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Impact of phenolic compounds on free amino acid contents and electrophoretic patterns of the Nile Tilapia, *Oreochromis niloticus*

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ABSTRACT

The present research was designed to determine the free amino acids (FAAs) composition and electrophoretic protein patterns by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in *Oreochromis niloticus* exposed to 1/10 LC_{50}-96 hrs of phenol and nonylphenol ethoxylate 9 (NPEO 9) under laboratory condition for 4 weeks, in an attempt to develop sensitive bio-indicators for evaluation of pollution effects. Variations of FAAs were apparent in both treated groups. Significant increases were recorded in all individual amino acids including essential and non-essential ones, except for proline for both treated groups and leucine for phenol exposed fish group where they decreased significantly. The electrophoretic analysis of protein of the skeletal muscle homogenate using SDS-PAGE indicated that the control group yielded 10 fractions of molecular masses between 166 and 23 kDa. A total number of 9 bands were separated from fish of phenol group; they were varied from 168 to 23 kDa. However, 6 bands only were separated from fish of the NPEO 9 group; they were varied from 157 to 28 kDa. The muscle protein extract from fish of phenol group showed five unique bands (168, 96, 68, 52, and 39 kDa). Moreover, fish that were exposed to NPEO 9 group showed three unique bands (157, 92, and 72 kDa). The highest similarity index "S" value observed between phenol and control groups was 0.37, followed by 0.33 between NPEO 9 group and the control one. This could be an indicator of changes in protein metabolism due to phenol and NPEO 9 pollution.

INTRODUCTION

The contamination of water by emerging contaminants such as, drugs, diagnosis products, steroids, hormones, and personal care products are matters of growing concern (Boleda *et al.*, 2009; Santos *et al.*, 2010). Organisms in aquatic environments are usually subjected to a complex mixture of chemicals including parent compounds and their transformation products causing numerous harms to the organisms, population and ecosystem, organs' function, reproductive cycle, and biological diversity (Vorosmarty *et al.*, 2010, Ginebreda *et al.*, 2014;).

Phenols are released into water from the effluents of a variety of industries for example, coal refineries, phenol manufacturing, industries of resin, paint, dyeing, textile, leather, petrochemical, and pulp mill. Natural processes such as the decomposition of plant matter additionally add to phenol accumulations in the aquatic environment (Ali *et al.*, 2011). Contingent upon the environmental concentration, phenol can lead to death to fishes (Moraes *et al.*, 2015). Multiple mechanisms of action of phenol or some phenolic compounds have been reported, including drug antagonists (Roche and Bogé, 2000), carcinogenesis, and mutagenesis (Yin *et al.* 2006), and metabolic interruption (Hori *et al.* 2006). Also, phenolic residues in the fish flesh may prompt a danger of stomach cancer for the consumers (Moustafa *et al.*, 2007).

Phenol is among the first compounds depicted as toxic by the Environmental Protection Agency - United States (US EPA*)* and because of its relevance as an ecotoxin, it has been maintained in the priority list (Varadarajan *et al.*, 2014). Nonylphenol ethoxylate (NPEO) is one of the most perilous chemicals that are recorded in aquatic environments (Tsuda *et al.*, 2000; Rivero *et al.*, 2008). Such chemical is generally used in the production and formulation of many commercially sold products (e.g. industrial and commercial detergent, polymer resin, and cosmetic items).

Amino acids are considered one of the most reliable procedures for the recognition of changes in protein synthesis in cells (Shruti *et al.*, 2014). Amino acids fundamental intermediates in protein synthesis and its degradation products show up as various nitrogenous substances (Jobgen *et al*., 2006). Amino acid and some nitrogenous compounds play an important role during osmotic stress subsequently increment or reduction in free amino acid (FAA) content provide valuable information during stress phenomenon at the tissue level. Two sorts of changes in the FAA pool of organisms normally happen under toxicant-induced stress. The first includes either an increase or decrease in the total FAA concentration. The second kind of response brings about alterations in specific amino acids (either increases or decreases), which may or may not cause changes in the total amino acid concentration (Matta *et al.*, 2007).

