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Biochemical changes in proteins and DNA in *Ctenopharyngodon idella* in response to treatment with the biopesticide "Triology®".

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ABSTRACT

This work was planned to study the biochemical effects of neem biopesticide "Triology®" on plasma albumin, plasma total protein, heat shock protein70, and DNA content in liver of the fresh water fish, *"Ctenopharyngodon idella"*

Fish were divided into 3 groups. The first group was exposed to 1/10 LC_{50} (11.2 mg/L) of the pesticide "neem". Fish were sampled at intervals of 5, 10 and 15 days post exposure. Some fish were left for recovery, and sampled at the intervals of 5 and 10 days. The second group was exposed to 1/2 LC_{50} (56 mg/L) of the pesticide and were sampled after successive intervals of 2, 4 and 6 days of exposure. Some fish were left for recovery, and sampled after the intervals of 3 and 6 days. The third group served as control.

The biochemical results revealed a great decline in plasma albumin, plasma total protein, and DNA content in the liver, as well as an enhancement of hsp70 gene which translated to increased amounts of heat shock protein (hsp70) in the neem-treated groups. The recovery studies showed a partial to complete recovery in the investigated parameters, but not in the same manner.

Key words: Neem, Triology®, liver, *Ctenopharyngodon idella*, heat shock protein (hsp70), plasma albumin, plasma total protein, DNA.

INTRODUCTION

The pecticides derived from the neem tree (*Azadirachta indica)* are considered to be relatively safe and promising biopesticides (Anon, 1992). Azadirachtin is a secondary plant product of the neem tree (*Azadirachta indica* A. Juss). It is the main active component of neem seeds and a prime example of biobotanical insecticides that disturb an insect's development rather than the biochemical or metabolic activity of enzymes that are found more ubiquitously in nature (Schmutterer, 1990). However, these pesticides have been found to be toxic to fish (Mondal *et al*., 2007 and Winkaler *et al*., 2007).

In Egypt, the neem oil extract (trade name: Triology) is used to control the pests in the economic crops such as red spider mite on Strawberry crop, white fly on Cucumber crop and aphis on Allspice crop. However, neem may enter aquatic systems such as streams, rivers, and lakes if used in the adjacent areas, or if an accidental spill occurs (Hassanein *et al*., 2006). Thus, the present work was planned to investigate the effect of this pesticide on some biochemical parameters such as heat shock protein70, plasma albumin, plasma total protein, and DNA content in liver of the fresh water fish, *" Ctenopharyngodon idella"*

Heat shock proteins are a group of highly conserved proteins that play a major role in cellular stress response. The naming of these proteins is based on their molecular mass (kilodaltons, KDa). Thus, they are divided into 5 families: hsp 100, hsp 90, hsp 70, hsp 60, and the low molecular weight (LMW) family, hsp 16-30 (Morimoto *et al*., 1994).

These proteins function as molecular chaperones to protect the cells from the damage caused by environmental stress. They protect other proteins from unfolding or refold denatured proteins, or obligate them for degradation (Morimoto *et al*., 1994).

The members of hsp 70 family of stress proteins are known to be inducible by a variety of stressors, including temperature shock, somatic shock and exposure to heavy metals or toxic organo-chemicals (Schröder *et al*., 1998, Hassanein *et a*, 1999; Hassanein and Abu-Amra, 2001; Badria *et al.*, 2004 and Hassanein *et al*., 2005 & 2006).

Moreover, variety of neem products was found to alter the total proteins either in fish or other experimental animals. Thus, the effects of fruit methanolic extract of the neem tree as well as water suspension of its milled dried leaves on serum protein pattern of the freshwater catfish, *Clarias lazera*, were studied over a period of 4 weeks (Adham, 1992).The results indicated that *C. lazera* provokes disturbances in the levels of circulating proteins during the administration of both neem products. These are expressed as hyperproteinemia as well as disproteinemia. The higher intensity of hyperproteinemia was evoked by the application of leave water suspension. The highest significant hyperproteinemia was recorded after 3 weeks of treatment. Moreover, disturbances in albumin levels (hyper-& hypoalbuminemia) were exhibited by both neem-fed sets. However, the disturbance in the total proteins level due to different neem extracts has been reported (Ibrahim *et al*., 1992 (a&b); Kasturi *et al*., 1995; Joshi and Nazeer , 1996; Rahman *et al.* ,1996; Kasturi *et al*., 1997; Mahdi *et al*. ,2003 and Hassanein *et al*., 2006).

