



ORIGANUM MAJORANA ESSENTIAL OIL AMELIORATED THE BEHAVIORAL, BIOCHEMICAL, PHYSIOLOGICAL AND PERFORMANCE PERTURBATIONS INDUCED BY AFLATOXIN B1 IN GROWING RABBITS

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Abstract

The current study assessed the benefits of marjoram essential oil (MEO) in reducing the aflatoxin B1 (AFB1) toxicity in growing rabbits. Forty-eight rabbits, aged 4 weeks, were randomly assigned to four groups (4 replications of 3 rabbits/replicate) as follows: BD group (control); AFB1 group (supplemented with AFB1 0.3 mg/kg diet); MEO-supplemented group (1 g MEO/ kg diet); AFB1 + MEO group (AFB1 0.3 mg/kg diet + 1 g MEO/kg diet). AFB1 exposure resulted in reduction of rabbit growth and total protein and albumin content in serum; increased liver function related enzymes (ALP, ALT and AST); induced oxidative stress displayed by lower level of antioxidant enzymes (CAT and GSH), increased biomarkers of DNA and lipid oxidative damage with significant upregulation of the tumor suppressor protein interferon- γ , interleukin 1 β , tumor necrosis factor- α and heat shock protein-70 when compared to the control ($P < 0.05$). Moreover, exposure to AFB1 increased the bioaccumulation of AFB1 residues in rabbit liver. Supplementation of MEO in the AFB1-exposed rabbits alleviated all of its negative effects. In conclusion, dietary addition of 1 g MEO could effectively ameliorate the adverse impacts of AFB1 on rabbit growth; enhanced their antioxidants and reduced oxidative stress and inflammatory response indicators, as well as the AFB1 residues in liver.

Key words: aflatoxins, marjoram, rabbit, growth, behavior, antioxidant, liver

One of the main challenges facing the feed manufacture and animal production sectors all over the world is the contamination of the feedstuffs and feeds with mycotoxins which are secondary metabolites of fungi such as *Penicillium*, *Aspergillus* and *Fusarium*. Mycotoxins are characterized by low molecular weights, thermal stability, high potential to accumulate in feeds (Mousavi Khaneghah et al., 2019). Several factors contribute to the incidences and concentrations of mycotoxins in cereal products including chemical and physical characterization of food (composition, pH, and moisture), production conditions (storage, harvesting method and processing), environment (rainfall, temperature, humidity and insects) (Reyneri, 2006; Udomkun, et al., 2018; Zain, 2011). The biological effects and high toxicity of aflatoxin B1 (AFB1) and ochratoxin A have made them the most important and frequently detected mycotoxins (Tinelli et al., 2019).

Rabbits are one of the most sensitive animals to toxins such as aflatoxins, zearalenone, deoxynivalenol (DON),

and trichothecene (T-2) toxins (Zhang et al., 2022). Aflatoxins are one of the most frequently occurring metabolites with the highest toxigenic effects (Richard, 2007). They can lead to oxidative damage by enhancing the ROS (reactive oxygen species) production causing alterations in cellular contents (proteins, DNA and lipids) (Mary et al., 2012). Feeding on aflatoxin-contaminated diets resulted in several negative impacts including decreased feed intake (FI) and utilization, which in turn adversely affected growth performance, immune response, raised susceptibilities to diseases and stressors, induced high mortality rates, altered reproduction which consequently resulted in economic losses in the livestock production sector (Reda et al., 2020; Tinelli et al., 2019; Zain, 2011).

Several strategies have been developed for mycotoxin decontamination or detoxification including physical (separation and sorting, heating, solvent extraction, washing, adsorption, and irradiation), chemical (ozone and alkaline treatments), biological (bacteria, yeast, food fermentation, fungi) and enzymatic detoxification as well

as nutritional approaches (addition of some antioxidants and plant extracts) as reviewed by Agriopoulou et al. (2020). With all of these approaches which have been established for the control of mycotoxins, they are still insufficient to ensure food safety; as a result, there is an urgent need for finding other alternatives to decrease and/or eliminate the occurrence of mycotoxins in food or their detrimental impacts on animals. Among the environmentally acceptable alternatives being developed and investigated for antifungal activity against seed borne infections are natural compounds, such as plant extracts and essential oils (Moumni et al., 2021). Essential oils are a rich source of plant-derived metabolites which perform several biological functions with antifungal activity which could prevent both fungal growth and their production of toxic metabolites (Ebani and Mancianti, 2020).

Marjoram (*Origanum majorana* L.), widely called sweet marjoram, belongs to the Lamiaceae family. This herb has long been used extensively as a medicinal plant and food flavoring ingredient (Ghazal et al., 2022). It has a lot of industrial uses due to its antioxidant and antibacterial components (Amor et al., 2019; Bina and Rahimi, 2017). Their essential oil, dried leaves and their extracts were utilized for treating gastrointestinal, respiratory, and urinary tracts diseases due to its spasmolytic, anti-rheumatic, diuretic, and anti-asthmatic effects (Bina and Rahimi, 2017). *O. majorana* has been shown to possess a number of pharmacological actions such as antibacterial, antioxidant, cardioprotective, hepatoprotective, anti-ulcer, anti-inflammatory anticoagulant, antifungal and gastric secretory actions (Bina and Rahimi, 2017; Ghazal et al., 2022). Numerous investigations also reported the essential oil's antifungal and antibacterial properties against various strains of Gram-positive and Gram-negative bacteria as well as some fungi (Amor et al., 2019; Ben Salha et al., 2019; Ragab et al., 2019). In the present study, the protective mechanism of marjoram essential oil against aflatoxin B1 (AFB1) adverse effects was

studied. This was assessed through investigating rabbits' growth performance, behavioral parameters, blood biochemistry changes, antioxidant status, bioaccumulation of AFB1 residues in liver tissues as well as the expression of some stress and inflammatory-related genes.