Electrophoresis is the capacity to isolate a polypeptide of interest and to have an indication of its molecular size. It is very important in any investigation including mixtures of proteins (Bakry *et al.*, 2011). The most relatively powerful and basic method involves sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). In this technique, separation of proteins based on their molecular size; where, the SDS–protein complexes are sieved through a polyacrylamide gel matrix (Bakry *et al.*, 2013).

The present work aims to investigate the impact of sub-lethal concentrations of phenol and nonylphenol ethoxylate 9 (NPEO 9) on the free amino acids content and the electrophoretic protein patterns (using SDS polyacrylamide gel electrophoresis) of *Oreochromis niloticus* under laboratory conditions.

MATERIALS AND METHODS

Phenol and NPEO 9

Phenol: Technical grade of phenol (C_6H_5OH) was obtained from El-Gomhorria Pharmaceutical Chemical Company, Cairo, Egypt. Phenol solutions were prepared by dissolving the estimated weights in water upon usage.

NPEO 9: Technical grade of NPEO 9 [$C_9H_{19}C_6H_4$ $(OCH_2CH_2)_nOH$] was obtained from Sigma-Aldrich Company (St. Louis, MO, USA).

Maintenance of Nile tilapia, *O. niloticus* fish

Healthy Nile tilapia, *O. niloticus*, samples were obtained alive from World Fish Center Farm (WFC) in El-Abbassa, El-Sharkeya governorate, Egypt. Fish were transported to the laboratory in plastic bags containing de-chlorinated water and aerated with oxygen. All fish were acclimated in a 50 L tank with de-chlorinated tap water and supplied with compressed air from air pumps *via* air-stones for two weeks prior to the experiment (Khalil *et al.*, 2017). Water temperature was maintained at 25 ± 2°C.

Toxicity screening

After acclimation, the fish were submitted to static acute toxicity tests (24, 48, 72, and 96 hrs). The concentrations used for phenol were (10, 20, 30, 40, and 50 mg/L) and for NPEO 9 were (100, 200, 400, 800, and 1600 µ/L). The tests were carried out in glass aquaria of 30 L, with five fish in each aquarium. A control group exposed only to water was contrasted with treated ones at each experimental time. All toxicity tests were carried out in triplicate. The exposure periods were with the same temperature, dissolved oxygen, and pH as in the acclimatization period. The dead fish was recorded in each concentration to estimate the LC_{50} values. Computation of LC_{50} values was determined utilizing the statistical program SPSS v. 17.0 for Windows (SPSS Inc. 2008).

Design of experiments

The fish were transferred from the initial acclimation tank to exposure tanks (30 L capacity). The acclimated fishes about 19 g in weight and 10 cm in length) were randomly divided into three groups; phenol, NPEO 9, and control groups. Triplicates were kept for both the treated groups and the respective control. Sub-lethal concentrations of phenol (2.0 mg/L) and NPEO 9 (10 µ/L), corresponding to 1/10 LC_{50}-96 hrs of the respective compounds against fish were used. The exposure period was for 28 days. During experiments, the ambient temperature was 25 ± 2ºC and all aquaria supplied by aerators. Fish were fed daily on the commercial fish diet with a rate of 3% of live body weight divided into 2 equal daily meals (Eurell *et al.*, 1978). The experiments were designed to determine the possible protein changes in the muscles of the treated fish compared to the control ones.

Determination of free amino acids content

Extraction procedures

By the end of the experiment, fish (5 animals/replicate) were sacrificed and 1 g of the dorsal muscle was mixed with 10 mL of 6 mol HCl and heated at 100°C for 24 hrs and then filtered (Campanella *et al.*, 2002; Laurens *et al*., 2012; Jajić *et al*., 2013).

