

Aflatoxin B₁ Induced-Changes in Protein Electrophoretic Pattern and DNA in *Oreochromis niloticus* with Special Emphasis on the Protective Effect of Rosemary and Parsley Extracts

Manal I. El-Barbary

Aquatic Pathology Laboratory, National Institute of Oceanography and Fisheries,
Inland Branch Water, Cairo, Egypt

Abstract: The adverse effects of two concentrations (0.25 and 0.50 of LC₅₀) of aflatoxin B₁ (AFB₁) on protein electrophoretic pattern and DNA strands of *Oreochromis niloticus* (• 22 g) muscles was investigated. Evaluation of the possible protective effects of rosemary and parsley extracts against the adverse effects of AFB₁ was another target. Sixteen groups of fish were tested; groups A and B were injected with saline and dimethylsulphoxide (DMSO), as control and control solvent groups, respectively. Groups F₁ and F₂ were injected with AFB₁ alone (9 and 18 mg kg⁻¹ B.W., respectively). While rosemary (R) and parsley (P) were injected (I.P.) either alone, at two levels of 2 and 4 g/Kg B.W. in groups (R₁ and R₂) and (P₁ and P₂), respectively, or in combination with AFB₁ at their different levels, (F₁R₁, F₁R₂, F₂R₁ and F₂R₂) and (F₁P₁, F₁P₂, F₂P₁ and F₂P₂ groups). Herbs extract and AFB₁ were dissolved in DMSO (25%). Samples were collected from muscles; protein was extracted and subjected to electrophoresis. DNA was extracted and purified. Results showed that the LC₅₀ was 36 mg AFB₁. Protein analysis showed remarkable variation in the number of bands and their genetic similarity in the phylogenetic tree. Rosemary in groups F₁R₂, F₂R₁ and F₂R₂ led to the pronounced highest numbers of protein bands (78, 77 and 74, respectively) among all fish groups injected with AFB₁. Although these groups fall into the same cluster, they were the farthest to control groups (A), genetically. Concerning parsley, F₂P₁ group showed increased number of detected bands (73) as compared to the other AFB₁ and parsley groups. DNA damage was clearly observed in F₂ then F₁ group. In conclusion, the damage due to AFB₁ was repaired or reduced by using rosemary and parsley, particularly with the lowest level of AFB₁. Also, rosemary may be more effective in reducing the DNA damage than parsley, especially with the highest level of rosemary. Yet, the low level of parsley was better than the high level.

Key words: Aflatoxin B₁ % Nile tilapia % Rosemary % Parsley % Electrophoretic protein % DNA damage

INTRODUCTION

Fish are important protein source for human beings in many countries. Most countries of the world care for increasing the fish production whether naturally or via aquaculture. Aflatoxin is among the most common contaminants causing great economic losses in aquacultural enterprises [1, 2]. It is a mycotoxin produced by certain fungal species, mainly *Aspergillus flavus* and *A. parasiticus*. Fish that exposed to chronic or acute toxicity of aflatoxin develop various health problems including reduction of growth performance and feed utilization, increased mortality [3-6], immunosuppression with consequent enhanced susceptibility to infectious

diseases [7] and deleterious effects on the reproductive traits [8,9]. Aflatoxin also causes dangerous histopathological changes in internal organs in addition to the mutagenic and carcinogenic effects [10-13]. The metabolic activation of AFB₁ results in the formation of toxic metabolites, such as AFB₁-8, 9-epoxide. The epoxide subsequently covalently binds to DNA to form AFB-DNA adducts in the liver [14, 15]. The mechanism of action of aflatoxin on the cell is mediated through the production of free radicals and reactive oxygen species, ROS [16, 17]. An *in vitro* study showed that AFB₁ could stimulate the release of free radicals resulting in chromosomal damages [18]. So, ROS may in part be responsible for the carcinogenic activity of AFB₁ [19].