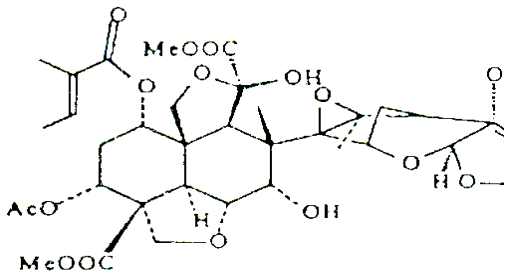
MATERIALS AND METHODS

Experimental animals:

Healthy samples of grass carp "*Ctenopharyngodon idella*" were collected from fish culture station at El-Ahaiwa, Sohag Governorate to be used for the present study. They were transported in well aerated large containers to the laboratory, then were kept in well aerated aquaria for 10 days to get acclimatized for the laboratory conditions before the start of the experiments. The fish were fed on grass during this period.

Tested pollutant:

The compound used in the present investigation is the neem oil extract (trade name: **Triology**®), a product of Thermo Triology Corp., USA. It was used as a commercial material of a concentration of 90 g /100 ml from which different dilutions were prepared by emulsification in water on the basis of LC_{50} (112 mg/L) which was previously determined in our laboratory.



Structure of Azadirachtin

Experimental design:

The experimental fish were divided into 3 groups. The first group was exposed to 1/10 LC_{50} (11.2 mg/L) of the pesticide "neem". Fish were sampled at intervals of 5, 10 and 15 days post exposure. Some fish were left for recovery, and sampled at the intervals of 5 and 10 days. The second group was exposed to 1/2 LC_{50} (56 mg/L) of the pesticide and were sampled after successive intervals of 2, 4 and 6 days of exposure. Some fish were left for recovery, and sampled after the intervals of 3 and 6 days. The third group served as control. At the end of each exposure time 10 samples of treated as well as control fish, were used for different investigations.

Biochemical parameters:

The levels of hsp expression were measured using dot blot technique according to the method of David (1991). The blots were incubated with mc anti-hsp70 and anti-mouse alkaline phosphatase conjugated antibody according to Backmann *et al*. (1986). The level of control dot was set to 100% and all other dots were compared to this ratio.

Plasma albumin concentration was determined according to Doumas *et al*. (1971). Plasma total protein concentration was determined according to Josephson *et al*. (1957). Deoxyribonucleic acid (DNA) was visualized by using the standard Feulgen's method (Drury and Wallington, 1980). The carnoy's fixed sections of liver were placed in N-HCl at 60°C for hydrolysis, followed by staining in Schiff solution then immersed in prepared bisulphate solution. The appearance of purple coloration indicated the presence of DNA.

Statistical analysis:

The data obtained in the present work were expressed as mean ± SE and were statistically analyzed using student t-test (Milton and Tsokos, 1983) to compare means of treated data against their control ones and the results were considered significant at ($P<0.05$).

RESULTS

Effect of Triology® on Liver hsp70

Effect of 1/10 LC_{50} (Group I)

Expression of hsp70 in fish liver exhibited marked increase after exposure to Triology® pesticide for 15 days Fig.(1A). The percentages of this increase were 211.72%, 248.71% and 239.15% after 5, 10 and 15 days respectively. Statistically, the increase after 5 days was highly significant ($P< 0.01$), while after 10 and 15 days of exposure was significant ($P< 0.05$) (Fig. 2A).

At the end of the recovery periods, 5 and 10 intervals, the expression of hsp70 in fish liver was decreased to 221.14%, and 189.83% respectively. All the observed changes were statistically highly significant ($P < 0.01$) (Fig. 2A).

Effect of 1/2 LC_{50} (Group II)

Administration of Triology® for 6 days resulted in the increase of hsp70 expression in the liver of treated fish as shown in Fig. (1B).

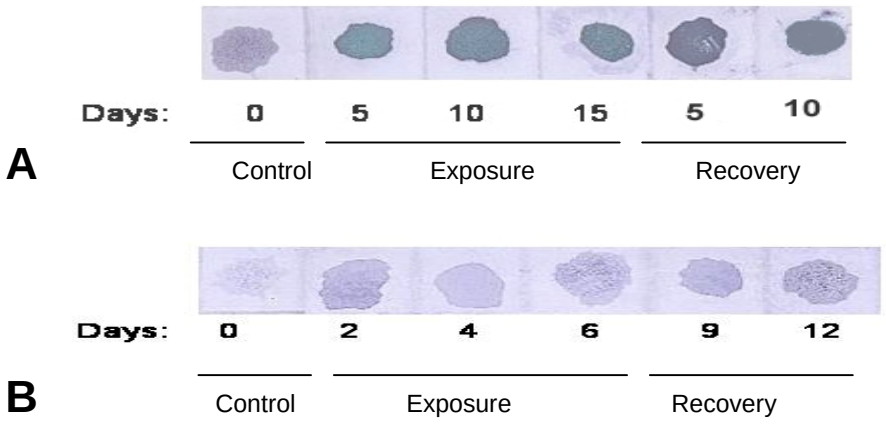
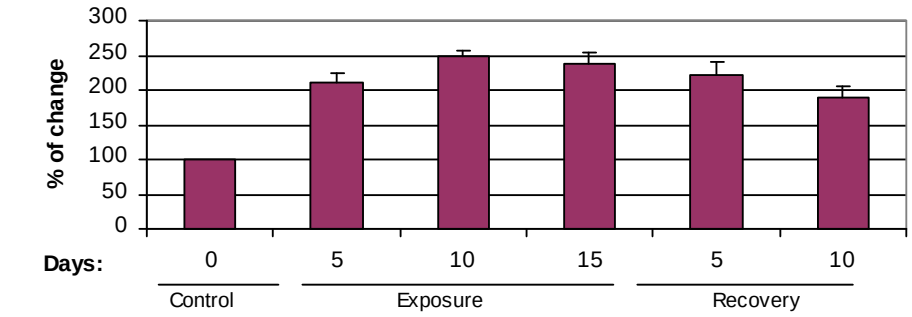