HPLC conditions

- HPLC analysis was carried out using an Agilent 1260 series. The separation was carried out using ZORBAX Eclipse Amino Acid Analysis (AAA) C18 column (4.6 mm x 150 mm, 5 μm). The mobile phase consisted of phosphate-buffered saline, pH 7.8 (A), and acetonitrile: methanol: water 50:45:5 (B) at a flow rate of 2 mL/min. The mobile phase was programmed consecutively in a linear gradient as follows:
- **Mobile phase A:** 1.0 liter of Na_2HPO_4 (dissolve 5.5 g of NaH_2PO_4 in 1.0 L distilled water and adjust pH to 7.8 by 10 mol NaOH)
- **Mobile phase B:** acetonitrile: methanol: water (45:45:10)
  The separation was done with the following gradient at 0.0 – 1.9 min 100% A, at 1.9 – 18.1 min, 43% A, 57% B, at 18.6 – 22.3 min, 100% B, at 23.2 – 26.0 min, 100% A, the solvent flow rate was 2 mL/min and the separation was at 35°C, and the injection volume was 10 μL of the standards and extracts.
- The diode-array detection was monitored at 338 nm (band width 10 nm) and 262 nm (band width 16 nm).

Protein analysis by SDS-PAGE

SDS-PAGE was used to investigate any possible variations that would be found in the muscle proteins (sarcoplasmic proteins) of the tested fish by the end of the 4^{th} week of exposure. Briefly, after sacrificing fish, a sample of 1.0 g white dorsal epiaxial muscle from 5 fish (0.2 g from each fish) was isolated and then immediately stored at –20°C until the processing. Tissue extracts were prepared by homogenizing the samples in a Tris-HCl buffer solution (10% SDS, 20% glycerol, 0.2 mol Tris pH 6.8, 10 mmol beta-mercaptoethanol, and 0.05% bromophenol blue) in a 1:4 (w/v) ratio, then centrifuged at 4000 rpm for 15 minutes at 4ºC in cooling centrifuge. The supernatants were pipetted into Eppendorf vials and stored at –20°C until used for analysis. Protein samples were then boiled in a water-bath for 5 min at 100°C to obtain soluble protein. This procedure was done according to the method described by Jesslin *et al.* (2013).

Electrophoretic analysis of soluble tissue proteins

The technique of Laemmli (1970) was used to separate proteins according to their molecular weights. The soluble proteins were subjected to 15% SDS-PAGE gel (prepared on the SDS-PAGE system) using a vertical slab gel unit, followed by staining with Coomassie stain. Electrophoresis was carried out at a constant volt (50 V) in an electrode

buffer composed of 200 nmol glycine, 25 mmol Tris-HCl and 0.1% SDS, pH 8.3 (Sambrook and Russell, 2001).

Data analysis

Data are expressed as mean ± standard error (SE). Data of three different groups were subjected to one-way analysis of variance (ANOVA) to compare between them. If ANOVA indicated significance, the least significant difference (LSD) test was used to determine differences among means (Snedecor and Cochran, 1989). Differences were considered significant at $P < 0.05$. Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) software (version 17, SPSS Inc., Chicago, USA).

RESULTS

Effects of phenol and NPEO 9 on amino acids content in the skeletal muscles of *O. niloticus*

The amino acid profiles of *O. niloticus* continuously treated with phenol (2 mg/L) and NPEO 9 (10 µ/L) after 4 weeks, as well as control fish, are summarized in Table (1). All amino acids were significantly increased when *O. niloticus* fish exposed to phenol (2 mg/L) and NPEO 9 (10 µ/L), except for proline for both treated groups and leucine for phenol exposed fish group where they showed significant decreases as compared with the control group. The maximum increase in non-essential amino acids was noticed in tyrosine content in muscles of *O. niloticus* fish exposed to phenol and NPEO 9 (76.35 and 188.24%, respectively). The maximum increase in essential amino acids was noticed in isoleucine content (55.86%) and valine content (74.60%) in muscles of *O. niloticus* fish exposed to phenol and NPEO 9, respectively (Table 1).

The essential amino acids increased with 21.20% and 28.49% for phenol and NPEO 9 fish groups, respectively, than the control one. Furthermore, the non-essential amino acids elevated with 32.56% in phenol-treated fish and 42.76% in NPEO 9-treated fish group. Also, the estimated total FAAs content of the phenol fish group increased by 27.33% than control fish, meanwhile NPEO 9-treated fish have 36.14% increase in total FAAs content (Table 1).

Effects of phenol and NPEO 9 on tissue soluble proteins in the skeletal muscles of *O. niloticus*

The SDS-PAGE of the skeletal muscle homogenate of *O. niloticus* fish of different experimental groups are presented in Figure (1) and Table (2). The electrophoretic analysis of protein of the control group yielded 10 fractions of molecular masses between 166 and 23 kDa. A total number of 9 bands were separated from fish of phenol group; they were varied from 168 to 23 kDa. However, 6 bands only were separated from fish of the NPEO 9 group; they were varied from 157 to 28 kDa.