Overproduction of such free radicals can cause oxidative damage to biomolecules (e.g. lipids, proteins, DNA) leading eventually to many chronic diseases as well as cancer. The defense is provided by antioxidants, many herbs may contain a wide variety of free radical scavenging molecules, such as phenolic compounds which are rich in antioxidant activity [20]. Previous studies referred to that leaf extract of *Ocimum sanctum* provides protection against AFB₁ carcinogenesis by acting as an antioxidant [21]. Therefore, *Amrita bindu*, herbal food supplements, was evaluated by aflatoxic fish (*Labeo rohita*) and proved that it has a potential role in ameliorating the AFB₁-induced DNA damage, thus suggesting its applicability in protecting the vital macromolecule DNA. While [22] reported that *Melampodium divaricatum*, medicinal plant, extract had an antigenotoxic action towards the DNA damage induced by AFB₁. On the same trend, the effect of AFB₁ on electrophoretic patterns of muscular protein of *O. niloticus* was investigated with emphasis on the possible protective effects of *Nigella sativa* [23] and ginger and chamomile [9]. The presence of phenolic antioxidant has been reported in rosemary *Rosmarinus officinalis* [24-26] and parsley [27-29]. Therefore the objective of this study was to evaluate the possible protective effects of rosemary and parsley extracts against the adverse effects of AFB₁ as monitored by an electrophoretic pattern of muscular protein and DNA damage in *O. niloticus*.

MATERIALS AND METHODS

Preparation of Aflatoxin B₁: AFB₁ was produced on liquid medium (potato dextrose) by *A. parasiticus* (NRRL 2999) according to Ready *et al.* [30]. AFB₁ was dissolved in chloroform and quantitatively estimated by thin layer chromatography, TLC [31]. So, chloroform was evaporated to dryness on a rotary vacuum evaporator at 40°C and redissolved in aqueous dimethylsulfoxide (DMSO) 25% (1:3 water) to the requirement of each aflatoxin concentration. AFB₁ was freshly dissolved in DMSO before injection.

Herbal Materials and Preparation of Their Extracts: Fresh rosemary and parsley leaves were obtained from a local farm and carefully washed with tap water then left to dry in the dark at room temperature. Twenty gram of the ground leaves were extracted for 24 hr by soaking in 500 ml of methanol (70%). The extract was then filtered

and the filtrate was divided into two amounts (one part and its double) before evaporating till dryness in a rotary evaporator (45°C). The residues of the two amounts were dissolved in constant volumes of 25% DMSO to obtain the two concentrations of herbs extract. The dose levels of 0, 2 and 4 g/kg B.W. were divided into two equal doses, the first was injected at the start of the experimental period and the second was injected one week later.

Determination of Polyphenols: The solvent of herbs extract was evaporator under vacuum and the dried residues containing the phenol compounds were dissolved in a solution consists of methanol: water: acetic acid (40: 59.3: 0.7 v/v/v) and stored in vials. The high performance liquid chromatographic (HPLC) method suggested by Christian [32] was used.

Determination of Lethal Concentration 50 (LC₅₀) of AFB₁ in *O. niloticus*: Fish, *O. niloticus*, were obtained from El-Serw (governmental fish farm), where the experiment was carried out in 2007, with an average body weight of 22 g to determine the LC₅₀ of AFB₁. The fish were acclimated for 2 weeks in aquaria supplied with dechlorinated tap water. After the acclimatization period, the fish were divided randomly into 6 groups, 24 fish in each group maintained in three aquaria per AFB₁ level. These groups were injected I.P with 0, 20, 40, 60, 80 and 100 mg AFB₁/Kg B.W. and were observed for mortality for a 96 hr period. The control fish group was injected only with DMSO 25%.

Fish and Experimental Design: Two hundred and eighty eight fingerlings of *O. niloticus* were acclimated to aquaria conditions for 2 weeks before the experiment was initiated. Six fish (approximately the same size, 20g in average) were stocked into each of the 48 aquaria, three glass aquaria (70X40X30 cm and contained 50 l of water) for each treatment. The aquaria were provided with continuous aeration and their water was changed partially every daily and totally a week. All fish were received their diet twice daily at a daily feeding rate of 3% of the actual body weight, six days weekly for two weeks. Fish were divided into 16 groups and were administered the test compounds I.P. and the effect was studied at the end of the 2nd week. The experimental setup used is shown in Table 1. AFB₁ was tested at three levels, being 0, 0.25 and 0.50 the LC₅₀, in a single dose, while either of rosemary and parsley extract was used at three levels also (0, 2 and 4 g/kg B.W.), each was divided into 2 doses (pretreatment at the

