Shankar K, Hennings L, Albano E, Badger TM, Ronis MJ (2008) N-acetylcysteine attenuates progression of liver pathology in a rat model of nonalcoholic steatohepatitis. *The Journal of nutrition,* 138, 1872-1879.

[9] Burnett KG (2005) Impacts of environmental toxicants and natural variables on the immune system of fishes. *Biochemistry and molecular biology of fishes,* 6, 231-253.
[10] Carlson E, Li Y, Zelikoff J (2002a) Exposure of Japanese medaka (*Oryzias latipes*) to benzo [a] pyrene suppresses immune function and host resistance against bacterial challenge. *Aquatic Toxicology,* 56, 289-301.
[11] Carlson E, Li Y, Zelikoff J (2002b) The Japanese medaka (*Oryzias latipes*) model: applicability for investigating the immunosuppressive effects of the aquatic pollutant benzo [a] pyrene (BaP). *Marine environmental research,* 54, 565-568.
[12] Carlson E, Li Y, Zelikoff J (2004) Suppressive effects of benzo [a] pyrene upon fish immune function: evolutionarily conserved cellular mechanisms of immunotoxicity. *Marine environmental research,* 58, 731-734.
[13] Chambras C (1999) Xenobiotic-metabolizing enzymes in carp (*Cyprinus carpio*) liver, spleen, and head kidney following experimental *Listeria monocytogenes* infection. *Journal of Toxicology and Environmental Health Part A,* 56, 205-219.
[14] Christybapita D, Divyagnaneswari M, Dinakaran Michael R (2007) Oral administration of *Eclipta alba* leaf aqueous extract enhances the non-specific immune responses and disease resistance of *Oreochromis mossambicus*. *Fish and shellfish immunology,* 23, 840-852.
[15] Dalmo R, Ingebrigtsen K, Bøgwald J (1997) Non-specific defence mechanisms in fish, with particular reference to the reticuloendothelial system (RES). *J Fish Dis,* 20, 241-273.
[16] Dey P, Khaled KL (2013) An Extensive Review on Allium ampeloprasum A Magical Herb. *International Journal of Science and Research,* 4, 371-377.
[17] Douglas G, Prince R, Butler E, Steinhauer W (1994) The use of internal chemical indicators in petroleum and refined products to evaluate the extent of biodegradation. Lewis Publishers: Ann Arbor, MI, pp. 219-236.
[18] Ellis A (1999) Immunity to bacteria in fish. *Fish Shellfish Immunol,* 9, 291-308.
[19] Ellis AE (1987) Inhibition of the *Aeromonas salmonicida*, extracellular protease by α 2-macroglobulin in the serum of rainbow trout. *Microbial pathogenesis,* 3, 167-177.
[20] Fatima M, Ahmad I, Sayeed I, Athar M, Raisuddin S (2000) Pollutant-induced over-activation of phagocytes is concomitantly associated with peroxidative damage in fish tissues. *Aquatic Toxicology,* 49, 243-250.
[21] Fleischauer AT, Poole C, Arab L (2000) Garlic consumption and cancer prevention: meta-analyses of colorectal and stomach cancers. *The American journal of clinical nutrition,* 72, 1047-1052.
[22] Goksøyr A (1991) A semi-quantitative cytochrome P450IA1 ELISA: a simple method for studying the monooxygenase induction response in environmental monitoring and ecotoxicological testing of fish. *Science of the total environment,* 101, 255-262.
[23] Gorbi S, Regoli F (2004) Induction of cytochrome P4501A and biliary PAH metabolites in European eel *Anguilla anguilla*: Seasonal, dose-and time-response variability in field and laboratory conditions. *Marine environmental research,* 58, 511-515.
[24] Guarrera P, Savo V (2013) Perceived health properties of wild and cultivated food plants in local and popular traditions of Italy: a review. *Journal of Ethnopharmacology,* 146, 659-680.
[25] Habig WH, Pabst MJ, Jakoby WB (1974) Glutathione S-transferases the first enzymatic step in mercapturic acid formation. *Journal of biological Chemistry,* 249, 7130-7139.
[26] Hardin JA, Hinoshita F, Sherr DH (1992) Mechanisms by which benzo [a] pyrene, an environmental carcinogen, suppresses B cell lymphopoiesis. *Toxicology and applied pharmacology,* 117, 155-164.
[27] Harikrishnan R, Heo J, Balasundaram C, Kim M-C, Kim J-S, Han Y-J, Heo M-S (2010) Effect of *Punica granatum* solvent extracts on immune system and disease resistance in *Paralichthys olivaceus* against lymphocystis disease virus (LDV). *Fish Shellfish Immunol,* 29, 668-673.
[28] Hassanain M, Abdel-Rahman EH, Abo-Hegab S, Tawfik M, Abbas WT (2007) Induction of cytochrome P450 1A1 as a biomarker of Benzo-a-pyrene pollution in Egyptian fresh water fish. *Pakistan journal of biological sciences: PJBS,* 10, 1161-1169.
[29] Hodson P, Kloepper-Sams P, Munkittrick K, Lockhart W, Metner D, Luxon P, Smith I, Gagnon M, Servos M, Payne J (1991) Protocols for measuring mixed function oxygeases of fish liver *Canadian Technical Report of Fisheries and Aquatic Sciences*
[30] 33-38.
[31] Holladay S, Smith S, Besteman E, Deyab A, Gogal R, Hrubec T, Robertson J, Ahmed S (1998) Benzo [a] pyrene-induced hypocellularity of the pronephros in tilapia (*Oreochromis niloticus*) is accompanied by