Table (1): Free amino acids (FAAs) contents in muscles of *O. niloticus* fish exposed to phenol and nonylphenol ethoxylate 9 (NPEO 9) after 4 weeks in comparable to the control fish.

Amino acids	Concentrations (µg/g)				
	1/10 LC_{50}-96 hrs of Phenol (2 mg/L)	% of change	1/10 LC_{50}-96 hrs of NPEO 9 (10 µ/L)	% of change	Control
Arginine	276.55 ± 1.155 a	48.18	282.33 ± 1.67 a	51.27	186.63 ± 1.63 b
Valine	389.88 ± 1.88 a	49.32	455.88 ± 0.88 b	74.60	261.09 ± 1.09 c
Methionine	221.49 ± 0.51 a	6.48	268.68 ± 1.31 b	29.17	208 ± 1.41 c
Phenylalanine	135.66 ± 0.66 a	7.66	133.19 ± 1.19 a	5.70	126 ± 2.00 b
Lysine	682.01 ± 2.01 a	2.42	729.44 ± 2.56 b	9.54	665.89 ± 3.11 c
Leucine	59.35 ± 0.64 a	-22.10	128.5 ± 0.5 b	68.65	76.19 ± 2.19 c
IsoLeucine	368.5 ± 1.5 a	55.86	263.84 ± 1.16 b	11.59	236.42 ± 1.42 c
ΣEAA	**2133.44 ± 8.355**	**21.20**	**2261.86 ± 9.27**	**28.49**	**1760.22 ± 12.85**
Proline	446.24 ± 1.24 a	-4.42	335.04 ± 0.95 b	-28.24	466.90 ± 0.9 c
Aspartic acid	433.24 ± 3.24 a	36.82	468.59 ± 0.9 b	47.99	316.63 ± 2.63 c
Glutamic acid	652.65 ± 2.35 a	53.65	672 ± 2.99 b	58.21	424.75 ± 1.25 c
Serine	110.58 ± 0.57 a	24.72	195.78 ± 1.77 b	120.82	88.66 ± 0.66 c
Glycine	313.18 ± 0.81 a	14.39	311.80 ± 0.19 a	13.88	273.78 ± 0.78 b
Alanine	573.57 ± 1.57 a	52.62	624.58 ± 1.41 b	66.20	375.8 ± 1.19 c
Tyrosine	205.15 ± 1.85 a	76.35	335.31 ± 1.31 b	188.24	116.33 ± 1.33 c
ΣNEAA	**2734.61 ± 11.63**	**32.56**	**2943.1 ± 9.52**	**42.67**	**2062.85 ± 8.74**
ΣEAA/ ΣNEAA	**0.78**		**0.76**		**0.85**
Total FAAs	**4868.05 ± 19.985**	**27.33**	**5204.96 ± 18.79**	**36.14**	**3823.07 ± 21.59**

- EAA; Essential amino acids, NEAA; Non-Essential amino acids.
- Data are presented as mean ± standard error, n=5
- The mean values in the same row with different small letters are significantly different at ($P < 0.05$).

Protein of muscle extract from fish of phenol group (2 mg/L) showed five unique bands (168, 96, 68, 52, and 39 kDa). Moreover, fish that were exposed to NPEO 9 (10 µ/L) showed three unique bands (157, 92, and 72 kDa). Concerning similarity index "S" value observed between phenol and control groups, it was 0.37, followed by 0.33 between NPEO 9 and the control one, and 0.20 between phenol and NPEO 9 groups (Table 3).