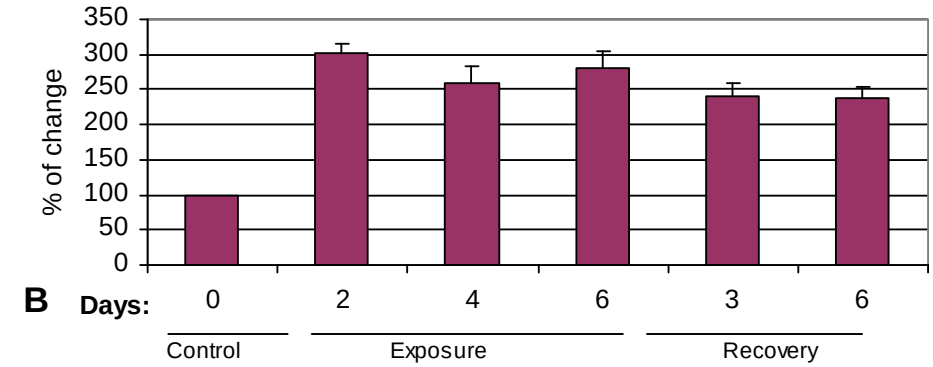
Fig. (1): Dot blot showing the expression of hsp70 in the liver of *C. idella* after exposure to Triology® A:1/10 LC_{50} for 15 days followed by 10 days recovery and B: 1/2 LC_{50} of Triology® for 6 days followed by other 6 days recovery.

The level of hsp70 was increased than its normal value during the whole experimental period, this increase was statistically highly significant (P< 0.01) after 2 and 6 days, while after 4 days it was significant (P< 0.05). The maximum expression level was observed after 2 days recording 301.34% compared to control value (100%) as represented in Fig. (2B).

In samples that were left for recovery for 3 and 6 days, the expression level of hsp70 recorded 239.4% and 237.94% respectively. Statistically, these changes were significant (P< 0.05) after 3 days, and highly significant (P< 0.01) after 6 days (Fig. 2B).



A



B

Fig.(2): Percentage of changes in the expression of hsp70 in the liver of *C. idella* after exposure to Triology® A:1/10 LC_{50} for 15 days followed by 10 days recovery and B: 1/2 LC_{50} of Triology® for 6 days followed by other 6 days recovery.
** highly significant *significant ° non-significant

Plasma albumin

Effect of 1/10 LC_{50} (Group I)

The level of plasma albumin recorded highly significant decrease (P<0.01) and P<0.001) after both 10 and 15 days of exposure to Triology® (11.2 mg/L), but the mean plasma albumin level was not significantly (P > 0.05) decreased after 5 days of exposure to Triology® (Fig 3). These mean levels were 1.143 $\pm$ 0.08, 0.898 $\pm$ 0.38 and 0.598 $\pm$ 0.049 g/dl after 5, 10 and 15 days of treatment, respectively. Also, fish groups exposed to 1/10 LC_{50} of neem for 10 days recovered and showed non-significant change (P > 0.05) in the plasma albumin level during the recovery period. The mean levels of plasma albumin were 1.205 $\pm$ 0.12 and 1.163 $\pm$ 0.097 g/dl after 5 and 10 days of recovery period, respectively.

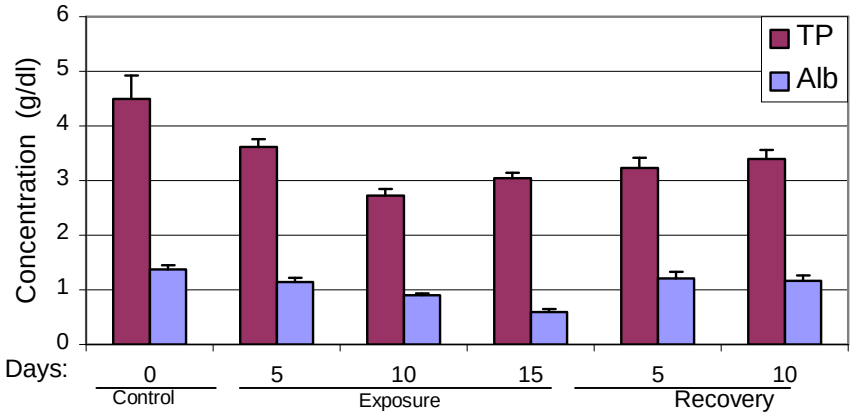


Fig.(3): Changes in plasma total protein and albumin of *C. idella* after exposure to 1/10 LC_{50} of Triology® for 15 days followed by 10 days recovery.