Table 1: Explanation of the experimental groups

Groups	Pretreatment first week	Second week
A	Saline	Saline
B	DMSO 25%	DMSO 25%
F ₁	DMSO 25%	AFB ₁ 9 mg kg ¹ B.W.
F ₂	DMSO 25%	AFB ₁ 18 mg kg ¹ B.W.
R ₁	Rosemary 1g/kg B.W.	Rosemary 1g/kg B.W.
F ₁ R ₁	Rosemary 1g/kg B.W.	Rosemary 1g/kg B.W. + AFB ₁ 9mg kg ¹ B.W.
F ₂ R ₁	Rosemary 1g/kg B.W.	Rosemary 1g/kg B.W. + AFB ₁ 18mg kg ¹ B.W.
R ₂	Rosemary 2g/kg B.W.	Rosemary 2g/kg B.W.
F ₁ R ₂	Rosemary 2g/kg B.W.	Rosemary 2g/kg B.W. + AFB ₁ 9mg kg ¹ B.W.
F ₂ R ₂	Rosemary 2g/kg B.W.	Rosemary 2g/kg B.W. + AFB ₁ 18mg kg ¹ B.W.
P ₁	Parsley 1g/kg B.W.	Parsley 1g/kg B.W.
F ₁ P ₁	Parsley 1g/kg B.W.	Parsley 1g/kg B.W. + AFB ₁ 9mg kg ¹ B.W.
F ₂ P ₁	Parsley 1g/kg B.W.	Parsley 1g/kg B.W. + AFB ₁ 18mg kg ¹ B.W.
P ₂	Parsley 2g/kg B.W.	Parsley 2g/kg B.W.
F ₁ P ₂	Parsley 2g/kg B.W.	Parsley 2g/kg B.W. + AFB ₁ 9mg kg ¹ B.W.
F ₂ P ₂	Parsley 2g/kg B.W.	Parsley 2g/kg B.W. + AFB ₁ 18mg kg ¹ B.W.

Table 2: Survival rate (SR %) of aflatoxicated fish at different concentration after 96 hr of I.P. injection

AFB ₁ Concentration (mg kgG ¹)	No. of fish at different intervals h.									
	0	12	24	36	48	60	72	84	96	SR%
0	24	24	24	24	24	24	24	24	24	100
20	24	24	23	22	22	21	20	18	17	70.8
40	24	22	20	17	16	15	13	12	11	45.8
60	24	18	14	13	12	10	6	6	5	20.8
80	24	15	13	9	8	6	4	3	2	8.30
100	24	12	10	8	7	5	3	2	1	4.17

start of the experiment and one week later). AFB₁ and herbs extract were mixed together directly before administration.

At the end of the experimental period (2nd week), appropriate samples of skeletal muscles were cut off from three immediately killed fish of each group, put in Eppendorf tubes with saline solution (0.85% of NaCl and 70% ethanol alcohol) and kept in a deep freezer till the preparation and extraction of the protein and DNA to electrophoresis.

Protein Extraction and Electrophoresis: Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE) technique was used to compare sample protein patterns. Three muscle samples of each fish group were extracted. Samples of gel preparation, electrophoresis conditions, staining and destining gels were done according to Laemmli [33]. Hence, proteins extract samples were analyzed using 17-well gel (for all fish groups in three gels for analysis).

DNA Extraction and Purification: DNA was prepared from muscles tissue according to the method of Iwasa *et al.* [34]

Statistical Analysis of Protein Gels: All gels resulted from protein electrophoresis was analyzed using Total

Lab Ver. 2.01 software. SPSS software package (Ver.15) was used to infer similarities and genetic distances. Among groups similarity was calculated as described by Lynch [35]. The dendrogram was constructed according to Bardakci *et al.* [36].

RESULTS

The Lethal Concentration 50 (LC₅₀) of AFB₁ in *O. niloticus*: The LC₅₀ was calculated according to the relationship between the survival rate and different doses of AFB₁ at the sequent periods (0-96 hr). Table 2 showed SR% at 96 hr of the experiment. Fig. 1 showed that the LC₅₀ for I.P. injected aflatoxin in *O. niloticus* was 36 mg kg¹B.W.