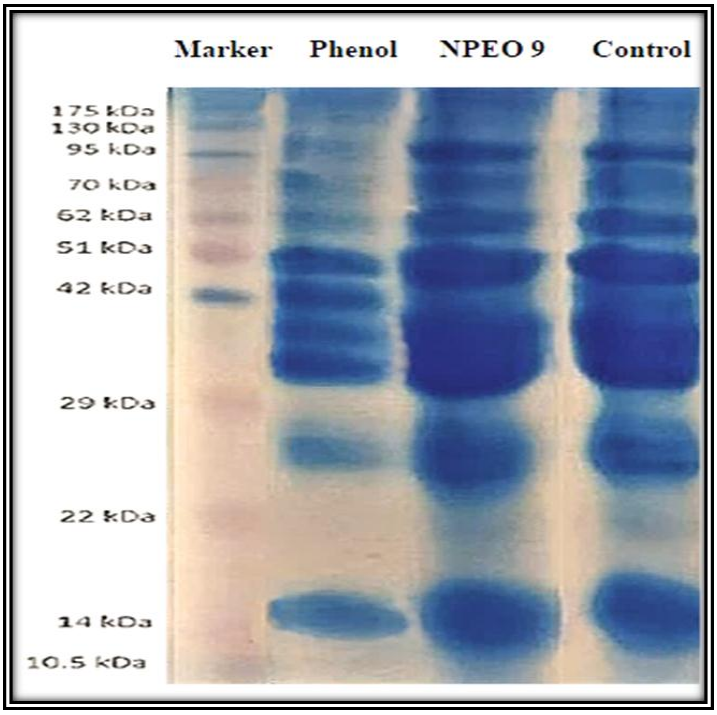

Fig. (1): SDS-PAGE showing the impact of phenol and nonylphenol ethoxylate 9 (NPEO 9) on protein profile of *O. niloticus* after 4 weeks exposure to sub-lethal concentrations of phenol and NPEO 9.

Table (2): Electrophoretic separation of tissue soluble protein from the muscle of *O. niloticus* exposed to sub-lethal concentrations of phenol and nonylphenol ethoxylate 9 (NPEO 9) after 4 weeks of exposure.

Bands	Marker (kDa)	1/10 LC_{50}-96 hrs of Phenol (2 mg/L) (kDa)	1/10 LC_{50}-96 hrs of NPEO 9 (10 µ/L) (kDa)	Control (kDa)
1	175			
2		168		
3				166
4			157	
5	130			
6				99
7		96		
8	95			
9			92	
10				76
11			72	
12	70			
13		68		
14	62			
15			54	54

16		52		
17	51			
18	42			
19			40	40
20		39		
21		34		34
22				30
23	29			
24		28	28	
25				25
26		24		24
27		23		23
28	22			
29	14			
30	10.5			
No. of bands	11	9	6	10

Table (3): Similarity index of different protein patterns of muscle extract of *O. niloticus* after 4 weeks exposure to sub-lethal concentrations of phenol and nonylphenol ethoxylate 9 (NPEO 9).

Variables	**1/10 LC_{50}-96 hrs of Phenol (2 mg/L) (kDa)**	**1/10 LC_{50}-96 hrs of NPEO 9 (10 µ/L) (kDa)**	**Control**
1/10 LC_{50}-96 hrs of Phenol (2 mg/L) (kDa)	1.00		
1/10 LC_{50}-96 hrs of NPEO 9 (10 µ/L) (kDa)	0.20	1.00	
Control	0.37	0.33	1.00

DISCUSSION

The free amino acid composition is a vital indicator of muscle quality, the amount of free amino acid is the basis for the increase in muscle protein content (Yang *et al.*, 2018). The present study indicates that all amino acids were significantly increased when *O. niloticus* fish exposed to phenol (2 mg/L) and NPEO 9 (10 µ/L), except for proline for both treated groups and leucine for phenol exposed fish group, where they decreased significantly as compared with the control group. In harmony with this observation, Tripathi *et al.* (2003) reported that the enhanced FAAs might be because of consumption

of reserved glycogen so that the fish can try to yield metabolic energy by gluconeogenesis process. It may also be ascribed to the lesser utilization of amino acids and their involvement in the maintenance of acid-base balance (Moorthy *et al.*, 1984). Matta *et al.* (2007) reported that the total FAAs concentrations were significantly ($p < 0.05$) higher in testes and ovaries of *O. niloticus* fish collected from the two polluted locations in Lake Mariut than that from the reference area. In addition, De Smet and Blust (2001) found that the concentrations of FAAs and the activities of proteases and aminotransferases were increased at day 4 in gills, liver, and kidney of common carp "*Cyprinus carpio*" exposed to 4 and 20 µmol of cadmium, and in gills and kidney at day 29 in carp exposed to 4 µmol cadmium. They suggested that the observed proteolysis was intended to increase the role of proteins in energy production due to cadmium stress. Senthil Elango and Muthulingam (2014) recorded a decrease in protein patterns and an increase in amino acid contents in brain and muscle of *Oreochromis mossambicus* that exposed to sublethal concentrations of chromium.