Material and methods

Preparation of the aflatoxin

The preparation of AFB1 and the studied concentration were based on the previous findings of Hassan et al. (2023). In brief, *Aspergillus flavus* MD 341 was provided by the Center of Agricultural Pesticide Residues, Dokki, Egypt. It was exposed to eight days cultivation on a liquid medium with yeast extract (2%) and sucrose (20%) to yield AFB1 which was calculated in the cultivation media by HPLC analysis according to AOAC (1990). The standard of aflatoxin was purchased from Sigma-Aldrich (St Louis, Missouri, USA). The media was found to contain AFB1 only.

Marjoram (*Origanum majorana*) essential oil extraction and analysis (MEO)

The fresh marjoram plant (*Origanum majorana*) was purchased from a local shop in Zagazig, Al-Sharkia, Egypt. The plant's botanical identity was confirmed by a taxonomist from Zagazig University's Faculty of Agriculture's Botany Department. The fresh leaves were divided into small pieces and hydro-distilled (HD) for three hours using Clevenger-style equipment, as per the method described in Farouk et al. (2017). Following extraction, anhydrous sodium sulphate (El-Gomhouria Co, Zagazig, Egypt) was used for drying of the oil which was then kept in airtight glass vials, lined with aluminum foils, and kept at -20°C till analyzed. The active constituents of MEO were identified by gas chromatography-mass spectroscopy (GC-MS) analysis (Scheubert et al., 2013) and are presented in Figure 1.

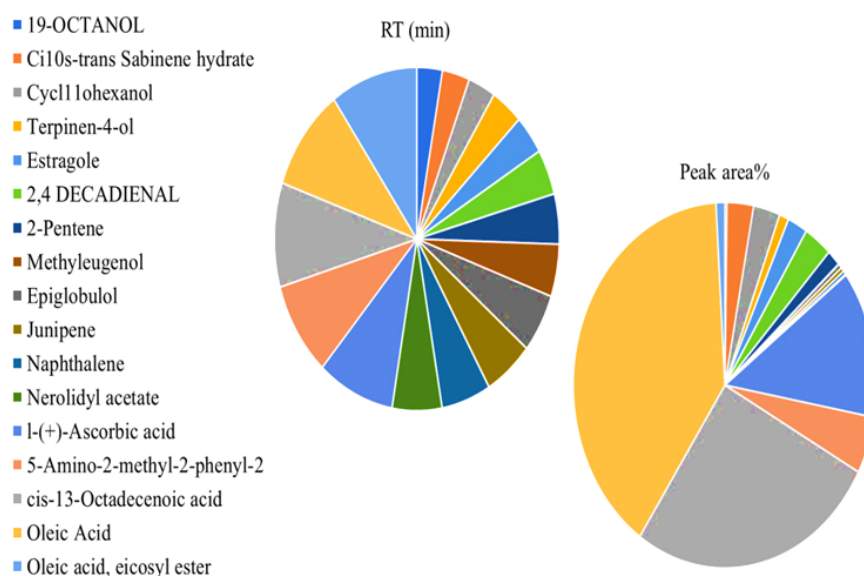


Figure 1. The main bioactive components in *O. majorana* essential oil

Experimental design and rabbits

Forty eight growing NZW rabbits, 4 weeks old, were divided into 4 groups at random, each with 4 duplicates and 3 animals. These were the experimental groups: 1) The basic diet; 2) The basic diet plus 0.3 mg AFB1/kg; 3) The basal diet with 1 g of MEO/kg; and 4) The basal diet with 0.3 mg AFB1/kg diet plus 1 g of MEO/kg. The rabbits were housed in cages that were 30 cm wide, 50 cm long and 40 cm high for the duration of the 8-week trial. Rabbits were raised under the same managerial and sanitary circumstances, and with food that satiated their nutritional requirements (Supplementary Table 1). The animal study was reviewed and approved by the institutional Ethics Committee of Zagazig University, Egypt (ZU-IACUC/2/F/387/2022).

Growth performance and carcass parameters

Live body weight (LBW), body weight gain (BWG), FI, and feed conversion ratio (FCR) were calculated. Six rabbits per group were captured, weighed, and slaughtered at the end of the experiment to determine the carcass traits. We evaluated the hot carcass weight and dressing percentage (including the main body, liver, heart, kidneys and all other edible portions).

Observations of rabbit behavior in cages

The last three days of the experiment were chosen to document the rabbits' behavioral traits. Three kinds of behavior were observed: comfort, resting, and maintenance behavior (eating and drinking). The definitions of these behavioral patterns are shown in Supplementary Table 2 and come from previous studies (Gunn and Morton, 1995; Morisse and Maurice, 1997). Four infrared cameras (TX-14300A, Turbo-X) were employed to record the rabbit's movements. A digital video recorder with a hard drive stored the recorded data (TX168; Telexper Inc., USA). For all of the behavioral measurements, one person was blind to group and decoded every recording.

