Influence of Rosemary Extract on Immune Responses and Oxidative Stress in Mice Intoxicated by Aflatoxins

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Abstract: A research study was conducted to investigate the impact of rosemary extract (RM) as a natural antioxidant on immune responses and oxidative stress in mice challenged with aflatoxins (AFB1). Seventy five samples of feeds included poultry ration, processed animal meal and bone and blood meal (25 of each), were subjected for mycological and aflatoxin detection. The results revealed that 8 genera of fungi and one genus of yeast were recovered. *Aspergillus flavus (A. flavus)* was recovered from all tested feed samples of poultry ration, processed animal meal and bone and blood meal in a significant frequency (72%, 64% and 60%) respectively. Significant levels of aflatoxins were produced by *A.flavus* that recovered from the feed samples included in this work , where the maximum toxin level was obtained from *A. flavus* (86.6%)that isolated from bone and blood meal which produced the mean level of 70 ± 0.2ppb aflatoxins, followed by those isolated from poultry ration (66.6‰ with mean level of 33.5±0.61ppb). Whereas, *A. flavus* that recoverd from processed animal meal produced the lower level of aflatoxins with a relatively lower incidence (56.2‰ with mean level of 7.6±0.1ppb). A total of 60 BALB/c mice were randomly assigned into three equal groups, 1st gp as control, 2nd gp orally treated with AFB1 (50 ug/0.1ml/mice/day) and 3rd gp orally treated with AFB1 (50 ug/0.1ml/mice/day) plus RM extract (1000 mg/ kg b.wt./day) for 3 weeks. The peritoneal macrophages were isolated from these animals and mononuclear cells activation was determined. Immunological parameters indicated that proliferation of peritoneal macrophages was higher in the 3rd gp than 2nd gp. It is interesting to report that the releases of nitric oxide (NO) and tumor necrosis factor-α (TNF-α) in serum have been regulated against AFB1 toxicity. MAD and GSH levels and catalase activity in hemolysate have provoked a high correlation between RM extract intake and the improved functions of total antioxidant enzymes under oxidative stress induced by the aflatoxin challenge. From the foregoing results it was concluded that antioxidant components of RM extract were able to improve the impaired immune responses and oxidative disorders from oxidizing agents which produced during experimental aflatoxigenesis. Therefore, the RM extract could be used as an alternative compound to antioxidants which have dangerous side effects to human and animal health. The significance of our results was fully discussed.

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1. Introduction

Up to date the fungal and mycotoxins contamination of human food and animal feeds gained a wide field of research due to their dangerous effects on vital internal organs of consumers (Benkerronum and Tantawi, 2001; Hassan *et al.,* 2004; 2007; 2008; 2009; 2010 a, b; 2011 a, b) who recovered different genera of fungi and mycotoxins from foods and feeds in association to liver and kidney cirrhosis. They added that the mould contamination, particularly with mycotoxin producing fungi causes significant economic losses due to the dangerous effect on the animal production included reproductive disorders , diminution in production of milk, meat and leathers (Hassan *et al.,* 2010 a,b ; Awaad *et al.,* 2011 Hassan *et al.* 2011 a, b,). The effects of mycotoxins Particularly aflatoxins on animals include hepatotoxicity, nephrotoxicity, immunotoxicity, oncogenesis and genotoxicity (Bullerman,1986;Dierhiemer,1998; Beutner *et al.,* 2001; Sudakin,2003; Bouket and Oswald,2005; Awaad *et al.,* 2011; Hassan 2011 a, b).However, the majority of the toxic species belongs to the genera Aspergillus, Penicillium and Fusarium were recovered previously by many authors (Kuasal and Sinha 1993 ; Hassan *et al.,* 2003b ; Hassan *et al.,* 2007 ; 2009 ; 2010 a, b). These fungi when contaminated food and feed and the adverse conditions of storage including high temperatures and humidity could be enhanced the mycotoxins production by these mould causing many mycotic diseases and mycotoxicosis in animals and human consumed these food (Awaad *et al.,* 2011; Hassan *et al.,* 2011 a, b).The most dangerous types of all mycotoxins were aflatoxins B1,B2,G1 and G2. Aflatoxin B1 was responsible for aflatoxicosis in different types of animals. This toxin was a toxic metabolite produced *by Aspergillus flavus and Aspergillus parasiticus* which enhanced a carcinogenic effects on the liver and kidney

(Willkinson et al., 2003; Verma 2004; Hassan et al., 2004; 2010 a, 2011 a ,b). The most significant effects of AFB1 was its diminutions the acquired immunity resulted from animal vaccination against infectious viral and bacterial diseases, complement activity, decreasing interferon production and bactericidal activity. These effects could be attributed to the depression of antibody formation by immune systems of animals due to chronic consumption of toxicated feeds ((Ritchard *et al.,* 1975; Giambrone *et al.,* 1978; Pondy and Pestka, 2000 ; Hassan *et al.,* 2003 a , b and 2009).

