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Aflatoxin B_1 Induced-Changes in Protein Electrophoretic Pattern and DNA in *Oreochromis niloticus* with Special Emphasis on the Protective Effect of Rosemary and Parsley Extracts

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Abstract: The adverse effects of two concentrations (0.25 and 0.50 of LC_{50}) of aflatoxin B_1 (AFB_1)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 AFB_1 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 F_1 and F_2 were injected with AFB_1 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 (R_1 and R_2) and (P_1 and P_2), respectively, or in combination with AFB_1 at their different levels, (F_1R_1, F_1R_2, F_2R_1 and F_2R_2) and (F_1P_1, F_1P_2, F_2P_1 and F_2P_2 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 LC_{50} was 36 mg AFB. Protein analysis showed remarkable variation in the number of bands and their genetic similarity in the phylogenetic tree. Rosemary in groups F_1R_2, F_2R_1 and F_2R_2 led to the pronounced highest numbers of protein bands (78, 77 and 74, respectively) among all fish groups injected with AFB_1. Although these groups fall into the same cluster, they were the farmost to control groups (A), genetically. Concerning parsley, F_2P_1 group showed increased number of detected bands (73) as compared to the other AFB_1 and parsley groups. DNA damage was clearly observed in F_2 then F_1 group. In conclusion,the damage due to AFB_1 was repaired or reduced by using rosemary and parsley, particularly with the lowest level of AFB_1. 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 B_1 · 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 producted 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 AFB_1 results in the formation of toxic metabolites, such as AFB_1-8, 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 AFB_1 could stimulate the release of free radicals resulting in chromosomal damages [18]. So, ROS may in part be responsible for the carcinogenic activity of AFB_1 [19].

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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 *Ocimus sanctum* provides protection against AFB_1 carcinogenesis by acting as an antioxidant [21]. Therefore, *Amrita bindu*, herbal food supplements, was evaluated by aflatoxic fish (*Labeo rabita*) and proved that it has a potential role in ameliorating the AFB_1-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 AFB_1. On the same trend, the effect of AFB_1 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 officlnalis* [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 AFB_1 as monitored by on electrophortic pattern of muscular protein and DNA damage in *O. niloticus*.

MATERIALS AND METHODS

Preparation of Aflatoxin B_1: AFB_1 was produced on liquid medium (potato dextrose) by *A. parasiticus* (NRRL. 2999) according to Ready *et al*. [30]. AFB_1 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. AFB_1 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 tow 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 (LC_{50}) of AFB_1 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 LC_{50} of AFB_1. 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 AFB_1 level. These groups were injected I.P with 0, 20, 40, 60, 80 and 100 mg AFB_1/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. AFB_1 was tested at three levels, being 0, 0.25 and 0.50 the LC_{50}, 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_1R_1	Rosemary 1g/kg B.W.	Rosemary 1g/kg B.W. + AFB_1 9mg kg^{-1}B.W.
F_2R_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_1R_2	Rosemary 2g/kg B.W.	Rosemary 2g/kg B.W. + AFB_1 9mg kg^{-1}B.W.
F_2R_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_1P_1	Parsley 1g/kg B.W.	Parsley 1g/kg B.W. + AFB_1 9mg kg^{-1}B.W.
F_2P_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_1P_2	Parsley 2g/kg B.W.	Parsley 2g/kg B.W. + AFB_1 9mg kg^{-1}B.W.
F_2P_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	No. of fish at different intervals h.									
(mg kg^{-1})	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). AFB_1 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 (LC_{50}) of AFB_1 in *O. niloticus*: The LC_{50} was calculated according to the relationship between the survival rate and different doses of AFB_1 at the sequent periods (0-96 hr). Table 2 showed SR% at 96 hr of the experiment. Fig. 1 showed that the LC_{50} for I.P. injected aflatoxin in *O. niloticus* was 36 mg kg^{-1}B.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, caffic 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

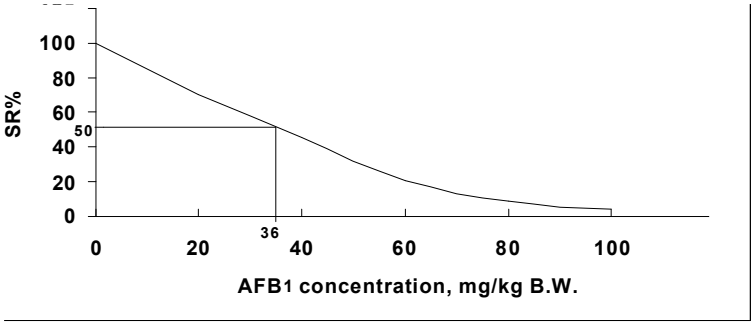

Fig. 1: The relationship between SR% and AFB1 concentrations after 96 h of I.P. injection of Nile tilapia fingerlings