Collection, preparation, and measurement of samples

At the completion of the study, rabbits (n=6 per group) were killed, and blood samples were collected in sterile tubes, left to coagulate, and then centrifuged at 4000 rpm for 10 minutes. The collected sera were kept until testing at -20°C. The lipid profile, 8-hydroxy-2-deoxyguanosine, and markers of liver function were all determined using the serum. Specimens of liver were dissected and stored at -20°C for detection of AFB1 residues. A second batch of liver samples was kept at -20°C for further biochemical testing. After being immediately frozen in liquid nitrogen, a third batch of liver samples was stored at 80°C till the extraction of RNA.

Antioxidant assays

Liver samples were centrifuged at 3000 rpm for 15 minutes after being homogenized (10% w/v) in potassium phosphate buffer (pH 7.4). Following the manufac-

turer's instructions, the generated supernatants were utilized to measure GSH (reduced glutathione) content and CAT (catalase), and SOD (superoxide dismutase) activity by kits from BioMérieux, Marcy l'Etoile, France.

Oxidative stress markers

The liver specimens (100 mg each) were homogenized by mechanical homogenizer with phosphate buffer (10 mM phosphate/20 mM tris-pH 7.0) at 4°C and then were centrifuged for 3 min at 600 × g. The supernatant was collected and used in the analysis of oxidative stress biomarkers in liver homogenate. Hepatic malondialdehyde (MDA), and protein carbonyl (PC) and serum 8-hydroxy-2-deoxyguanosine (8-OHdG) were used as biomarkers of lipid, protein and DNA oxidative damages respectively; they were determined by the use of ELISA kits from MyBiosource.com, San Diego, California (Cat No., MBS040484, MBS1601647, and MBS726394, respectively) following the manufacturer's protocol.

Markers of liver function

Albumin, total protein, AST (aspartate aminotransferase), ALT (alanine aminotransferase), and ALP (alkaline phosphatase) were measured by a Shimadzu spectrophotometer by the use of diagnostic kits (Bio-diagnostic Co., Giza, Egypt) following the manufacturer's protocol. AST was determined based on the principle that α -oxoglutarate + L aspartate in the presence of AST were converted to L-glutamate + oxaloacetate, the formed oxaloacetate was determined by monitoring the content of oxaloacetate hydrazine formed with 2, 4 dinitrophenyl hydrazine at wavelength 460 nm. While, ALT was determined based on the principle that oxoglutarate + DL-alanine in the presence of ALT were converted to L-glutamate + pyruvate, the formed pyruvate was determined by monitoring the content of pyruvate hydrozone formed with 2, 4 dinitrophenyl hydrazine at wavelength 460 nm. ALP was determined based on the principle that P-nitrophenyl phosphate + H₂O in the presence of ALP were converted to p-nitrophenol + phosphate and the yellow colored indicator p-nitrophenol was measured at 405 nm.

Lipid profile

Using commercial kits purchased from Bio-diagnostic Co (Giza, Egypt), the lipid profiles including triglycerides (TG), total cholesterol (TC), LDL (low-density lipoprotein) and HDL (high-density lipoprotein) were assessed by spectrophotometer.

Transcriptional evaluation of genes in the liver tissues that code for stress and inflammatory cytokines

RT-PCR (quantitative real-time polymerase chain reaction) was used to determine the mRNA expression levels for the targeted genes as previously described in Hassan et al. (2023) using the following primers tumor necrosis factor- α (TNF- α): F:5'-CTGCACTTCAGGGTGATCG-3' and R:5'-

CTACGTGGGCTAGAGGCTTG-3' (GenBank accession no. NM_001082263), interleukin-1 β (IL-1 β): F:5'-TTGAAGAAGAACCCGTCCTCTG-3' and R:5'-CTCATACGTGCCAGACAACACC-3' (GenBank accession no. NM_001082201), interferon gamma (IFN- γ): F:5'-TGCCAGGACACACTAACCAGAG-3' and R:5'-TGTCACCTCTCCTCTTTCCAATTCC-3' (GenBank accession no. NM_001081991), heat shock protein 70 (HSP70): F:5'-CGTGGAGTCCTACACCTACAAC-3' and R:5'-ACTCGTCTTTCTCGGCCATC-3' (GenBank accession no. XM_002719559.3) and the control gene (β -actin): F:5'-CTGGAACGGTGAAGGTGACA-3' and R:5'-CGGCCACATTGCAGAACTTT-3' (GenBank accession no. NM_001101683.1).

Detection of AFB1 residues in hepatic tissues

The Vicam AflaTest fluorometer method was used to identify the AFB1 residues in the hepatic tissues of rabbits as described in Abd-Elghany and Sallam (2015). AflaTest was calibrated by the use of mycotoxins calibration standards. For accuracy assurance, two ml of purified and the plain reagent (1 ml developer and 1 ml methanol) both should give 0 ppb reading on the calibrated fluorometer. Twenty five g of sample were blended for 1 min at high speed with 125 ml of methanol: water (60:40) and 5 g of NaCl. Then the mixture was filtered by fluted filter paper, and 20 ml of purified were used to dilute the obtained extract (20 ml) then filtered by the use of 1.5 μ m glass microfiber filter into a clean flask. Filtered extract (10 ml, represents 1 g sample) at a flow rate nearly 2 drops/s was passed through the affinity column followed by water (10 ml, 2 drops/s). The aflatoxin was eluted with HPLC methanol (1 ml, 1 drop/s), and then collected in a glass cuvette (VICAM part # 34000). The developer (1 ml) and elute were combined before being added to the calibrated fluorometer.