On the other hand, aflatoxins-induced immunosuppression may manifest as depressed phagocytic activity, intracellular killing and spontaneous superoxide production of macrophages (Cusumano *et al.,* 1990; Jakab *et al.,* 1994; Hinton *et al.,* 2003). Other study reported that AFB1 inhibited the production of tumor necrosis factor, interleukin-1 and interleukin-6 (Moon and Pyo, 2000). Therefore, the recent studies has been directed considerably in naturally occurring antioxidants to use in food or medicinal materials to replace synthetic ones which are being restricted due to their cumulative carcinogenic effects on internal vital organs of the body (Gűlcin *et al.,* 2004; Hassan *et al.,* 2010 a, b, c ; Hala, 2011) . Rosemary (*Rosmarinus officinalis*) is of medicinal plants which known by its powerful antioxidant activity, antibacterial activity, anti-mutagenic properties and as a chemo-preventive agent (Oluwatuya *et al.,* 2004 ; Hala, 2011).

The main compounds responsible for rosemary's antioxidant properties have been identified as phenolic diterpens such as carnosic acid, carnosol, rosemanol, epi and isorosmanol , rosmadial and methyl carnosate (Del Baño *et al.,* 2003; Ibańez *et al.,* 2003; Suhaj ,2006).The antioxidant activity of phenolic compounds is mainly due to their redox properties which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen ,or decomposing peroxides (Beutner *et al.,* 2001; Luiz *et al.,* 2002). Phenolic compounds also appear to have a protective effect on immune function, for instance leukocytes functions were improved in prematurely age mice after five weeks of diet supplemented with polyphenol-rich cereals. Moreover, they could increase macrophage chemotaxsis, phagocytosis, microbicidal activity,natural killer functions and increase lymphoproliferation and IL-2 release in response to conacavalin A and lipopolysaccharide(Alvarez *et al.,* 2006).

Aflatoxins are unique in being resistant to degradation under normal food processing conditions (Ciegler and Vesonder, 1983 ; Hassan *et al.,* 2010 a, b and c), this makes the selection of proper degradation methods that will effectively decompose aflatoxins, while retaining the nutritive quality and palatability of the treated food, a continuous challenge. Therefore, the experiment described in this paper was carried out to evaluate the immunoprotective effects of rosemary extract and its efficacy in alleviating the AFB1-induced oxidative stress against the toxigenicity of Aspergillus *flavus* and *Aspergillus parasiticus.*

2 .Materials and Methods

Feed samples.

A total of 75 samples of different feedstuffs were received by the animal Research Institute/ Mycology Department to investigate aflatoxin contamination. These samples included Poultry ration, processed animal meal and bone and blood meal (25 of each).

Mycotoxins standard solution for TLC and fluorometric analysis

Aflatoxins standard B1, B_2, G_1, G_2 and their immune-affinity column were purchased from Sigma Chemical Company, St. Louis U.S.A.

Production and estimation of aflatoxins (Gabal *et al.,* 1994)

Cultivation and extraction of aflatoxins

The isolated strains of *Aspergillus flavus* were inoculated into flasks Containing 50 ml of sterile yeast extract solution (2%): 20% sucrose (YES). Inoculated flasks were incubated at 25ºC for 10-15 days. At the end of the incubation period, 50 ml chloroform were added and the mixture was thoroughly mixed for one minute in electric shaker apparatus, then centrifuged (3000 r.p.m.) for 10 minutes after which the chloroform layer decanted. The Chloroform extraction was repeated for more time. One ml ethanol, 3 gm copper – (III)-hydroxide carbonate and 5 gm anhydrous sodium sulphate were added to the chloroform extract, mixed well and filtered. The filtrate was then evaporated in a rotatory vacuum evaporator, the residue cooled and re-suspended in 5 ml of chloroform.