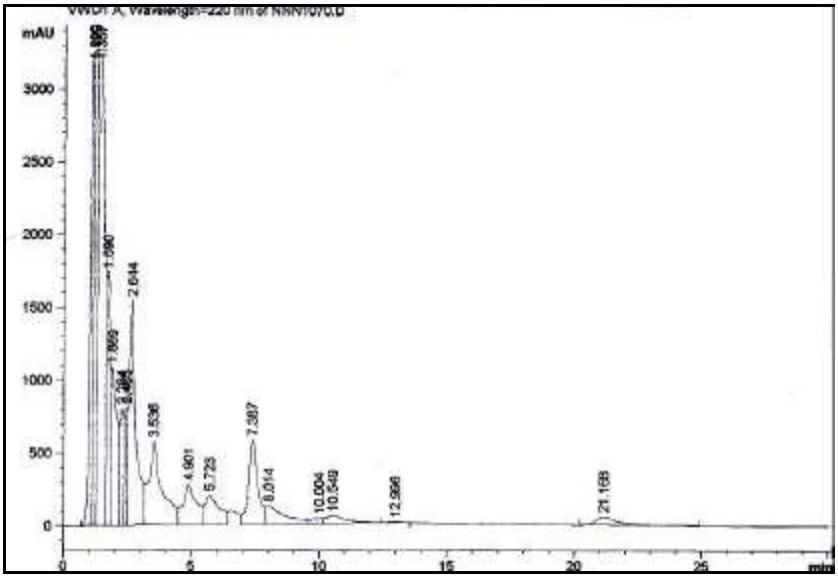

Fig. 2: Chromatogram of fractions of phenolic compounds of rosemary

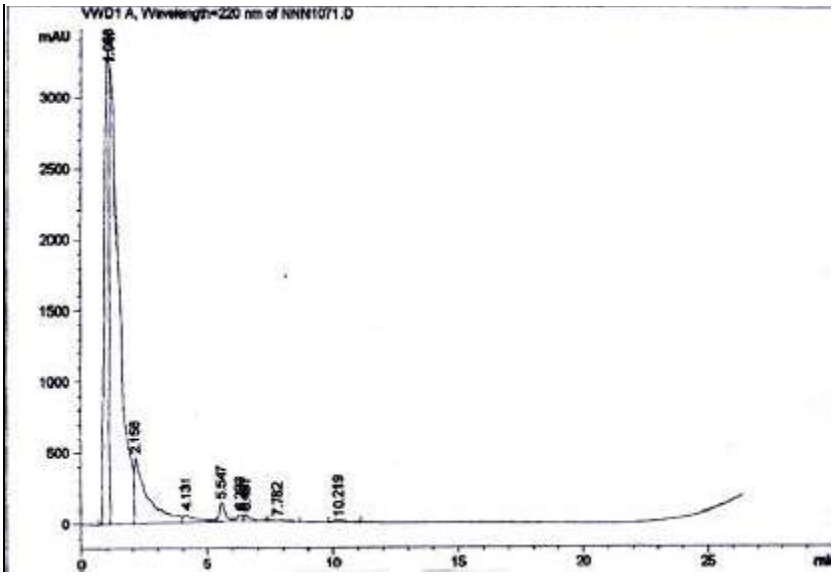

Fig. 3: Chromatogram of fractions of phenolic compounds of parsley

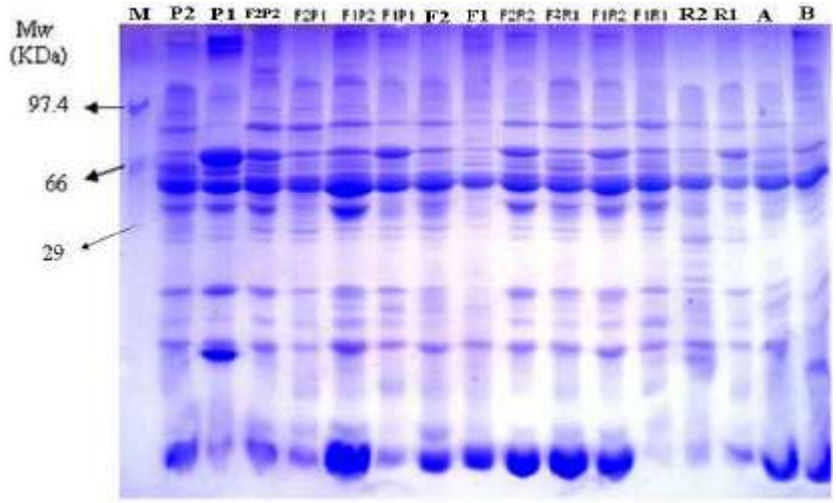

Fig. 4: Electrophoretic pattern of muscular protein of Nile tilapia fish tested

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
Cinnanic 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 AFB_1-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_1P_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 (R_1 and R_2) or parsley (P_1 and P_2) 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 F_1 group (9 mg kg^{-1}). Considering the combination between AFB_1 and the two herbs extract at the different levels, it showed that fish groups F_2R_1, F_1R_2, F_2R_2 (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 AFB_1.On contrary, in the case of AFB_1 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.

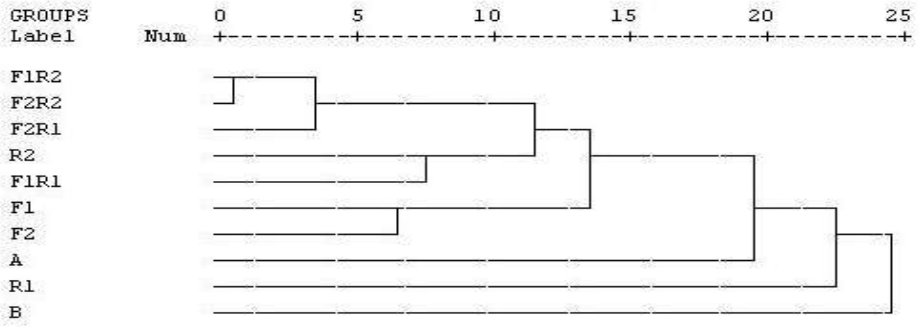

Fig. 5: Dendrogram of rescaled distance cluster combine presented as phylogenetic tree based on SDS-PAGE of the fish groups injected with AFB1 either with or without rosemary extract