Effect of 1/2 LC_{50} (Group II)

Exposing of grass carp *C. idella* to 56 mg/L of neem pesticide (Triology®) for 6 days resulted in a highly significant decrease ($P< 0.001$) in plasma albumin level. These levels were 0.777 ± 0.1, 0.743 ± 0.11 and 0.728 ± 0.085 g/dl after 2, 4 and 6 days of exposure respectively (Fig. 4).

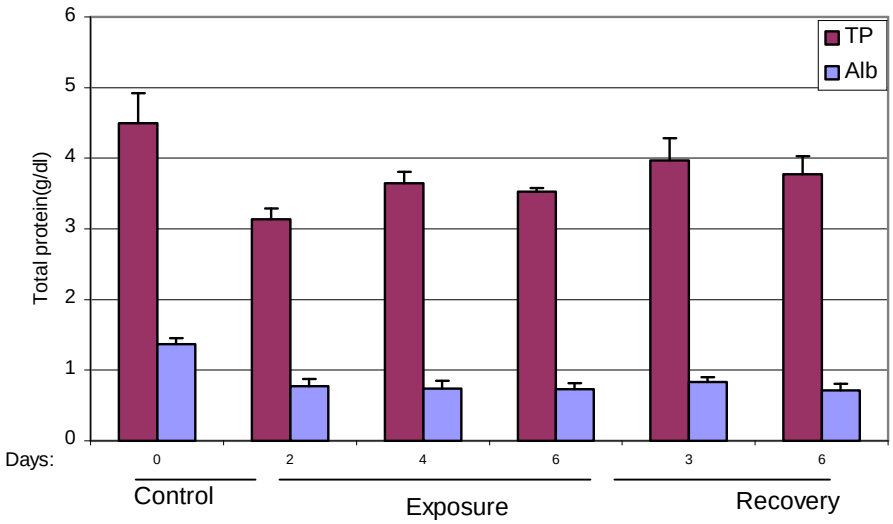


Fig. (4): Changes in plasma total protein and albumin of *C. idella* after exposure to 1/2 LC_{50} of Triology® for 6 days followed by 6 days recovery.
** highly significant *significant ° non-significant

However, fish groups exposed to 1/2 LC_{50} of neem for 6 days did not recover and showed highly significant reduction ($P< 0.001$) in the level of plasma albumin throughout the two different intervals of recovery period. These levels were 0.832 ± 0.07 and 0.71 ± 0.1 g/dl after 3 and 6 days of recovery period.

Plasma total proteins

Effect of 1/10 LC_{50} (Group I)

Plasma total protein was markedly decreased in fish treated with low dose concentration (11.2 mg/L) of Triology® for 15 days (Fig.3). These levels were 3.62 ± 0.14,

2.72 ± 0.13 and 3.04 ± 0.097 g/dl after 5, 10 and 15 days of treatment respectively. Statistically, these changes were non-significant (P> 0.05) after 5 days and highly significant (P<0.01) after 10 days while after 15 days, it was significant (P < 0.05).

In fish samples that were left for recovery for 5 and 10 days, the level of plasma total proteins was significantly (P< 0.05) lower than the corresponding data in the control group (3.23 ± 0.19 , 3.4 ± 0.16 g/dl and control 4.5± 0.42, respectively.

Effect of 1/2 LC_{50} (Group II)

The present data indicated that treatment with high concentration of Triology® (56 mg/ L) showed a non-significant decrease (P> 0.05) in plasma total proteins at all periods except after 2 days which decreased significantly (P < 0.05) as compared with the control value (Fig. 4).The mean level of plasma total proteins were 3.14 ± 0.15, 3.65 ± 0.16 and 3.53 ± 0.05 g/dl after 2, 4 and 6 days of exposure respectively.

Keeping the fish groups in untreated water for a period of 6 days for recovery showed a slight improvement in the plasma total protein levels. These levels were 3.97 ± 0.31 and 3.77 ± 0.26 g/dl after 3 and 6 days of recovery period respectively. However, these changes were statistically non- significant (P > 0.05).

Deoxyribonucleic acid (DNA) in liver:

Normal fish:

In the normal liver sections of untreated *Ctenopharyngodon idella*, stained with feulgen's reaction, the red-purple coloured nuclei represent the DNA-content (plate 1 a).

Treated fish:

Effect of 1/10 LC_{50} (Group I)

Histochemical examination of liver tissue treated with 1/10 LC_{50} for 5 days showed a slight decrease of DNA contents in the hepatocytes (plate 1 b). This decrease became more pronounced after exposing the experimental fish to the same concentration of the pesticide for10 and 15 days (plate 1 c & b).