Thin layer Chromatographic analysis of chloroform extract (Scott, 1990)

The concentrated extract was spotted onto activated thin layer chromatography plates coated with silica gel of 0.25 mm thickness. Standard solutions of aflatoxins B1, B2, G1and G2 were spotted on the plate using 10-20 µl capacity pipettes. The spots were air dried and the TLC plates out in the developing tank containing the developing solvent system (5 Toluene: 4 ethyl acetate: 1 of 90% formic acid (V/V/V) .When the solvent travels about

12 cm front, the plates were removed from the tank, air dried and inspected under the ultraviolet light lamp for examining the tested and standard spots and determining the rate of flow (Rf of the toxin) then the results recorded.

Quantitative estimation of aflatoxins by a fluorometeric method according to (Hansen, 1993)

After calibration of the fluorometer by using specific FGis Afla test standards. At the end of the inoculation period, 25 ml of fungal culture filtrate were extracted three times with 50 ml chloroform. The chloroform extract was collected and evaporated in a rotatory flash evaporator. The residue of chloroform extract was dissolved in 100 ml methanol: water (80:20 V/V) and filter through fluted filter paper. Then, 10 ml of filtrate were diluted with 40 ml distilled water. The diluted extract was filtered through microfiber filter paper. 4 ml of filtered diluted extract were completely passed through Afla-T-affinity column at a rate of 2 drops/second until air come through column. The column was washed twice with 10 ml distilled water at a rate of 2 drops/second. The affinity column was eluted by passing 1 ml HPLC grade methanol through column at a rate of 1-2 drops/second and the sample elute was collected in a glass cuvette. One ml of afla test developer was added to elute in the cuvette and mixed will. The cuvette was placed in a calibrated fluorometer and aflatoxin B1 concentration was read after 60 seconds.

Preparation of rosemary plant extract

The extract of rosemary leaf was obtained according to *Garima and Goyal (2007).*The leaves of the plant were carefully cleaned, shade dried and powdered in a grinder. The plant material was prepared by extracting 200 gm of leaf powder with double distilled water by refluxing for 36 hrs (12 hrs. x 3) at 55 ± 5°C. Pellets of the extract were obtained by evaporation of its liquid contents in the incubator. An approximate yield of 22 % extract (w/w) was obtained. The required dose for treatment was prepared by dissolving the pellets in double distilled water and administered by oral gavage with a micropipette (100 µl/ animal) at a dose of 1000 mg/ kg body wt./animal (1000 mg of 22% of original plant weight).

Determination of total phenolic compounds

The amount of total phenolics in the rosemary plant extract was determined with the folin-Ciocalteu reagent according to the method by Slinkard and Singleton (1977).

Animals

Apparently healthy 60 BALB/c mice weighted (30-50 g) were housed under hygienic conventional conditions in suspended stainless steel cages. Prior to experiment, mice were fed on healthy basal diet (free from any cause of disease). Drinking water was supplied in glass bottles, cleaned three times a week.

Experimental design

The mice were divided into three groups with 20 mic per group. 1st gp as control, 2nd gp orally treated with AFB1 (50 ug/0.1ml/mice/day) for 3 weeks and 3rd gp orally treated with AFB1 (50 ug/0.1ml/mice/day) plus RM extract (1000 mg/ kg b.wt./day) for 3 weeks.

Estimation of phagocytic activity

Peritoneal macrophages were collected from the experimental groups at first and third week post treatments to evaluate phagocytic activity as described by Victor *et al., (2003).* Briefly, five ml of hank's balanced Salt solution pH 7.4 was injected intraperitoneally /mice then peritoneal cells were collected, washed and resuspended in RPM1medium with 10% fetal calf serum. The peritoneal macrophages were incubated in cell culture staining chambers (ccsc) with cover slip for 2 hrs at 37°c in 5% CO_2 and 90% humidity, the non-adherent cells were removed, and the ccsc were reincubated overnight at the same conditions. Phagocytic activity of peritoneal macrophages was estimated using *Cadida albicans* according to De-La fuent *et al.,(2000).* Finally, cover slips were stained with Giemsa stain and 100 peritoneal cells were counted under oil immersion to determine phagocytic percentage and phagocytic index.

Serum samples

Serum samples were collected from all groups, stored at –20°C until further use for evaluation of nitric oxide activity at first and third week post treatment and for determination of tumor necrosis factor alpha at the end of the experiment.