Statistics

One-way ANOVA and the post-hoc Newman-Keuls test were employed in the analysis, which was done using SAS (SAS, 2004), to find statistical differences among groups. At P 0.05, significance was determined.

Results

Behavioral response, growth performance and carcass traits

Rabbit behavioral response to the different treatments is presented in Table 1. Rabbit comfort behavior was significantly affected as it was reduced in rabbits receiving the AFB1 and rabbits receiving the AFB1 and supplemented with MEO (P = 0.005) when compared to control rabbits. Other behavioral patterns including frequency and duration of eating and drinking as well as resting showed no differences among groups.

As illustrated in Table 2, rabbits that received AFB1 in their diet showed the lowest BWG (P = 0.002), FI (P = 0.017) and deteriorated FCR when compared to the other experimental groups (P<0.001). Supplementation with MEO to the rabbits fed AFB1 improved their BW, FI and FCR compared to the group received AFB1 alone. The highest performance was observed in rabbits fed on the diet containing MEO only when compared to the other groups.

Regarding the carcass measurements (Table 3), AFB1-fed rabbits showed the lowest carcass %, internal organs % (heart, kidney, and spleen), giblets % and the dressing % relative to control. The addition of MEO to the AFB1 containing feed reduced the negative effects on the carcass traits comparable to their control rabbits (P<0.001). Rabbits that received MEO in their diet showed the highest response in terms of all measured carcass traits when compared with other groups (P<0.001).

Table 1. Behavioral patterns of rabbits (min/h) after continuous 24-h recording on three consecutive days

	Frequency of eating	Frequency of drinking	Duration of eating	Duration of drinking	Comfort behaviors	Resting
Control	0.47 $\pm$ 0.01	0.19 $\pm$ 0.01	2.68 $\pm$ 0.16	0.31 $\pm$ 0.01	11.19 $\pm$ 0.03 a	36.41 $\pm$ 0.68
AFB1*	0.43 $\pm$ 0.01	0.18 $\pm$ 0.00	2.73 $\pm$ 0.10	0.32 $\pm$ 0.00	10.20 $\pm$ 0.04 b	34.09 $\pm$ 1.04
MEO	0.47 $\pm$ 0.01	0.19 $\pm$ 0.01	2.77 $\pm$ 0.12	0.31 $\pm$ 0.00	10.92 $\pm$ 0.31 a	35.90 $\pm$ 0.72
AFB1+MEO	0.46 $\pm$ 0.02	0.19 $\pm$ 0.00	2.66 $\pm$ 0.18	0.31 $\pm$ 0.01	10.23 $\pm$ 0.02 b	36.50 $\pm$ 0.61
P value	0.129	0.719	0.947	0.802	0.005	0.183

Means $\pm$ SEM in the same column with different letters are significantly different (P<0.05). *AFB1: 0.3 mg/kg diet; MEO group (1 g/kg diet); AFB1 + MEO group (AFB1 0.3 mg/kg diet + 1 g MEO/kg diet).

Table 2. Growth performance of NZW growing rabbits (n=5/group)

	Initial weight (g)	Final body weight (g)	Weight gain (g)	Feed intake (g)	FCR (g feed/g gain)
Control	661.50 $\pm$ 0.53	1973.03 $\pm$ 11.74 a	26.22 $\pm$ 0.64	60.68 $\pm$ 0.39 a	2.22 $\pm$ 0.05 b
AFB1	660.32 $\pm$ 1.09	1868.89 $\pm$ 5.44 b	25.37 $\pm$ 0.41	54.31 $\pm$ 2.13 b	2.81 $\pm$ 0.05 a
MEO	661.88 $\pm$ 0.63	1992.38 $\pm$ 3.85 a	27.58 $\pm$ 0.29	60.79 $\pm$ 0.88 a	2.06 $\pm$ 0.03 c
AFB1+ MEO	661.70 $\pm$ 0.33	1941.67 $\pm$ 27.23 a	26.17 $\pm$ 0.72	57.88 $\pm$ 0.70 ab	2.43 $\pm$ 0.06 bc
P value	0.437	0.002	0.106	0.017	<0.001

Means $\pm$ SEM in the same column with different letters are significantly different (P<0.05). * AFB1: 0.3 mg/kg diet; MEO group (1 g/kg diet); AFB1 + MEO group (AFB1 0.3 mg/kg diet + 1 g MEO/kg diet).