In fish samples that were left for recovery for 5 and 10 days, the histological preparations of the liver showed a marked increase in DNA contents (plate 1 e & f) as compared with the treated fish groups**.**

Effect of1/2 LC_{50} (Group II)

Histological observations revealed a decrease in DNA contents of hepatic cells after 2, 4 and 6 days of treatment with 1/2 LC_{50} (56 mg/L) of Triology® as compared with those of the normal control group (plate 2 a, b and c).

In addition, after 3 days of recovery period the decrease in DNA contents was continued (plate 2 d), but at the end of recovery period (6 days), the results revealed a slight improvement of DNA contents as compared with treated groups (plate 2 e).

DISCUSSION

The data obtained during the present work showed that exposing fresh water fish of the strain "*Ctenopharyngodon idella*" to 1/10 LC_{50} and 1/2 LC_{50} of the neem biopesticide Triology® has led to enhancement of hsp70 gene which translated to increased amounts of heat shock protein in the liver of the used fish at all intervals of exposure, but not in the same manner. However, these increased levels of expression of hsp70 were time dependant.

These results are in accordance with the results recorded by Hassanein (2004) who attributed the elevated levels of hsp70 in the tissues of *Tilapia zillii* to hormonal alterations induced by neem, where hormones are known to affect heat shock protein expression in fish as reported by Iwama *et al.* (1998), Deane *et al*. (2000) and Sathiyaa *et al.* (2001). Furthermore, these results may also be due to a damage occurred in the DNA. This suggestion is supported by the marked decrease DNA observed during the present study, as well as the results of Akudugu *et al*. (2001) who reported that DNA is a critical target for neem

intoxication. Moreover, expression of hsp70 is known to correlate with DNA damage in fish (Schröder *et al*., 2000). In addition, a published data provided that there are interactions between heat shock proteins and hormone-mediated, physiological processes such as the organismal stress response (cortisol and adrenaline), growth (growth hormone), and osmoregulation (prolactin and cortisol) as reviewed by Basu *et al*. (2002).

Although, the increased levels of the hsp70 which observed during the exposure period started to recover slowly after the elimination of the pesticide however, they will still showing high expression levels even till the end of two recovery periods. This is probably due to the fat that, heat shock proteins play a role in the long term adaptation of animals to their environment; their levels remain elevated in organisms long after the stressor removed (Parsell and Linddquist, 1993 and Morimoto and Santoro, 1998)

Proteins of blood serum are fairly labile biochemical system, precisely reflecting the condition of the organisms under the influence of internal and external changes.

The present data have revealed a marked decrease in the plasma albumin and total protein as well as in the liver DNA of *C. idella* treated with the pesticide Triology® at all intervals of treatments. At the end of two recovery periods, there was slight to marked recovery in these investigated parameters. The above mentioned changes were in accordance with the results offered by some investigators working on the impacts of different neem extracts on proteins. Tewari *et al*. (1989) found that neem oil decreased the total protein content in the uterus of rats. Also, there was a decrease in the total protein levels in serum of Brown Hisex chicks feeding with diets containing 2% and 5% *Azadirachta indica* leaf (Ibrahim *et al*. 1992a) as well as diets containing 2% and 5% *Azadirachta indica* ripe fruit (Ibrahim *et al*. 1992b). Moreover, Adham (1992) observed that the effects of fruit methanolic extract of the neem tree, *Melia azedarach* as well as water suspension of its milled dried leaves on serum proteins pattern of the freshwater catfish, *Clarias lazera* cuase disturbance in the levels of circulating proteins during the administration of both neem products. These are expressed as hyperproteinemia as well as disproteinemia. In addition, Kasturi *et al*. (1995) reported that the effects of dry, powdered *A.indica* leaf suspended in distilled water on adult male Wistar rats have caused decrease in total protein in both the caput and caudal regions of the epididymides of treated rats. Similarly, the effects produced by treating male wistar rats with powdered dry neem leaves for 24 days were characterized by protein reduction in the testes (Joshi and Nazeer., 1996). Also, there was a decrease in total protein in seminal vesicles and ventral prostate after oral administration of 20, 40 and 60 mg of dry *Azadirachta indica* leaf powder for 24 days (Kasturi *et al*., 1997).

The antioxidative potential of *Momordica charantia, Azadirachta indica, Allium sativum* and *Ocimum sanctum* was assessed in streptozotocin induced diabetic rats (Mahdi *et al*., 2003). Albumin was decreased in diabetic group as compared to normal control. Following treatment with herbal preparations, except in case of *A.indica*, significant increase in albumin level was recorded. On other plant extracts, exposure of fish over 96 hrs to 40 and 80% of LC_{50} (24 hrs) of aqueous latex extracts of *Euphorbia royleana* and *Jatropha gossypifolia*of family *Euphorbiaceae* significantly decrease the level of total protein, total free amino acids, and nucleic acids, in muscle, liver and gonadal tissue of the fish *Channa punctatus* (Singh and Singh, 2002).