Measurement of nitrite concentration

The nitrite accumulated in serum samples of all groups was measured as an indicator of nitric oxide production according to Rajaraman *et al.*, (1998). Briefly, 100ul of serum samples was incubated with an equal volume of Griess reagent into flat buttom 96 well Elisa plate at 25ºC for 10 min. The absorbance was measured at 550nm using Elisa reader and the concentration of nitrite was calculated from an Na No_2 standard curve.

Determination of Tumor necrosis factor –α

The TNF-α (mouse) EIA kit was used for the quantitative determintation of mouse TNF-α in serum samples of the three experimental groups. The kit was purchased from Ray Biotech, Inc.

Blood samples

At the end of the experiment,blood samples were collected from each gp into small labeled dry and clean vials with anticoagulant for lysated Red blood cells (RBCs).

Lysate preparation and assays of antioxidant parameters

RBCs were separated from plasma by centrifugation, washed three times with saline and lysed (Tietz, 1996) .For determination of antioxidant parameters, The lysate was mixed with an equal volume of Drabkin's reagent to haemoglobin determination levels by Van kampen and Zijlstra (1965).

Catalase activity; lipid peroxidation as malonaldehyde (MDA) and reduced glutathione (GSH) in lysated rbcs were determined according to Aebi (1974); Ohkawa *et al.*, (1979) and Ellman (1959), respectively.

Statistical analysis

Data were presented as means ± SE for the indicated number of independently performed experiments. Stastical significance (≤ o.o5) was assessed by student t-test.

3. Results and Discussion

Fungi were reported as potential pathogens and caused different disease conditions in human and animals, particularly after prolonged exposure to adverse environmental condition. The most pathogenic fungi were belonged to the mycotoxin producers particularly the aflatoxigenic mould (Hassan *et al.,* 2003 a and b; 2009; Awaad *et al.,* 2011). In the present work, the current data in table (1) showed that 8 genera of fungi and one genus of yeast were recovered. The different members of *Aspergillus* were the most prevalence fungi isolated from different feed samples, while other genera of mould were recovered in relatively lower frequency. On the other hand, the yeasts were isolated in higher frequency (20% in poultry ration, 8‰ in processed animal meal and bone and blood meal). Kuasal and Sinha, 1993; Hassan, 1998 , 2003; Hassan *et al.,* 2007; 2009; 2010 a, b; 2011 a , b) reported that the majority of the toxic species belongs to the genera *Aspergillus, Penicillium* and *Fusarium* were recovered from different feed, air, water and worker hands, Whereas, Hala (2011), recovered several species of yeasts from clinical animal discharges in association with animal diseases. *Aspergillus flavus* was recovered from all tested feeds samples of poultry ration, processed animal meal and bone and blood meal in a significant frequency(72%, 64% , 60%) respectively. Similar results were obtained by (Hassan, 1998 and 2003 and Hassan *et al.,* 2007; 2009; 2010 a, b; 2011 a, b) who isolated the aflatoxigenic *A.flavus* from feed, food, meat and fish samples from animal cases suffered from signs of intoxication.However, significant levels of aflatoxins were produced by *A. flavus* that recovered from the feed samples included in this work (table, 2) where, the maximum toxin level was obtained from 86.6% of *A. flavus* that isolated from which produced the mean level of 70 ± 0.2ppb aflatoxins, followed by those isolated from poultry ration (66.6‰ with mean level of 33.5±0.61ppb). Whereas, *A. flavus* that recoverd from processed animal meal produced the lower level of aflatoxins with a relatively lower incidence (56.2‰ with mean level of 7.6±0.1ppb). Several authors were reported the aflatoxigenicity of isolated fungi from feeds in several farm suffered from problems of animal diseases and toxicities (Hassan, 1998; 2003; Hassan *et al.,* 2007; 2009; 2010 a, b; 2011 a, b).