Table 3. Carcass traits of NZW growing rabbits (n=5/group)

	Carcass %	Heart g/kg SW	Liver g/kg SW	Lung g/kg SW	Kidney g/kg SW	Spleen g/kg SW	Giblets %	Dressing %
Control	57.20±1.25 ab	3.15±0.02 c	30.84±0.42	6.46±0.03	7.08±0.04 b	1.40±0.06 a	4.86±0.03 a	65.19±0.10 a
AFB1	55.14±1.09 b	2.68±0.04 d	30.90±0.35	5.94±0.43	6.43±0.09 c	0.82±0.01 c	4.46±0.12 b	60.19±0.03 c
MEO	61.26±0.41 a	3.77±0.04 a	30.34±0.11	6.63±0.04	7.60±0.06 a	1.51±0.01 a	5.07±0.03 a	65.32±0.06 a
AFB1+ MEO	57.00±1.34 ab	3.43±0.13 b	30.40±0.06	6.10±0.40	7.07±0.03 b	1.17±0.09 b	4.17±0.09 b	64.50±0.14 b
P value	0.023	<0.001	0.416	0.388	<0.001	<0.001	<0.001	<0.001

Means ± SEM in the same column with different letters are significantly different (P<0.05). *AFB1: 0.3 mg/kg diet; MEO group (1 g/kg diet); AFB1 + MEO group (AFB1 0.3 mg/kg diet + 1 g MEO/kg diet).

Blood biochemistry

Serum biochemical changes of rabbits are presented in Table 4. AFB1 induced significant reduction in serum total protein and albumin concentrations, while significantly increased the liver function related enzymes (ALP, P = 0.011; ALT and AST; P<0.001) relative to other groups. Compared to AFB1 diet, AFB1 + MEO containing diet ameliorated the negative impacts as it enhanced the total protein and albumin and reduced the ALP, ALT and AST enzyme activities. Rabbits that received the MEO alone in their diet recorded the lowest levels of liver function enzymes along with higher total protein and albumin.

Regarding the serum lipid profile shown in Table 5, all parameters were significantly modified in response to the experimental treatments. Rabbits in AFB1 group showed higher serum TC, TG and LDL (P<0.001) and lower HDL (P = 0.011) concentrations compared to the control and the other treated groups. On the contrary, rabbits fed on AFB1 + MEO showed reduced levels of these

parameters with non-significant change in the serum HDL when compared to the AFB1 containing diet. The MEO fed rabbits showed non-significant difference in all lipid profile parameters compared to the control rabbits.

Antioxidant status, biomarkers of lipid peroxidation and protein and DNA oxidation

AFB1 significantly reduced the antioxidant enzymes (CAT and GSH) with higher levels of the MDA (P<0.001) and 8-OHdG (P = 0.005) biomarkers compared to control (Table 6). Non-significant difference was reported in the SOD enzyme activity and protein oxidation biomarker among groups (P = 0.434). The co-addition of MEO with AFB1 enhanced the GSH enzyme activity (P<0.001) while reduced the lipid and DNA oxidation biomarkers compared to AFB1 group. Supplementation of MEO to rabbit diet showed no difference in the stress biomarkers (MDA, 8-OHdG, and PC) while enhanced the antioxidant enzymes CAT (P = 0.006) and (GSH) when compared to control.

Table 4. Blood biochemistry of NZW growing rabbits (n=5/group)

	Total protein (g/dl)	Albumin (g/dl)	ALP (U/L)	ALT (U/L)	AST (U/L)
Control	6.64±0.12 a	3.85±0.03 a	10.05±0.58 b	33.51±3.05 b	13.45±0.49 c
AFB1	5.46±0.02 b	2.88±0.08 c	22.02±2.45 a	99.59±1.31 a	45.37±1.05 a
MEO	6.64±0.12 a	3.91±0.04 a	9.83±0.46 b	26.96±0.75 b	13.40±0.49 c
AFB1 + MEO	6.07±0.04 c	3.30±0.06 b	14.96±3.35 ab	33.52±3.05 b	25.98±2.37 b
P value	<0.001	<0.001	0.011	<0.001	<0.001

Means ± SEM in the same column with different letters are significantly different (P<0.05). *AFB1: 0.3 mg/kg diet; MEO group (1 g/kg diet); AFB1 + MEO group (AFB1 0.3 mg/kg diet + 1 g MEO/kg diet).

Table 5. Serum lipid profile of NZW growing rabbits (n=5/group)

	TC (mg/dl)	TG (mg/dl)	HDL (mg/dl)	LDL (mg/dl)
Control	52.14±0.01 c	72.24±0.13 b	44.18±0.55 a	7.30±0.21 c
AFB1	112.66±3.34 a	90.60±3.40 a	36.86±2.24 b	72.41±0.39 a
MEO	51.80±0.34 c	72.22±0.11 b	44.59±1.05 a	7.08±0.30 c
AFB1 + MEO	66.81±4.66 b	72.23±0.55 b	36.66±2.02 b	28.32±0.41 b
P value	<0.001	<0.001	0.011	<0.001

Means ± SEM in the same column with different letters are significantly different (P<0.05). *AFB1: 0.3 mg/kg diet; MEO group (1 g/kg diet); AFB1 + MEO group (AFB1 0.3 mg/kg diet + 1 g MEO/kg diet).