However, decrease of the protein contents was a clear response to different pesticides. In this regard, treatment of *Gambusia affinis* treated with malathion had induced a highly significant ($P<0.01$) decrease of the protein contents of the liver, testis and ovary as elucidated by Hassanein (1991). In addition, exposure of *Channa striatus* to sublethal concentrations of cypermethrin, permethrin and fenvalerate pesticides had resulted in a significant decline in the total proteins level of the liver and muscle tissues of the fish (Singh and Agarwal, 1994). Moreover, the Indian catfish, *Heteropneustes fossilis* exhibited marked alterations in its serum proteins after exposure to sublethal concentrations of malathion (Munshi *et al*., 1999).

Reductions of total proteins were also recorded in subsequent to pesticides treatment in mammals as postulated by Saleh *et al.* (1986) who observed a marked diminution of serum proteins and amino acids in rats treated with fenvalerate. Shaker *et al.* (1988) found that rabbit treatment with dimethoate had resulted in a significant depletion in serum total proteins and globulins. Moreover, Abu-El-Zahab *et al.* (1993) recorded a highly significant fall in serum total proteins and albumin concentrations in rats after application of pyrethroids pesticides.

In contrast, the present results disagree with those of Rahman *et al*. (1996) who concluded that serum levels of protein contents were significantly increased at all doses in male and female wistar rats administered with neem pesticide. Likewise, other pesticides rather than neem have caused an increase in serum, brain and liver total proteins in rabbits after exposure to cyanofenphos and profenphos pesticides (Enan *et al.*, 1987) and in total proteins and albumins in rats exposed to parallethrin (Seki *et al.*, 1987).

The decrease in the plasma total protein levels observed in the present study may be due to their degradation and to their possible utilization for metabolic purposes. In addition, these results may be attributed to DNA damage, destruction or necrosis of cells and consequent impairment of protein synthesis or may also be due to impaired incorporation of amino acids into polypeptide chains (Bradbury *et al.*, 1987 and Singh *et al.*, 1996). This disturbance might also be due to increased albumin catabolism, the decreased synthesis of albumins or as a result of the two simultaneously occurring processes. In general, hypoalbuminemia has been shown to be concomitant with liver damage, as the liver is known to play an essential role in protein synthesis (Ross *et al.*, 1966 and Adham, 1992).

In conclusion, data collected in the present investigation may suggest that these plant extracts are potential inhibitor of DNA synthesis, this result is supported by the marked decrease of total proteins and DNA observed during the present study, an may also be supported by the results of Chattopadhyay (1998) who found that the water soluble part of alcoholic extract of *A. indica* leaves at a dose of 200 mg/kg inhibited significantly DNA and RNA in rats, and the results of Akudugu *et al*. (2001) who reported that DNA is a critical target for neem intoxication. In addition, Singh and Singh (2002) reported that pesticides attack many enzymes responsible for normal metabolic pathway. Thus it is possible that extract of neem plants might has inhibited the enzymes necessary for DNA synthesis.