Aflatoxin B1 is among the most common mycotoxins that are extremely toxic to animals and humans because of their greatest effect on immunity probably is related to cell-mediated processes; impaired phagocytosis, suppression of lymphoblastogenesis and suppression of delayed type hypersensitivity (Cusumano *et al.,* 1990; Vesonder etal., *et al.,*1991 ; Hussein and Brasel,2001). Macrophages play a crucial role in both nonspecific and specific immune responses, they exhibit scavenger functions leading to direct destruction of microbes and tumor cell targets (Qureshi *et al.,* 1986, 1989; Qureshi and Miller, 1991).In the present work, the 2nd gp showed suppression of phagocytic activity (table,4) at 1st and 3rd week post treatment with AFB1 which might be caused by three possible assumptions: (i) AFB1could directly decrease macrophage functions whereas macrophages may accumulate AFB1 either alone due to its hydrophobic characteristics or after being metabolized in a sensitive immune system into its active form by microsomal mixed functional oxidase , such active metabolite proved to be more toxic to macrophage functional potentials in response to AFB1 toxicity. This comes in agreement with Moon *et al.,* 1999 who reported that since macrophages would be expected to encounter AFB1 metabolites during aflatoxicosis, a significant functional reduction would be evident. (ii) Indirect effect on macrophage general cellular response to this

mycotoxin may reflect suppression of proliferation of granulocyte macrophage (GM) progenitor cells to granulocyte, macrophage ,and GM colony (Dugyla *et al.,* 1994).(iii) Pretreatment with AFB1 may interfere or suppress signal transcription and translation of macrophage functions (Moon *et al.,* 1999). On the contrary, the significant improvement of phagocytic activity achieved in gp (3) at 1st and 3rd wk post treatment with AFB1 and RM extract confirmed the potent antioxidant capabilities and properties of rosemary extract by which cell functions are regulated against AFB1 induced toxicity (Han *et al.,* 2007).Similar results were mentioned by Collett *et al.,* 2004, Kunnumakkara *et al.,* 2007 who stated that polyphenolic contents of RM extract are able to increase macrophage chemotaxis, phagocytosis, microbicidal activity ,natural killer function and increase lymphoproliferation and interleukin-2 release.

Table (1): Mycoflora of poultry ration , processed animal feed and bone and blood meal

Fungal genera	Prevalence of fungal genera								
	Poultry ration (25)			Processed animal meal (25)			Bone and blood meal (25)		
	No. of +ve	%	Mean of count ± SE	No. of +ve	%	Mean of count ± SE	No. of +ve	%	Mean of count ± SE
Aspergillus sp.	25	100	2.1 x 10^2 ± 0.3 x 10	22	88	4.0 x 10 ± 0.76	21	84	6.0x 10^2± 1.0
Penicillium sp.	18	72	1.1 x 10 ± 0.3 x 10	19	76	1.5 x 10± 0.1x10	15	60	1.1x 10± 0.2
Fusarium sp.	9	36	1.5 x 10 ± 0.2 x 10	2	8	1.1 x 10± 0.3	3	12	2.0x 10± 0.1
Cladosporium sp.	2	8	2.1 x 10 ± 0.1 x 10	1	4	0.5x 10 ± 0.1	1	4	1.0x 10± 0.1 x10
Mucor sp.	20	80	2.0 x 10 ± 0.1	14	56	1.8 x 10± 0.25	13	52	1.6x 10 ± 0.3 x10
Rhizopus sp.	3	12	0.5x 10 ± 0.2	5	20	1x 10 ± 0.3	1	4	1x10± 0.03 x10
Scopulariopsissp.	1	4	1x10 ± 0.12	-	-	-	-	-	-
Curvularia sp.	1	4	1x10 ± 0.3	-	-	-	-	-	-
Yeast sp.	5	20	1x 10^2 ± 0.03x10	2	8	2.1 x 10± 0.05x10	2	8	1.3 x10 ± 0.7x10

Table (2): Prevalence of *Aspergillus sp* in poultry ration, processed animal feed and bone and blood meal

Fungal genera	Prevalence of fungi								
	Poultry ration			Processed animal meal			Bone and blood meal		
	+ve	%	Colony count ± SE	+ve	%	Colony count ± SE	+ve	%	Colony count ± SE
A. flavus	18	72	2.5x 10± 0.4 x10	16	64	2.8x 10 ± 0.3	15	60	3x 10± 0.2x10
A. niger	10	40	2.8 x 10± 0.1 x10	9	36	0.5x 10^2 ± 0.03	10	40	0.7x 10± 0.3x10
A. candidus	8	32	1.0x10± 0.1	3	12	3x10 ± 0.1 x10	4	16	2.6x10± 0.7x10
A. fumigatus	8	32	1.6x 10± 0.1x10	7	28	0.5x10 ± 0.0	7	28	1.3x10± 0.5 x10
A.ochraceus	13	52	1.3x 10± 0.1x10	4	16	1x10^2± 0.3 x10	5	20	2.3x10± 0.2x10
A. terrus	6	24	1.0 x 10± 0.0	-	-	-	2	8	1.2x10± 0.03