Table 6. Antioxidant status, biomarkers of lipid peroxidation and protein and DNA oxidation in NZW growing rabbits (n=5/group)

	SOD ($\mu\text{g/g}$ tissue)	CAT ($\mu\text{g/g}$ tissue)	GSH ($\mu\text{g/g}$ tissue)	MDA (nmol/g tissue)	PC (nmol/g tissue)	8-OHdG (ng/ml)
Control	0.57 $\pm$ 0.01	0.32 $\pm$ 0.01 ab	5.11 $\pm$ 0.06 b	0.15 $\pm$ 0.01 b	4.45 $\pm$ 0.23	0.15 $\pm$ 0.02 c
AFB1	0.55 $\pm$ 0.02	0.19 $\pm$ 0.06 b	2.22 $\pm$ 0.07 d	0.46 $\pm$ 0.01 a	4.53 $\pm$ 0.03	0.54 $\pm$ 0.05 a
MEO	0.57 $\pm$ 0.01	0.43 $\pm$ 0.02 a	6.11 $\pm$ 0.24 a	0.09 $\pm$ 0.01 c	4.08 $\pm$ 0.08	0.12 $\pm$ 0.01 c
AFB1 + MEO	0.56 $\pm$ 0.01	0.19 $\pm$ 0.05 b	4.05 $\pm$ 0.04 c	0.09 $\pm$ 0.01 c	4.45 $\pm$ 0.24	0.33 $\pm$ 0.02 b
P value	0.434	0.006	<0.001	<0.001	0.310	0.005

Means $\pm$ SEM in the same column with different letters are significantly different ($P < 0.05$). *AFB1: 0.3 mg/kg diet; MEO group (1 g/kg diet); AFB1 + MEO group (AFB1 0.3 mg/kg diet + 1 g MEO/kg diet).

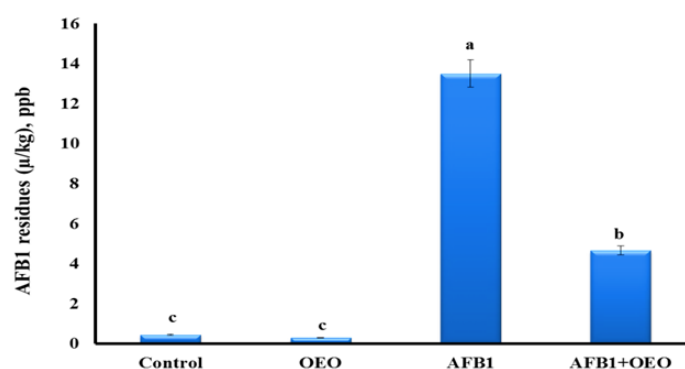


Figure 2. Effect of supplementation of MEO on the bioaccumulation of AFB1 residues in liver of rabbits. Values are mean $\pm$ SE, bars not sharing a common superscript letter (a,b,c) differ significantly at $P < 0.05$

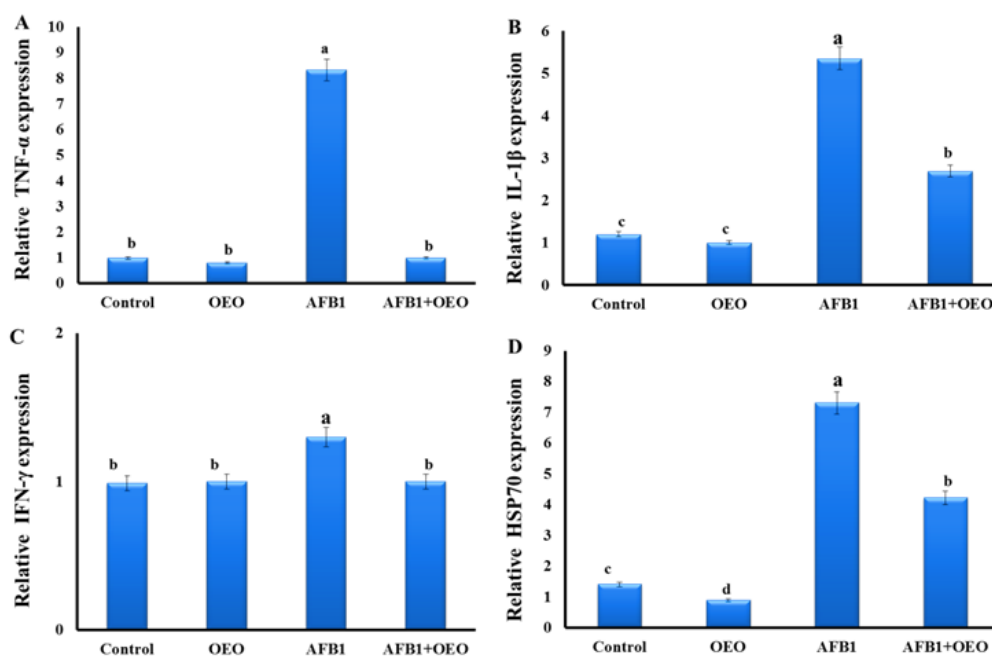


Figure 3. Effect of supplementation of MEO (1 g/kg diet) on the expression pattern of stress-related genes and pro-inflammatory cytokines in liver of rabbits exposed to AFB1. Values are mean $\pm$ SE, bars not sharing a common superscript letter (a,b,c,d) differ significantly at $P < 0.05$

AFB1 residues in the liver tissue

Bioaccumulation of AFB1 residues in rabbit liver is illustrated in Figure 2. The AFB1 residues were the highest in rabbits that received the AFB1 when compared

to the control or rabbits that received the MEO only ($P < 0.05$). The co-addition of MEO with the AFB1 significantly reduced the AFB1 residues in the hepatic tissue compared to AFB1 group.

Expression of stress genes and inflammatory cytokines in the liver tissues

AFB1 containing diet upregulated the expression of IFN- γ , IL-1 β , TNF- α and HSP-70 while the lowest expression was shown in control and MEO group ($P < 0.05$). Compared to AFB1 group, rabbits of MEO + AFB1 group showed significantly lower expression of previously mentioned genes which showed nearly similar TNF- α and IFN- γ expression to control and MEO group (Figure 3).