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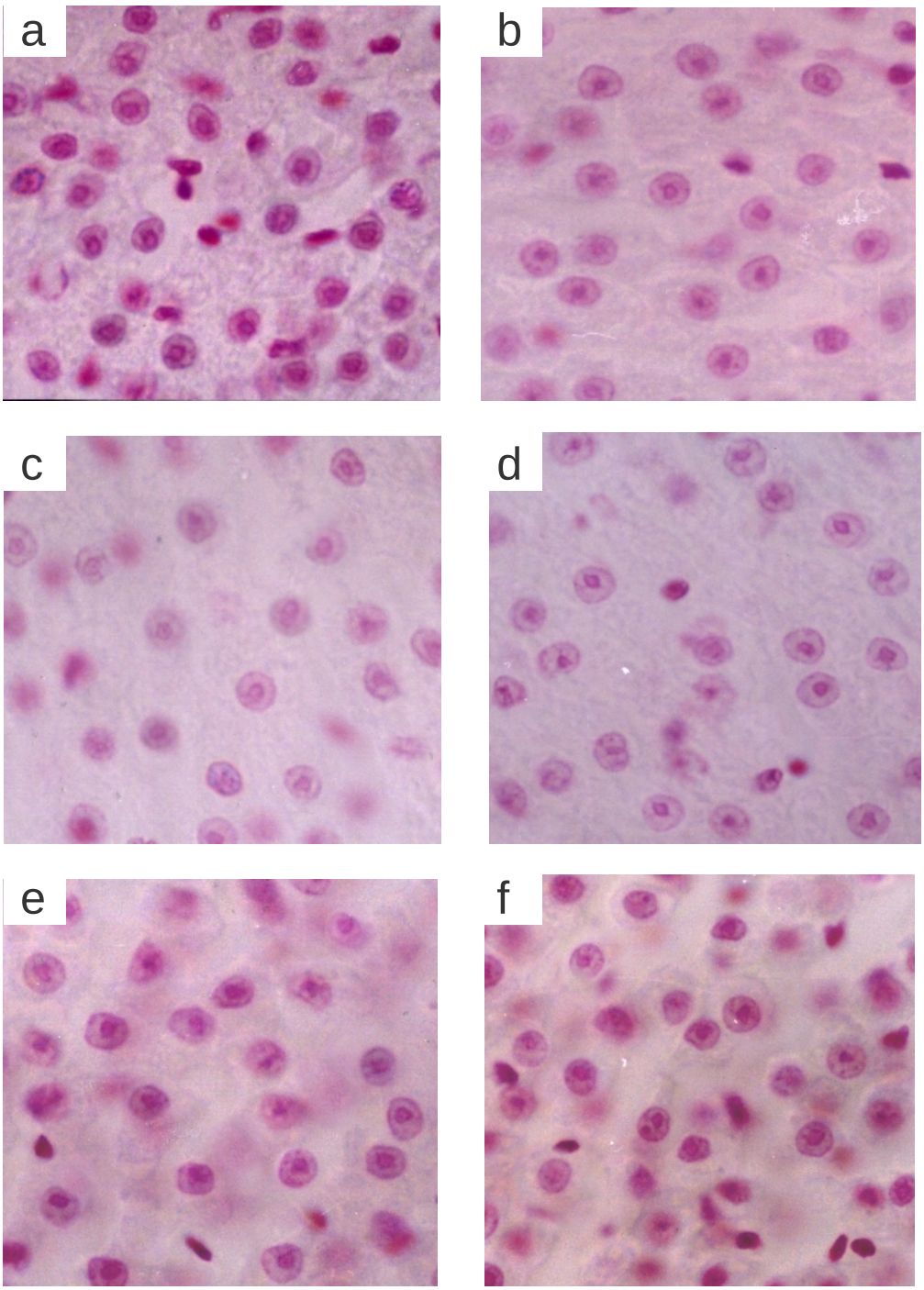

Plate 1: Sections through the liver of a: control fish stained with Feulgen's reaction, showing normal DNA content, **b:** the liver of fish treated with 1/10 LC_{50} of Triology® for 5 days, showing a slight decrease in the DNA content, **c:** after 10 days showing a decrease of DNA, **d:** after 15 days showing severe decrease of DNA content, **e:** after 5 days of recovery period showing an improvement of DNA content and **f:** after 10 days of recovery period, showing a rather complete recovery of the DNA content in the liver. X1000