Table(3): Rates of aflatoxins production by *A.flavus* isolated from examined samples

Source of isolates	No. of isolates	+ ve samples		Mean of count	Levels of AF ppb		
		No.	%		Max	Min	Mean ± SE
Poultry ration	18	12	66.6	10 ± 2.0	97.6	11.8	33.5 ± 0.61
Processed animal meal	16	9	56.2	10 ± 0.1	13.7	0.5	7.6 ± 0.1
Bone and blood meal	15	13	86.6	14 ± 0.03	650	109.3	70 ± 0.2

NO is an important physiological messenger and effectors molecule that can be synthesized from L-arginine by a family of enzymes called nitric oxide synthases (NOS) in a biological system whereas inducible nitric oxide synthase (iNOS) is induced quantitatively by macrophages and participating in the host immune defence system directed against pathogens (Bredt and Snyder, 1994; Dawson and Dawson,1995; Mayer and Hemens, 1997). Our experiment revealed that AFB1 was able to suppress NO concentration in serum of the 2nd gp when compared to its corresponded controls. However, less inhibitory response was recorded in association with RM extract treatment (table 4). This result can provide a reliable insight into impaired signal transduction generated by macrophages for transcription and translation of iNOS upon AFB1 exposure that clearly demonstrated by Moon *et al.,*

1999. Our model RM extract exhibited a relieve effect against NO down-regulation due to modulation of iNOS expression By carnosol which is also identified by Hsiang *et al.,* (2002), accordingly modulation of iNOS by the antioxidant effects of dietry polphenols seemed to be a useful strategy for treatment of NO related disorder like the undesired oxidative stressors as it possess beneficial roles in much of the microbicidal activity of macrophages against pathogens. (Smith and Moss, 1985 ; Hibbs *et al.,* 1987; Qureshi *et al.,* 1989 , Cook and Cattel, 1996; Kim *et al.,* 2003 ;Camacho-Barquero *et al.,* 2007; Nonn *et al.,* 2007 and Shakibaei *et al.,* 2007).

Table (4): Effects of rosemary extract on phagocytic activity and nitric oxide production in mice intoxicated with AFB1 (means±SE)

Immune parametrs	Cont/1st wk	AFb1/1st wk	Afb1+RM/1st wk	Cont/3rd wk	AFb1/3rdwk	Afb+RM/3rd wk
Phagocytic percent	85.3±1.76	58± 3.1	81.3± 0.8*	85.3±1.76	46.6 ± 3.1	87.3 ± 0.8**
Phagocytic index	0.53± 0.1	0.12±0.02	0.54± 0.05*	0.53± 0.1	o.12± 0.01	0.54± 0.005**
NO level (µM)	0.823±0.01	0.57± 0.01	0.648±0.05*	0.834±0.01	0.439±0.003	0.681±0.01*

Significant at p≥ o.o5*

Tumor necrosis factor-α is a potent immunoregulatory cytokine produced by several types of cells, especially macrophages which augments the production of other cytokines as well as enhances polymorphnuclear leukocytes (PMNLs) functions ,including O_{-2} and H2O2 production (Roilidies *et al.,* 1998). Additionally , TNF-α stimulates PMNLs to damage *Aspergillus* hyphae, enhances phagocytosis , augments PMNs oxidative respiratory burst and degranulation and its role in the immune response to bacterial and certain fungal, viral, and parasitic invasions as well as its role in the necrosis of specific tumors (Tracey and Cerami ,1990; Roilidies *et al.,* 1998). At the end of this study ,The examined serum samples taken from the 2nd gp expressed highly significant reduction of TNF- α release (table,5).In parallel to this respect, Dugyala *et al.,* 1994;Rodrique *et al.,* 1998 had demonstrated that inhibitory effects of AFB1 on macrophage mediators could be a result of suppressed proliferation of the granulocyte- macrophage (GM) progenitor cells to granulocyte, macrophage and GM-colonies which primes macrophage to release proinflamatory mediators including IL-1 and TNF-α. , hence, co-treatment with rosemary extract in the 3rd gp appears to enhance the production of TNF-α because polyphenolic contents of RM extract have the ability of antitumor activities through inhibition of cellular proliferation ,blockade of tumor cell cycle progression and induction of programmed cell death (Collett and Campbell, 2004 , Kunnumakkara *et al.,* 2007). Furthermore, polyphenols allow expression of genes such as interleukin-1Beta (IL-1B), IL-8 and TNF -α (Kim *et al.,* 2003;Camacho-Barquero *et al.,* 2007; Nonn *et al.,* 2007 ; Shakibaei *et al.,* 2007).