alterations in stromal and parenchymal cells and by enhanced immune cell apoptosis. *Vet Immunol Immunopathol,* 64, 69-82.

[32] Hudec J, Burdová M, Kobida Lu, Komora L, Macho V, Kogan G, Turianica I, Kochanová R, Lozek O, Habán M (2007) Antioxidant capacity changes and phenolic profile of *Echinacea purpurea*, nettle (*Urtica dioica* L.), and dandelion (*Taraxacum officinale*) after application of polyamine and phenolic biosynthesis regulators. *Journal of agricultural and food chemistry,* 55, 5689-5696.

[33] Jaffat HS, Hassan AS, A.B. N (2014) Protective Effect of *Allium Ampeloprasum* Against Toxicity Induced by CCL4 in Male White Rats. *International Journal of Scientific & Engineering Research,* 5, 852-858.

[34] Jha AK, Pal A, Sahu N, Kumar S, Mukherjee S (2007) Haemato-immunological responses to dietary yeast RNA, ω-3 fatty acid and β-carotene in *Catla catla* juveniles. *Fish Shellfish Immunol,* 23, 917-927.

[35] Kiruthiga P, Shafreen RB, Pandian SK, Arun S, Govindu S, Devi KP (2007) Protective effect of silymarin on erythrocyte haemolysate against benzo (a) pyrene and exogenous reactive oxygen species (H 2 O 2) induced oxidative stress. *Chemosphere,* 68, 1511-1518.

[36] Lange S, Guđmundsdottir BK, Magnadottir B (2001) Humoral immune parameters of cultured Atlantic halibut (*Hippoglossus hippoglossus* L.). *Fish Shellfish Immunol,* 11, 523-535.

[37] Laskowski Jr M, Kato I (1980) Protein inhibitors of proteinases. *Annual review of biochemistry,* 49, 593-626.

[38] Levine SL, Oris JT (1997) Induction of CYP1A mRNA and catalytic activity in gizzard shad (*Dorosoma cepedianum*) after waterborne exposure to benzo [a] pyrene. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology,* 118, 397-404.

[39] Morita T, Ushiroguchi T, Hayashi N, Matsuura H, Itakura. Y., Fuwa T (1988) Steroidal saponins from elephant garlic, bulbs of Allium ampeloprasum L. *Chemical and Pharmaceutical Bulletin,* 36, 3480-3486.

[40] Mudzinski S (1993) Effects of Benzo [a] pyrene on Concanavalin A-Stimulated Human Peripheral Blood Mononuclear cells in Vitro: Inhibition of Proliferation but No Effect on Parameters Related to the G1 Phase of the Cell Cycle. *Toxicology and applied pharmacology,* 119, 166-174.

[41] Nemeth K, Piskula M (2007) Food content, processing, absorption and metabolism of onion flavonoids. *Critical reviews in food science and nutrition,* 47, 397-409.

[42] Okey AB, Riddick DS, Harper PA (1994) The Ah receptor: mediator of the toxicity of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) and related compounds. *Toxicology letters,* 70, 1-22.

[43] Oldham KM, Bowen PE (1998) Oxidative stress in critical care: is antioxidant supplementation beneficial? *Journal of the American Dietetic Association,* 98, 1001-1008.

[44] Parente TE, De-Oliveira AC, Silva IB, Araujo FG, Paumgartten FJ (2004) Induced alkoxyresorufin-O-dealkylases in tilapias (*Oreochromis niloticus*) from Guandu river, Rio de Janeiro, Brazil. *Chemosphere,* 54, 1613-1618.

[45] Pastor A, Collado PS, Almar M, González-Gallego J (1997) Antioxidant enzyme status in biliary obstructed rats: effects of N-acetylcysteine. *Journal of Hepatology,* 27, 363-370.