Phenolic Compounds Identification in Rosemary and Parsley Extracts: According to the retention time (Table 3), rosemary extract presented a chemical profile composed of seven identified phenolic compounds including ferulic acid, syringic acid, cinnamic acid, protocotechuic acid, coumarin, caffeic acid and P-coumaric acid. The chromatogram also shows some other peaks, apart from the twelve standards studied (Fig. 2). The analysis of the typical HPLC chromatogram depicted that syringic acid, P-coumaric acid and ferulic acid are the major phenolic compounds. While the parsley showed

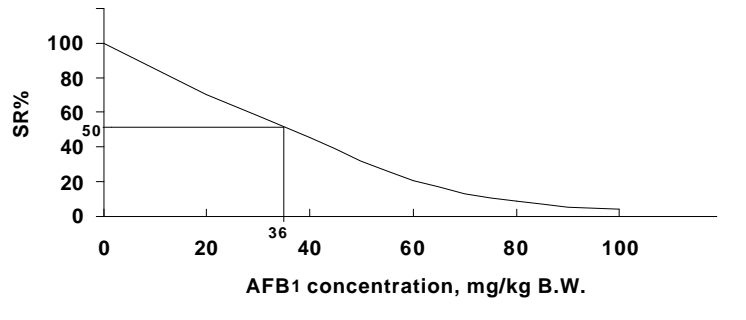


Fig. 1: The relationship between SR% and AFB1 concentrations after 96 h of I.P. injection of Nile tilapia fingerlings