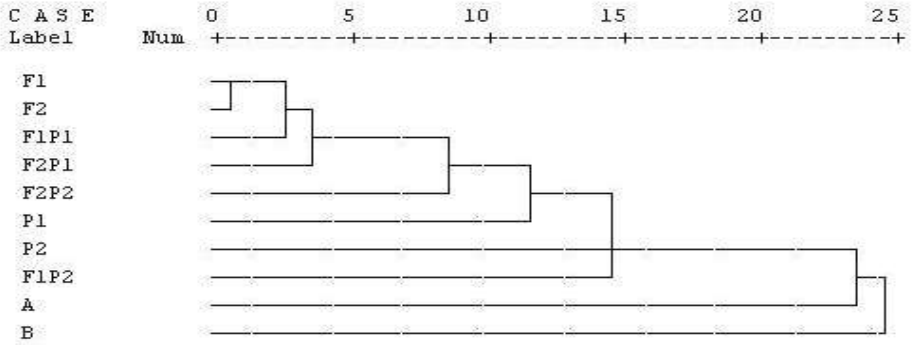

Fig. 6: Dendrogram of rescaled distance cluster combine presented as phylogenetic tree based on SDS-PAGE of the fish groups injected with AFB1 either with or without parsley extract

Figure 5 and 6 showed resealed 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, R_1 and A). The first main cluster grouped samples F_1R_2, F_2R_2, F_2R_1, R_2 and F_1R_1, respectively. The first cluster might be grouped to two subclusters (F_1R_2, F_2R_2 and F_2R_1) and (R_2 and F_1R_1).

The second cluster grouped samples F_1 and F_2 (fish injected with AFB_1 alone at the two levels 9 and 18 mg kg^{-1}B.W), respectively to each other. While fish group F_1R_1 was genetically the nearest to fish group (R_1) 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 (F_1, F_2, F_1P_1 and F_2P_1). The first cluster might be grouped to two subclusters included F_1 and F_2 and then the two levels of AFB_1 with the low level of parsley (F_1P_1 and F_2P_1), respectively the later subclusters. The second cluster grouped samples F_2P_2, P_1, P_2 and F_1P_2 to each other.