[46] Quade MJ, Roth JA (1997) A rapid, direct assay to measure degranulation of bovine neutrophil primary granules. *Vet Immunol Immunopathol,* 58, 239-248.

[47] Rao Y, Das B, Jyotyrmayee P, Chakrabarti R (2006) Effect of Achyranthes aspera on the immunity and survival of *Labeo rohita* infected with *Aeromonas hydrophila*. *Fish and shellfish immunology,* 20, 263-273.

[48] Rao Y V, Chakrabarti R (2005) Stimulation of immunity in Indian major carp *Catla catla* with herbal feed ingredients. *Fish Shellfish Immunol,* 18, 327-334.

[49] Reynaud S, Raveton M, Ravanel P (2008) Interactions between immune and biotransformation systems in fish: A review. *Aquatic Toxicology,* 87, 139-145.

[50] Risjani Y, Couteau J, Minier C (2014) Cellular immune responses and phagocytic activity of fishes exposed to pollution of volcano mud. *Marine environmental research,* 96, 73-80.

[51] Sahu S, Das B, Mishra B, Pradhan J, Sarangi N (2006) Effect of Allium sativum on the immunity and survival of Labeo rohita infected with Aeromonas hydrophila. *Journal of Applied Ichthyology,* 23, 80-86.

[52] Sahu S, Das BK, Pradhan J, Mohapatra B, Mishra B, Sarangi N (2007) Effect of Magnifera indica kernel as a feed additive on immunity and resistance to Aeromonas hydrophila in Labeo rohita fingerlings. *Fish and shellfish immunology,* 23, 109-118.

[53] Sakai M (1999) Current research status of fish immunostimulants. *Aquaculture,* 172, 63-92.

[54] Shaarawy SM, Tohamy AA, Elgendy SM, Elmageed Z, Bahnasy A, Mohamed MS, Kandil E, Matrougui K (2009) Protective effects of garlic and silymarin on NDEA-induced rats hepatotoxicity. *Int J Biol Sci,* 5, 549-557.

[55] Široká Z, Drastichova J (2004) Biochemical marker of aquatic environment contamination-cytochrome P450 in fish. A review. *Acta Veterinaria Brno,* 73, 123-132.

[56] Smith A, Schurig, GG. Smith, SASD, Holladay, D (1999) The hemolytic plaque-forming cell assay in tilapia (*Oreochromis niloticus*) exposed to benzo [a] pyrene: enhanced or depressed plaque formation depends on dosing schedule. *Toxicology Methods,* 9, 57-70.

[57] Stegeman JJ (1989) Cytochrome P450 forms in fish: catalytic, immunological and sequence similarities. *Xenobiotica,* 19, 1093-1110.

[58] Tuvikene A (1995) Responses of fish to polycyclic aromatic hydrocarbons (PAHs). In: *Annales Zoologici Fennici*. JSTOR*,* pp. 295-309.

[59] Vendemiale G, Grattagliano I, Caruso ML, Serviddio G, Valentini AM, Pirrelli M, Altomare E (2001) Increased oxidative stress in dimethylnitrosamine-induced liver fibrosis in the rat: effect of N-acetylcysteine and interferon-α. *Toxicology and applied pharmacology,* 175, 130-139.

[60] Villa-Cruz V, Davila J, Viana M, Vazquez-Duhalt R (2009) Effect of broccoli (*Brassica oleracea*) and its phytochemical sulforaphane in balanced diets on the detoxification enzymes levels of tilapia (*Oreochromis niloticus*) exposed to a carcinogenic and mutagenic pollutant. *Chemosphere,* 74, 1145-1151.

[61] Vuorinen PJ, Keinänen M, Vuontisjärvi H, Baršienė J, Broeg K, Förlin L, Gercken J, Kopecka J, Köhler A, Parkkonen J (2006) Use of biliary PAH metabolites as a biomarker of pollution in fish from the Baltic Sea. *Marine Pollution Bulletin,* 53, 479-487.

[62] Wedemeyer GA, Yasutake WT (1977) Clinical methods for the assessment of the effects of environmental stress on fish health. US Fish and Wildlife Service.

[63] Wolkers J, Jørgensen E, Nijmeijer S, Witkamp R (1996) Time-dependent induction of two distinct hepatic cytochrome P4501A catalytic activities at low temperatures in Arctic charr (*Salvelinus alpinus*) after oral exposure to benzo (a) pyrene. *Aquatic Toxicology,* 35, 127-138.

[64] Zhu S, Li L, Thornton C, Carvalho P, Avery BA, Willett KL (2008) Simultaneous determination of benzo [a] pyrene and eight of its metabolites in *Fundulus heteroclitus* bile using ultra-performance liquid chromatography with mass spectrometry. *Journal of Chromatography B,* 863, 141-149.