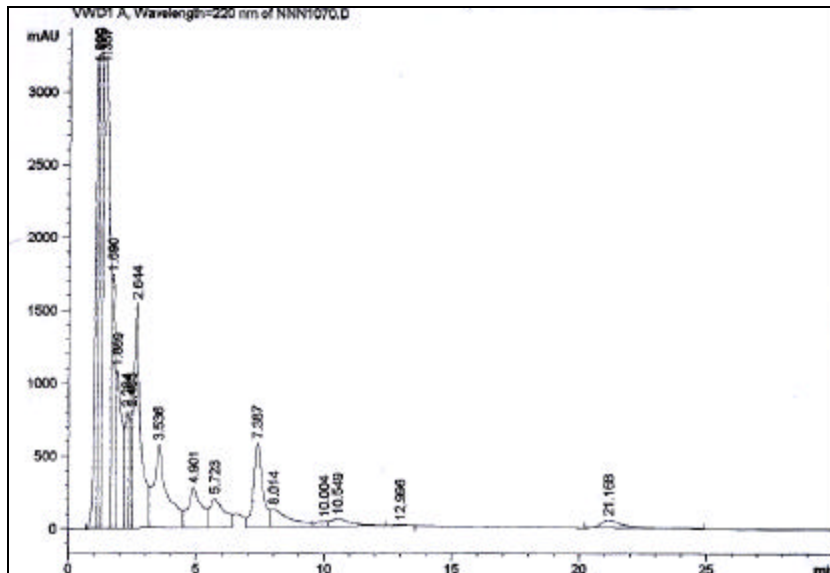


Fig. 2: Chromatogram of fractions of phenolic compounds of rosemary

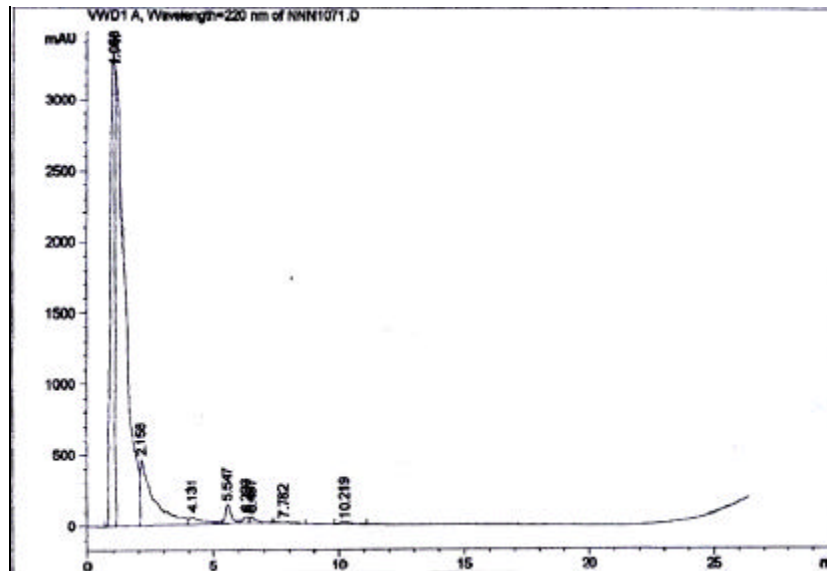


Fig. 3: Chromatogram of fractions of phenolic compounds of parsley

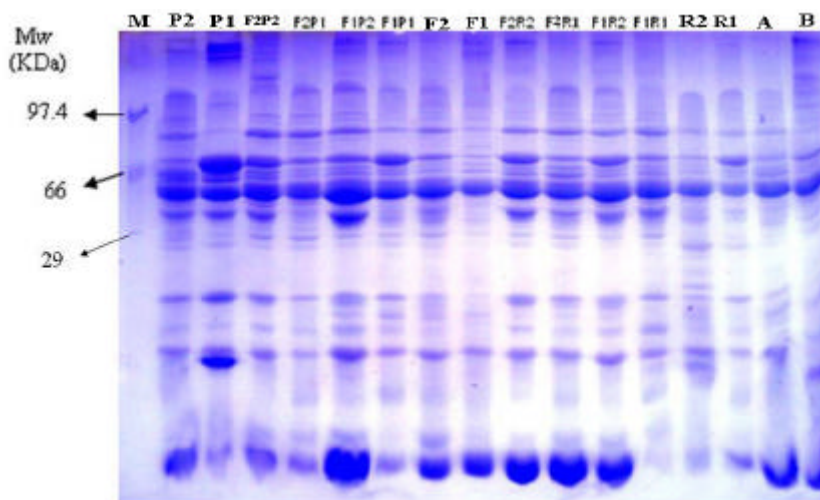


Fig. 4: Electrophoretic pattern of muscular protein of Nile tilapia fish tested

Table 3: Retention time of twelve standards phenolic compounds

Standards	R. time	Rosemary area (%)	Parsley area (%)
Ferulic acid	1.08	10.76	22.80
Syringic acid	1.40	21.72	54.93
Cinnamic acid	1.76	6.31	-
Protocatec	1.80	6.72	-
Vaniline	2.00	-	-
Coumarin	2.01	3.40	11.66
Caffic acid	2.40	2.33	-
P-coumaric acid	2.62	10.85	-
Rosocinol	3.39	-	-
Salicylic acid	3.92	-	1.65
Benzoic acid	4.29	-	-
Apigenin	4.46	-	-

Table 4: Descriptive analysis of different treatments of rosemary against Aflatoxin versus both solvent and control

Groups	No. of total bands	Means ± SE
A	64	0.52±0.29
B	57	0.47± 0.29
R ₁	57	0.47±0.29
R ₂	52	0.42±0.29
F ₁ R ₁	59	0.48± 0.29
F ₂ R ₁	77	0.63±0.28
F ₁ R ₂	78	0.64± 0.28
F ₂ R ₂	74	0.61±0.28
F ₁	64	0.52± 0.29
F ₂	69	0.57± 0.29

Where the mean equal No. of total bands of each group /121 (total number of bands of the three gels)

four compounds only including; ferulic acid, syringic acid, coumarin and salicylic acid where syringic acid and ferulic acid are the major compounds (Fig. 3).

Electrophoretic Patterns of Muscular Proteins: The total number of detected bands among all fish groups was 121 bands (per 3 gels), in the AFB₁-injected fish with or without rosemary or parsley at their different levels (Table 4 and 5, and Fig. 4). The bands number of the

Table 5: Descriptive analysis of different treatments of parsley against Aflatoxin versus both solvent and control

Groups	No. of total bands	Means ± SE
A	64	0.52±0.29
B	52	0.47±0.29
F ₁	64	0.52±0.29
F ₂	69	0.57±0.29
F ₁ P ₁	69	0.57± 0.29
F ₂ P ₁	73	0.60±0.28
F ₁ P ₂	65	0.53±0.29
F ₂ P ₂	62	0.51±0.29
P ₁	57	0.47± 0.29
P ₂	61	0.50±0.29