Discussion

The rabbit meat constitutes one of the important animal protein sources in diets of humans. So, the feed ingredients utilized by rabbits and their contaminations with unwanted compounds are important for the meat quality and the human health (Mézes and Balogh, 2009). The inclusion of mycotoxin contaminated feed ingredients in rabbit feed adversely affects their performance and immunity. Despite the great efforts in controlling this problem or reduction of its detrimental effects, it is a major concern still facing the animal and poultry production sectors worldwide. The aim of current experiment was to evaluate the protective effects of marjoram essential oil against the AFB1-induced effects in growing rabbits.

In the current investigation, the AF-fed rabbits showed reduced comfort behavior and impaired growth performance (lower final BW, WG, FI as well as deteriorated FCR) compared to the control rabbits. The obtained results are in line with Reda et al. (2020) who reported similar adverse effects with aflatoxins in the growing rabbits. Also, Prabu et al. (2013) fed the New Zealand White rabbits aflatoxin B1, ochratoxin A containing diets and their combinations for 60 days and reported the highest mortality and reduced BW in the interaction group suggesting additive effect of both mycotoxins. Moreover, Salem et al. (2001) stated that rabbits treated with aflatoxin B1 at sublethal doses (15 or 30 $\mu\text{g/kg}$ of body weight) showed reduced LBW and feed consumption. The lower growth performance reported in the AFB1-fed rabbits could be related to lower FI and the significant alteration in the activity of liver oxygenase enzymes required for protein, carbohydrate and lipid metabolism and could also alter the absorption of essential nutrients along with patho-anatomical changes in vital organs, particularly liver (Abdelhamid et al., 1990; Clark et al., 1980; Shabani et al., 2010) which in turn affected dietary nutrients utilization. Besides, lower BW in the AFB1 treated rabbits may be due to their toxic effects on the cellular contents occurring through reduction in RNA and DNA and protein syntheses (Marai and Asker, 2008). The deteriorated FCR in the AFB1-fed rabbits is consistent with results of previous studies in rabbits (Hassan et al., 2019; Reda et al., 2020).

Additionally, results also showed that AFB1-fed rabbits demonstrated negative effects on carcass traits

shown by lower carcass % and giblet % and internal organs relative weight including heart, kidney and spleen. The obtained results are in line with Reda et al. (2020) who attributed the obtained effects to reduction in FI and protein synthesis that occur during AF exposure, which are necessary for some damaged organs to regenerate. On the contrary, Ibrahim (2000) found that the relative weights of the internal organs (spleen, brain, liver and kidneys) were not affected in mature NZW rabbits orally treated with AFB1 (15 or 30 $\mu\text{g/kg}$ BW every other day). The inclusion of 1 g MEO/kg diet to the AFB1 containing diet effectively reduced negative impact of AFB1 on rabbit growth parameters and some carcass traits. MEO addition in rabbit diet alone or added to the AFB1 fed rabbits improved their BW, FI and FCR to normal values. In support, Bakr et al. (2017) and Seleem et al. (2007) reported enhanced final BW and WG in growing rabbits fed on diets supplemented with marjoram. Similar improved growth performance response was obtained by Ahmed and Abdel-Ghany (2015) and Ali (2014) in broiler and turkey respectively. The enhanced performance with MEO feeding could be associated with their content of the active components (as shown in Figure 1), which was documented to have antimicrobial, antioxidant and antifungal activities (Bina and Rahimi, 2017; Kaskatepe et al., 2022). These active substances positively improved the lower gut health, enhanced the efficiency of digestion and nutrients and this reflected on rabbit growth performance (Bakr et al., 2017). In the same context, some recent studies documented the antifungal activities of essential oils of *O. majorana* (Amor et al., 2019; Ben Salha et al., 2019; Chaudhari et al., 2020) which accounted this effect to its contents of oxygenated monoterpenes particularly terpinen-4-ol, α -terpinol and α -pinene. The antifungal mechanism of MEO could be related to the alterations of ergosterol (important cellular component of fungal cells) biosynthesis and membrane integrity, which could affect internal homeostasis and the release of cellular constituents (Chaudhari et al., 2020).

The impaired performance in the AFB1-fed rabbits was accompanied by alteration in the blood biochemistry represented by lower total protein, albumin and HDL, higher activities of liver function related enzymes (ALT and AST) and concentrations of lipid profile parameters (TC, TG and LDL). The obtained results agree with Atef et al. (2016) who found lower levels of TP and albumin in the aflatoxin exposed rabbits indicating protein synthesis inhibition, increasing catabolism of protein and/or kidney dysfunction (Jindal et al., 1994).

The serum values of ALT, AST, and ALP are frequently utilized as key indicators to determine the liver functions (Hu et al., 2014). The liver, as a detoxifying organ, is one of the target organs of aflatoxin toxicity. In the current study, AFB1 administration in rabbits was associated with a significant elevation of AST, ALT, and ALP enzymes suggesting damage of the hepatic tissue which in turn causes the releases of such intracellular enzymes into blood. The attained results suggested that AFB1 ex-

posure in rabbits stimulated an oxidative stress condition which resulted in liver injury accompanied with the interference in the liver functions including the lipid metabolism. The damage of the liver could be also related to the bioaccumulation of the AFB1 residues which was the highest in this group. As previously reported, feeding on a diet naturally contaminated with 50 $\mu\text{g kg}^{-1}$ AFB1 resulted in liver lesions with absence of lobular architectures (Abdelhamid et al., 2002). In support, Atef et al. (2016) reported similar increases in AST, ALT and ALP serum activities indicating initial hepato-cellular damages as a consequence of AFB1 treatment in rabbits. In the same context, Oraby et al. (2022) observed large prevalence of liver cells cirrhosis and necrosis in the aflatoxin subjected rabbits.