Table(5): Effects of rosemary extract on Tumor necrosis factor-α in mice intoxicated with AFB1 at the end of experiment (means±SE).

Treatment Groups	Tumor necrosis factor-α (pg/ ml)
Control	26 ± 1.09
AFB1	15.5±1.7
RM+ AFB1	18.5±2.3 **

Significant at p≥ o.o5**

The yield of plant RM water extract and the concentration of total phenolic content is 494mg/100 g fresh weight. A significant increase was observed in MDA level and significant decrease in GSH concentration and catalase enzyme activity at the last of experiment in comparison to the control group (table, 6).AFB1 mediated cell injury may be due to the release of free radicals which react with the lipid complex of the cell membrane and cause the peroxidation of lipid membrane (Comporti, 1993; Kohen and Nyska, 2003).

The cytotoxic nature of AFB1 may be the underlying reason for the increase in MDA level. It is a well-known fact that AFB1 inhibit protein synthesis (Ke eci ,1998*).* Due to this fact, a decrease may occur in seruloplasmin and transferrin levels synthesised in the liver. This may cause an increase in free copper and iron ions found in the organism, and therefore lead to deficiency of the defence system against lipid peroxidation. Iron plays a

particularly important role in the Fenton reaction, which is one of the phases in lipid peroxidation (Agil 1995; Kohen, and Nyska, 2003). Since the binding of iron with endogenous and exogenous chelators causes a decrease in the level of free iron ions, this also acts as a mechanism to prevent lipid peroxidation (Gutterridge and Halliwell, 1990; Comporti, 1993).

Table (6): Effects of rosemary extract on antioxidant enzymes in mice intoxicated with AFB1 (means±SE)

Treatments	GSH	Lipid peroxidase nmol/g Hb	Catalase u/mg Hb
control	13.10±0.96	30.51±1.36	20.86±1.24
AFB1	9.54±0.53*	38.96±1.94*	15.62±1.03*
RM+ AFB1	11.96±0.39	33.75±1.41	19.68±1.00

Significant at p≥ o.o5*

Glutathione is an important cellular reductant, which offers protections against free radicals. The decline in Catalase activity observed upon administration may be related to the consumption of highly active components during conversion into H_2O_2 due to the effect of AFB1 and utilization of these enzymatic antioxidants to scavenge the products of lipid peroxidation (Eraslan *et al.,* 2005; Balaji *et al.,* 2009*)*. Rosemary pretreatment significantly reduced the levels of thiobarbituric acid reactive substances, an indicator of lipid peroxidation, whereas increased the level of reduced glutathione and catalase enzyme.This result may be due to that carnosic acid (CA) and carnosol are the major polyphenolic compound present in rosemary plants reduced membrane damage by 40−50% when stressed by aflatoxinB1.

Carnosic acid is a lipophilic antioxidant that scavenges singlet oxygen, hydroxyl radicals, and lipid peroxyl radicals, thus preventing lipid peroxidation and disruption of biological membranes *(*Haraguchi *et al.,* 1995*)*. Both compounds significantly lowered DNA damage induced by AFB1. The antioxidant activities of carnosic acid and carnosol could be partly due to their ability *to* increase or maintain glutathione peroxidase and superoxide dismutase activities*(* Subhashinee *et al.,* 2007 ; Lee *et al.,* 2010*)*.

Therefore, it can be concluded that the results obtained in the present study drew our attention towards the possibilities of using new natural antitoxin for treatment together with the commercial conventional ones for achieving the synergistic action or even used as a new alternative treatment in the near future to overcome the cumulative side effects of the chemical commercial preparations and also due to the emergence of multi-drug resistance, and the development of antifungal resistant strains suggest that continued investigation is necessary to devise immunotherapeutic strategies and /or drug targets to combat fungal infection and mycotoxicosis.

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