Where the mean equal No. of total bands of each group/121 (total number of bands of the three gels)

muscular proteins of fish groups ranged from 52 to 78 for rosemary treatments (Table 4). Yet, this range was from 57 to 73 for parsley treatments (Table 5). Fish injected with either rosemary (R₁ and R₂) or parsley (P₁ and P₂) alone showed a decrease in average of bands number of protein (57 and 52) and (57 and 61), respectively compared to the control fish group (64). The high level of AFB₁ (18 mg kg⁻¹) revealed an additive impact on muscular protein fractions and higher number of protein bands (69) than F₁ group (9 mg kg⁻¹). Considering the combination between AFB₁ and the two herbs extract at the different levels, it showed that fish groups F₂R₁, F₁R₂, F₂R₂ (Table 4) pronounced the highest number of protein bands (77, 78 and 74, respectively) This might be attributed to the effect of rosemary on enhancement or stimulation of new proteins induction to face the deleterious effects of AFB₁. On contrary, in the case of AFB₁ plus parsley (Table 5), the highest number of detected bands (73) was obtained in fish group F₂P₁, where the level 2% of parsley led to appearance of a new fraction.

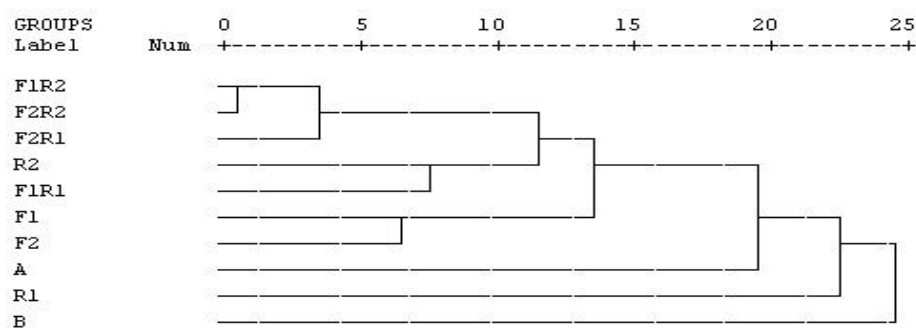


Fig. 5: Dendrogram of rescaled distance cluster combine presented as phylogenetic tree based on SDS-PAGE of the fish groups injected with AFB1 either with or without rosemary extract

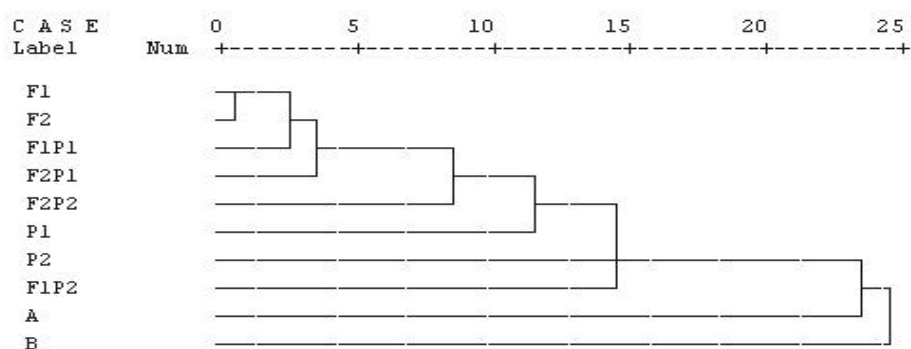


Fig. 6: Dendrogram of rescaled distance cluster combine presented as phylogenetic tree based on SDS-PAGE of the fish groups injected with AFB1 either with or without parsley extract

Figure 5 and 6 showed rescaled distance cluster combine represents samples grouping. The phylogenetic tree represented in Fig. 5 divided the fish samples into two main clusters and three outgroup samples (B, R₁ and A). The first main cluster grouped samples F₁R₂, F₂R₂, F₂R₁, R₂ and F₁R₁, respectively. The first cluster might be grouped to two subclusters (F₁R₂, F₂R₂ and F₂R₁) and (R₂ and F₁R₁).

The second cluster grouped samples F₁ and F₂ (fish injected with AFB₁ alone at the two levels 9 and 18 mg kg⁻¹B.W), respectively to each other. While fish group F₁R₁ was genetically the nearest to fish group (R₁) injected with rosemary alone at the low level (2 g/Kg B.W). The phylogenetic tree represented in Fig. 6 divided the fish samples into two main clusters and two outgroup samples (A and B). The first main cluster grouped samples (F₁, F₂, F₁P₁ and F₂P₁). The first cluster might be grouped to two subclusters included F₁ and F₂ and then the two levels of AFB₁ with the low level of parsley (F₁P₁ and F₂P₁), respectively the later subclusters. The second cluster grouped samples F₂P₂, P₁, P₂ and F₁P₂ to each other.