The supplementation of MEO to the AFB1 containing diet ameliorated these undesirable impacts on the hepatic tissue which was reflected on its function. MEO addition resulted in lower levels of liver function enzymes and AFB1 residues accumulation in liver and decreased lipid profile parameters in the serum (TG, TC and LDL) suggesting that the MEO has a hepatoprotective and hypolipidemic effect in the present study. These results are in line with Mossa et al. (2013).

AFB1 exposure significantly reduced the CAT activity and GSH content while increased the DNA and lipid oxidative damages. Similar results were obtained by Reda et al. (2020) as dietary aflatoxin induced a significant decrease in the serum total antioxidant capacity while increased the ROS, DNA and lipid oxidative damages in rabbits. Also, Prabu et al. (2013) reported higher levels of MDA in rabbits in response to aflatoxin. These adverse effects of AFB1 may be attributed to AFB1 biotransformation to intermediate metabolites of high reactivity (AFB1 8, 9 epoxide) and production of free radicals including hydrogen peroxide (H_2O_2), superoxide anions (O_2^-), and hydroxyl radicals ($\cdot\text{OH}$) implicated in oxidative damages (Shen et al., 1995). Moreover, Atef et al. (2016) found significant increases in the levels of MDA in lysate red blood corpuscles besides significant decreases in antioxidant enzymes activities (SOD, CAT and GPx) and suggested the occurrence of oxidative stress in response to the effects of aflatoxin or by the formed metabolites and the generated free radicals. An excessive buildup of oxidants like ROS in the body surpasses its capacity to scavenge them, which causes much higher levels of lipid peroxidation in several tissues, DNA damage and impairment of protein expression (Juan et al., 2021; Mary et al., 2012). The present results suggested that AFB1 induces excessive ROS production causing oxidative stress and impaired balance of the oxidation/antioxidant system inside the body which was confirmed by the reduced CAT activity and GSH content accompanied with increased lipid and DNA oxidative stress markers.

Raising rabbits on AFB1-containing diet supplemented with MEO alleviated these adverse effects as manifested by increased activity of their antioxidant enzymes and GSH content, and decreased MDA and DNA oxida-

tion indicators. The efficiency of the MEO in attenuating these toxic effects could be related to its antioxidant activity which was confirmed in previous studies (Bina and Rahimi, 2017; Mossa and Nawwar, 2011). They attributed this effect to their content of phenolic compounds which has a free radical scavenging nature, explained by their abilities in donating a hydrogen atom from their phenolic hydroxyl group which in turn inhibit the formation of ROS (Mossa and Nawwar, 2011).

Inflammation is an important marker of liver damage which results in response to toxic physical or chemical stressors. When the liver is injured, inflammatory cells are activated and inflammatory cytokines (TNF- α , and IL-6, and IL-1 β) are produced, which cause buildup of neutrophils in the liver and result in increased cytokine expression (Olteanu et al., 2012). In the current report, AFB1 feeding to rabbits was associated with upregulation of the stress and inflammatory cytokines including IFN- γ , IL-1 β and TNF- α and HSP-70 compared to other groups. These results are in line with Hassan et al. (2020) who detected that AFB1 induced hepatotoxic effects led to a significant elevation of IL-6 and TNF- α activity in rats. Also, Elbasuni et al. (2022) documented higher expressions of IL-1 β , TNF- α and IL-6 inflammatory cytokines in the aflatoxin-fed quails. Additionally, Hassan et al. (2023) reported upregulation of the stress and inflammatory cytokines in kidney of AFB1-exposed rabbits.

Co-addition of MEO to AFB1-containing diet, significantly downregulated the expression of these stress and inflammatory indicators, suggesting the anti-inflammatory activity of MEO. The obtained finding suggested that the MEO alleviated the AFB1 induced liver injury through reducing the inflammatory response. In the same respect, Arranz et al. (2015) indicated that marjoram essential oil (10 $\mu\text{g/mL}$) markedly suppressed the productions of pro-inflammatory cytokines (IL-1 β , TNF- α , IL-10 and IL-6) and downregulated expressions of cyclooxygenase 2 (COX2) and NFkB genes and devoted the anti-inflammatory effect to the sabinene hydrate and terpineol active compounds in marjoram.

Conclusion

From the aforementioned results, it could be concluded that the underlying mechanisms of AFB1-induced adverse effects could be associated with increased biomarkers of oxidative stress and decreased activity of antioxidants. This came in line with elevated AFB1 residues and higher expressions of inflammatory cytokines and genes of stress in the liver. The co-exposure to *Origanum majorana* essential oil attenuated these negative effects and had a clear modulatory effect. These beneficial effects might be correlated with its contents of anti-inflammatory and antioxidant components which could reduce oxidative damage and inflammatory response in liver and this reflected on the rabbit growth and behavior which was enhanced in the AFB1 + MEO co-exposed rabbits.

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