The electrophoretic analysis of protein of the control group yielded 10 fractions of molecular masses between 166 and 23 kDa. A total number of 9 bands were separated from fish of phenol group; they were varied from 168 to 23 kDa. While, 6 bands were separated from fish of the NPEO 9 group; they were varied from 157 to 28 kDa. In accordance, Sharaf-Eldeen and Abdel-Hamide (2002) investigated the exposure of *O. niloticus* to some pollutants and found that six protein fractions were missing because of treatment with a high level of copper. The disappearance of some protein bands, fading away of some existing bands, or the appearance of new bands, additionally influenced the relative quantities of protein fraction either by being increase or decline. However, this variance in protein bands might be considered as a reflection of stressor impact on ribosome and RNA levels, and therefore on protein synthesis (De Bruin, 1976).

CONCLUSION

In conclusion, the present study showed changes in protein metabolism due to phenol and NPEO 9 pollution.

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ARABIC SUMMARY

تأثير المركبات الفينولية على محتوي الأحماض الأمينية الحرة وأنماط الفصل الكهربي للبروتين في أسماك البلطي النيلي "*Oreochromis niloticus*" "

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أجريت الدراسة الحالية لتحديد تركيبات الأحماض الأمينية وتحليل الفصل الكهربي للبروتين باستخدام طريقة SDS-PAGE في أسماك البلطى النيلي "*Oreochromis niloticus*" التي تم تعريضها لـتركيز "-LC_{50} 1/10 96 hrs" من الفينول والنونيل فينول ايثوكسيلات (NPEO 9) تحت الظروف المعملية لمدة ٤ أسابيع وذلك لإيجاد مؤشرات بيولوجية حساسة لتقييم آثار التلوث. وقد كانت الاختلافات في الأحماض الأمينية الحرة (FAAs) واضحة في كلتا المجموعتين المعالجتين. حيث تم تسجيل زيادة معنوية في جميع الأحماض الأمينية الفردية بما في ذلك الأحماض الأمينية الأساسية وغير الأساسية، باستثناء البرولين لكل من المجموعتين المعرضتين والليوسين لمجموعة الأسماك المعرضة للفينول حيث أظهرا انخفاض معنوي. بينما يشير التحليل الكهربي للبروتين لمستخلص العضلات الهيكلية باستخدام طريقة "SDS-PAGE" أن المجموعة الضابطة أسفرت عن ١٠ حزم بروتينية تفاوتت بين ١٦٦ و ٢٣ كيلو دالتون. و كذلك تم فصل ٩ حزم بروتين من مجموعة أسماك الفينول حيث كانت متفاوتة من ١٦٨ إلى ٢٣ كيلو دالتون. بينما تم فصل ٦ حزم من البروتين من مجموعة الأسماك التي تعرضت إلى "NPEO 9" وكانت متفاوتة من ١٥٧ إلى ٢٨ كيلو دالتون. وقد أظهر بروتين مستخلص العضلات من مجموعة أسماك الفينول ٥ حزم بروتينية فريدة (١٦٨ ، ٩٦ ، ٦٨ ، ٥٢ ، ٣٩ كيلو دالتون). علاوة على ذلك، أظهرت الأسماك التي تعرضت إلى "NPEO 9" ٣ حزم بروتينية فريدة (١٥٧ ، ٩٢ ، ٧٢ كيلو دالتون). وقد وجد أن أعلى قيمة لمؤشر تشابه "S" بين مجموعة الفينول والمجموعة الضابطة كانت ٠,٣٧، يليها ٠,٣٣ بين NPEO 9 والمجموعة الضابطة. وقد يكون هذا مؤشرا على التغيرات في التمثيل الغذائي للبروتين بسبب التلوث بالفينول و NPEO 9 .