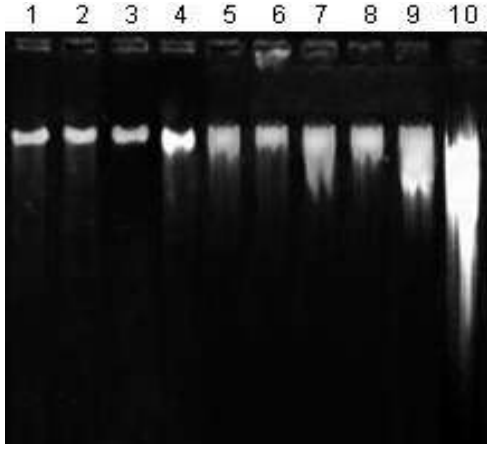

Fig. 7: The muscles DNA fragmentation on the 6th day after single i.p. administration of AFB_1 at the tow levels 9 and 18 mg kg^{-1}B.W. with or without rosemary

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

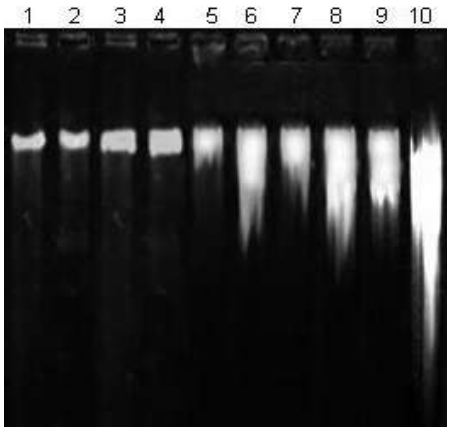

Fig. 8: The muscles DNA fragmentation on the 6th day after single i.p. administration of AFB_1 at the tow levels 9 and 18 mg kg^{-1}B.W. with or without parsley

the 6th day after single intraperitoneal administration of aflatoxin B_1 (at the two levels 9 and 18 mg kg^{-1}B.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 intacted 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 F_1 and became more serious with the increase of AFB_1 level, group F_2, (lanes 9 and 10; Fig. 7 and 8, respectively).

The DNA fragmentation was reduced in the case of fish injected with both of AFB_1 and rosemary in combination (Fig. 7) F_1R_1, F_1R_2, F_2R_1 and F_2R_2; lanes 5 to 8. An enhancement was occurred in this reduction by increasing the level of rosemary and reducing the level of AFB_1 (9 mg kg^{-1}B.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 AFB_1 (lanes 5 to 8 compared to lanes 9 and 10, Fig. 8).

DISCUSSION

Data in the present study showed that the LC_{50} for I.P. injected aflatoxin in *O. niloticus* is 36 mg kg^{-1}B.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 AFB_1 as monitored by on electrophortic 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-atheragenic, 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 AFB_1on protein bands revealed higher number of protein bands than the low level that may be attributed to the sensitivity of this fish group (F_2) to AFB_1, hence it produce a new protein fraction to resist the negative effect of AFB_1 on muscles protein. Thus, [23] found similar result where AFB_1-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 AFB_1 (1 µg/Kg), all bands pattern was recorded with high concentration of fractions. These findings mean that at such high level of AFB_1, 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 AFB_1 on electrophoretic patterns of the muscular proteins. [41] reported that AFB_1-administrated fish showed significant increase in protein carbonylation, this increase might be ascribed to the fact that free radical which are generated by AFB_1 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 F_1 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 AFB_1 on quantitative determination of nucleic acids, DNA and RNA in AFB_1-treated fish was reported by [4].While [42] found that *Labeo rohita* fish injected with AFB_1 showed severe damage of their liver DNA. In the current study, the DNA damage was more in group F_1 and became more serious with the increase of AFB_1 level, group F_2. This DNA damage led to significant reduction of blood total protein and significant increase of mortality rate in AFB_1-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 AFB_1 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, AFB_1 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 AFB_1 had better effect against AFB_1 more than its high level (4 g/Kg B.W). This effect of herbs extract on DNA damage of AFB_1-injected fish may be due to their inclusion of polyphenolic compounds which act as antioxidants. DNA adducts are formed by bulky genotoxins, such as AFB_1, where [44] reported that in the liver microsomes, AFB_1 is oxidised to its reactive epoxide forming exo AFB-8, 9 epoxide. This subsequently links itself to DNA and exhibits the mutagenicity [45]. Aflatoxin B_1-DNA adduct destabilities 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 AFB_1 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 AFB_1 and glutathione leading to reduce AFB -DNA adduction [48]. Similarly, [42] reported that the phytochemicals present in *Amrita bindu*, salt spice herbal mixture, could mitigate the aflatoxin B_1-induced free radicals and confer protection to DNA and prevent its subsequent adduct formation, thereby playing a major role in negating the aflatoxin B_1-induced toxicity.

It could be concluded that, the lethal concentration 50 of aflatoxin B_1 in *O. niloticus* (22 g) is 36 mg kg^{-1} B.W. Fish injected with both of 0.25 and 0.50 of LC_{50} of AFB_1 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 AFB_1.

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