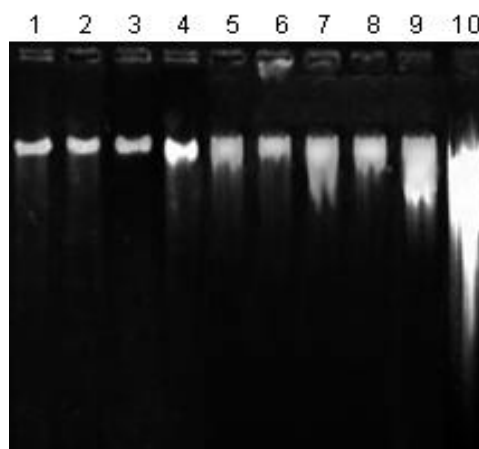


Fig. 7: The muscles DNA fragmentation on the 6th day after single i.p. administration of AFB₁ at the two levels 9 and 18 mg kg⁻¹B.W. with or without rosemary

DNA Damage: Figure 7 and 8 showed the agarose electrophoretic pattern of muscles DNA fragmentation on

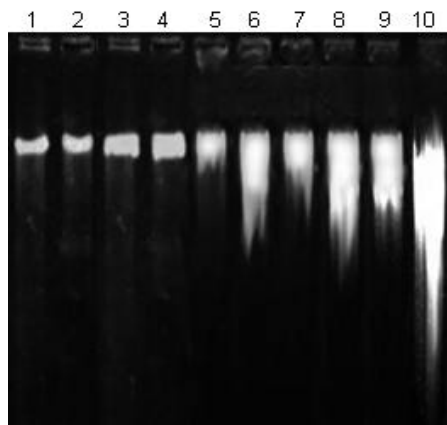


Fig. 8: The muscles DNA fragmentation on the 6th day after single i.p. administration of AFB₁ at the two levels 9 and 18 mg kg⁻¹B.W. with or without parsley

the 6th day after single intraperitoneal administration of aflatoxin B₁ (at the two levels 9 and 18 mg kg⁻¹B.W) with and without rosemary (Fig. 7) and parsley (Fig. 8) at the two levels of them (2 and 4 g/Kg B.W), all these groups were compared to the control group (A).

The solvent was tested also and it should be noted that DNA from the DMSO injected fish (group B) was intact and appeared similar to that of the control (compare lane 2 versus lane 1, Fig. 6 and 7). No change in DNA was observed in either rosemary or parsley injected fish groups at the two levels of them (lanes 3 and 4; Fig. 7 and 8, respectively). While DNA damage (DNA strand breaks) was more in group F₁ and became more serious with the increase of AFB₁ level, group F₂, (lanes 9 and 10; Fig. 7 and 8, respectively).

The DNA fragmentation was reduced in the case of fish injected with both of AFB₁ and rosemary in combination (Fig. 7) F₁R₁, F₁R₂, F₂R₁ and F₂R₂; lanes 5 to 8. An enhancement was occurred in this reduction by increasing the level of rosemary and reducing the level of AFB₁ (9 mg kg⁻¹B.W). On contrary, the DNA damage was less pronounced at the low level of parsley (2 g/Kg B.W) either with low or high level of AFB₁ (lanes 5 to 8 compared to lanes 9 and 10, Fig. 8).

DISCUSSION

Data in the present study showed that the LC₅₀ for I.P. injected aflatoxin in *O. niloticus* is 36 mg kg⁻¹B.W. and was calculated also in previous studies on *O. niloticus* [5, 37, 38]. Another aim of this study was to