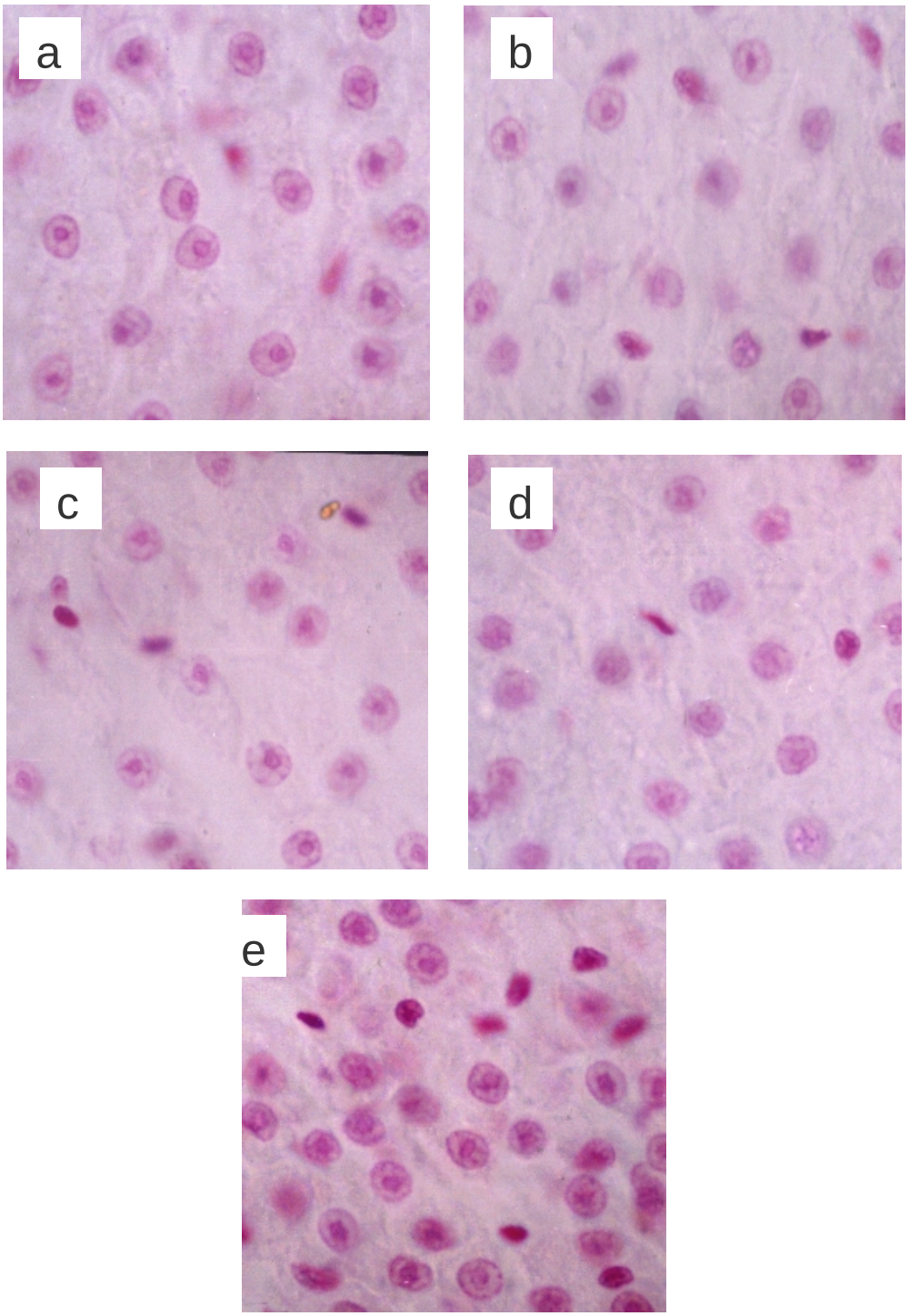

Plate 2: Sections through the liver of fish treated with 1/2 LC_{50} of Triology® a: after 2 days, showing a decrease of DNA, **b:** after 4 days showing severe loss of DNA, **c:** after 6 days, showing reduced DNA contents, **d:** after 3 days of recovery period, showing a decrease of the DNA content and **e:** after 6 days of recovery period, showing an improvement of the DNA content. X1000

ARABIC SUMMERY

التغيرات البيوكيميائية فى البروتينات و(دن.أ.)فى " تينوفارينجودون ايدلا" نتيجة المعاملة بالمبيد الحيوى (تريولوجي)

حمدى محمود أحمد حسانين و حنان على عقيل
قسم علم الحيوان- كلية العلوم - جامعة بسوهاج

لقد صمم هذا البحث لدراسة التأثيرات البيوكيميائية لمبيد النيم الحيوى (تريولوجى) على الزلال والبروتينات الكلية فى البلازما ,كذلك دراسة مقدار التعبير عن جين البروتين المستحث حراريا (hsp_{70}) و الحمض النووي الديوكسى ريبوزى (دن.أ.)في نسيج الكبد.

تم تقسيم الأسماك الى ثلاث مجموعات حيث عرضت المجموعة الأولى إلى التركيز (1/10 LC_{50}) من مبيد النيم الحيوي" تريولوجى" لمدة 15يوم ثم نقلت الأسماك إلى ماء بدون مبيد لمدة 10 أيام أخرى كفترة لاستعادة . تم اخذت العينات كل 5 أيام على مدار التجربة. و تم تعرض المجموعة الثانية إلى التركيز (1/2 LC_{50}) من مبيد النيم الحيوي " تريولوجى" لمدة 6 أيام مع اخذ العينات بعد 2و4و 6ايام. ثم نقلت الأسماك إلى ماء بدون مبيد لمدة 6 أيام أخرى كفترة استعادة ثم اخذت العينات كل 3 أيام. المجموعة الثالثة كانت مجموعة ضابطة.

أظهرت النتائج أن مستخلص النيم سبب نقص واضح في مستوى البروتينات الكلية و الزلال في البلازما وأيضا نقص في كمية الحمض النووي الديوكسى ريبوزى (دن.أ.) في نسيج الكبد. وقد لوحظ أيضا أن النقص في مستوى البروتينات الكلية و الزلال في البلازما خلال فترة التعرض بدأ في الاستعادة متراجع إلى حداما إلى المستوى الطبيعي بعد نقل الأسماك إلى ماء بدون مبيد خطة بعد التعرض للجرعة المنخفضة. ولوحظ أيضا تحسن في كمية الحمض النووي الديوكسى ريبوزى في نسيج الكبد.

أوضحت النتائج ايضا أن مستخلص النيم سبب زيادة مضطردة في مستوى (hsp_{70}) في الكبد بعد التعرض لتركيزين مختلفين (1/10 LC_{50}) و(1/2 LC_{50}) من مبيد النيم الحيوي" تريولوجى". هذا وقد وجد أن الكميات المتزايدة من (hsp_{70}) و التي لوحظت خلال فترة التعرض بدأت في الاستعادة متراجعة إلى حد ما إلى المستوى الطبيعي بعد نقل الأسماك إلى ماء بدون مبيد.