evaluate the possible protective effects of rosemary and parsley extracts against the adverse effects of AFB₁, as monitored by an electrophoretic pattern of muscular protein and DNA damage in *O. niloticus*. The Phenolic compounds identification in rosemary and parsley extracts (Table 3) indicated that these polyphenolic compounds are rich in antioxidant activity they exhibit also a wide range of physiological properties, such as anti-allergic, anti-atherogenic, anti-inflammation, antimicrobial, antioxidant, anti-thrombotic, cardio-protective and vasodilator effects [39]. The action of herbs extract alone on muscular proteins of Nile tilapia fish was observed. They led to a decrease in average of bands number of protein compared to the control fish group, this means that synthesis of such peptides was affected by the treatments. These inhibitory effects of herbs extract may be attributed to the presence of various bioactive phenolic components (Table 3) that can act as chainbreaking antioxidants by scavenging chain-propagating reactive, endogenous free radical sources may damage protein around and inside cells [40]. While the effect of the high level of AFB₁ on protein bands revealed higher number of protein bands than the low level that may be attributed to the sensitivity of this fish group (F₂) to AFB₁, hence it produce a new protein fraction to resist the negative effect of AFB₁ on muscles protein. Thus, [23] found similar result where AFB₁-treated fish (0.15 µg/Kg B.W.) showed disappearance of some fractions of muscles protein appearing in the control, while in the case of high level of AFB₁ (1 µg/Kg), all bands pattern was recorded with high concentration of fractions. These findings mean that at such high level of AFB₁, fish became adapted to the stress since an adaptation mechanism may be evolved to preserve the number of protein fractions. Also, [9] reported a negative effect of AFB₁ on electrophoretic patterns of the muscular proteins. [41] reported that AFB₁-administrated fish showed significant increase in protein carbonylation, this increase might be ascribed to the fact that free radical which are generated by AFB₁ may oxidise some of the side chain amino acids yielding carbonyl derivatives. In addition to the basis of grouping or clustering method depends on the genetic assumption. The genetically closest samples fall into the same cluster or such group. The phylogenetic tree led to the conclusion that the muscles protein fractions showed severe fluctuation since the number of bands was similar in group F₁ to that of control group (64 bands in Table 4,5), although they fall into different cluster because they were genetically different.

The effect of AFB₁ on quantitative determination of nucleic acids, DNA and RNA in AFB₁-treated fish was reported by [4]. While [42] found that *Labeo rohita* fish injected with AFB₁ showed severe damage of their liver DNA. In the current study, the DNA damage was more in group F₁ and became more serious with the increase of AFB₁ level, group F₂. This DNA damage led to significant reduction of blood total protein and significant increase of mortality rate in AFB₁-injected fish groups compared to control group in the complementary study [43] of the present study where protein synthesis usually depends on DNA and RNA. Additionally this DNA fragmentation was reduced in the cases of fish injected AFB₁ with both of rosemary or parsley extract in combination. This consists with several other findings in the complementary study [43] Including results of mortality rate, some blood parameters, AFB₁ residues and histopathological studies of liver of *O. niloticus* showed that the low level of parsley (2 g/Kg B.W) either with low or high level of AFB₁ had better effect against AFB₁ more than its high level (4 g/Kg B.W). This effect of herbs extract on DNA damage of AFB₁-injected fish may be due to their inclusion of polyphenolic compounds which act as antioxidants. DNA adducts are formed by bulky genotoxins, such as AFB₁, where [44] reported that in the liver microsomes, AFB₁ is oxidised to its reactive epoxide forming exo AFB_{8, 9} epoxide. This subsequently links itself to DNA and exhibits the mutagenicity [45]. Aflatoxin B₁-DNA adduct destabilizes the N-glycosidic bond of nucleotide leading to depurination and DNA strand scission [46]. So reducing the bioavailability and preventing its adduct formation is considered to be the primary choice to combat AFB₁ toxicity. DNA adduct could be repaired primarily through a complicated system called excision repair [47]. So this system may be activated via antioxidants which catalize formation of polar, excitable conjugate between the epoxide intermediate of AFB₁ and glutathione leading to reduce AFB₁-DNA adduction [48]. Similarly, [42] reported that the phytochemicals present in *Amrita bindu*, salt spice herbal mixture, could mitigate the aflatoxin B₁-induced free radicals and confer protection to DNA and prevent its subsequent adduct formation, thereby playing a major role in negating the aflatoxin B₁-induced toxicity.

It could be concluded that, the lethal concentration 50 of aflatoxin B₁ in *O. niloticus* (22 g) is 36 mg kg⁻¹ B.W. Fish injected with both of 0.25 and 0.50 of LC₅₀ of AFB₁ showed negative effects on electrophoretic patterns of muscles protein and DNA damage. Both rosemary and parsley have a positive effect on overcoming the side effects of AFB₁.

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