The Efficacy of Thyme Oil as Antitoxicant of Aflatoxin(s) Toxicity in Sheep

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Abstract: This study is an attempt to prevent or minimize the negative effects due to ingesting feed contaminated with aflatoxins (AFs). An exposure study extended for two different stages was conducted using eighteen Egyptian male sheep. The 1st stage (pre-treatment) was suggested to compare the performance of animal groupings under the normal conditions before receiving any treatment, either level of contamination(s) or dosage(s) of additive, such stage extended for 2 weeks. The 2nd stage (treatment), animals received aflatoxin(s)-contaminated diets (10 mg/kilogram concentrated diet) and / or the thyme oil at two levels (250 and 500 mg / head / day), such stage extended for 4 weeks. The average body weights, body weight gains and feed conversion ratios were dramatically affected during the exposure stage to AFs. The thyme oil treatment reduced the adverse effects of aflatoxins and improved the rumen activity and motility and animal performance in general, either at low or high dose. The activities of gamma-glutamyl transferase (γGT); aspartate aminotransferase (AST) and alkaline phosphatase (ALP) enzymes, were significantly elevated during exposure to aflatoxins. Also, serum uric acid level was increased; but the levels of serum glucose and albumin were decreased by dietary aflatoxin. On the other hand, the use of thyme oil either 250 or 500 mg / head / day; approximately normalizing such levels. The major excretory route of aflatoxins and their metabolites was found to be the urine (accounting for 39.71% to 68.32% of the tatoal AFs-excretion forms, whereas less than 10% of these forms were excreted in the feces. Only about 52% of the dose was recovered in the feces and urine in identifiable forms, mainly aflatoxin $B_{2\alpha}$ ($AFB_{2\alpha}$) and unchanged aflatoxin B_1(AFB1) without any detectable amount of aflatoxicol. In conclusion, our results may lead us to suggest that there is a significant liver dysfunction in these groups, 10 mg of aflatoxin/kg of diet was sufficient to impair performance and cause liver damage in male sheep, the thyme oil could be considered a potential natural antitoxicant for sheep and could protect sheep from hepato-toxicities which induced by aflatoxin-contaminated diet when used as a sole diet. [Journal of American Science 2010;6(10):948-960]. (ISSN: 1545-1003).

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1. Introduction:

Aflatoxins (AFs), a group of closely related, biologically active mycotoxins, are produced by strains of *Aspergillus flavus* and A. *parasiticus* and occur naturally in several important animal feeds, including corn, cotton seed, and peanuts (Moos, 2002). Acute aflatoxicosis causes hepatitis, icterus, hemorrhage, and death. Reduced growth rate is the most sensitive clinical sign of chronic aflatoxicosis, and it may be the only readily detectable abnormality (Pier, 1992; Casado *et al.,* 2001). Although the exact mechanism by which aflatoxins reduce growth rate is not known, disturbances in protein, carbohydrate, and lipid metabolism are probably involved (Cheeke and Shull, 1985). More attention has focused on the alleviation or prevention of aflatoxicosis by manipulation of dietary nutrients (Abdel-Wahab *et al.,* 2007; Saad and Abdel-Fattah, 2008) or the inclusion of chemiadsorptive compounds (Kubena *et al.,* 1991; Schell *et al.,* 1993a,b). Reports concerning aflatoxicosis in ruminants, in sheep in particular, vary. Some experiments showed that ruminants are more resistant to AF poisoning than monogastric animals (Pier, 1992). However, the data in the literature on the extent of ruminal degradation of AF and its effects on rumen microbial activity are not consistent (Jouany and Diaz, 2005). Harvey *et al.*, (1991) reported a dose of 2.6 mg of AFB_1 kg of diet significantly reduced feed intake and BW gain and increased serum enzyme activities indicative of liver damage.

Essential oils are one of the three main groups produced as a plant secondary metabolites. Some essential oils have antimicrobial activities and are currently considered safe for human and animal consumption, and are categorized as GRAS. Rrecently, the potential use of essential oils in ruminant diets has been reviewed by Ultee *et al.,* (2002); Calsamiglia *et al.*, (2007) and Benchaar *et al.*, (2007) and some reported that the compounds with phenolic structures, such as thymol and carvacrol, are more effective as antimicrobials in

comparison with other non-phenolic secondary plant metabolites due to the presence of a hydroxyl group in the phenolic structure.

Ruminal ciliate protozoa play an important role in biodegradation of plant toxins and mycotoxins (Yiannikouris and Jouany, 2002). Moreover, Ruminal ciliates play an important role in regulation of ruminal condition such as pH. Ruminal ciliate protozoa eliminate certain pathogens from the digestive tract of ruminant, protecting them from disease and so improving the food safety of edible animal products (McIntosh *et al.,* 2003).

There is no available data dealing with the efficacy of essential oils on the protozoal activities and its ability to biodegradation of chemicals that have negative effect on rumen ecosystems (such as mycotoxins). So, the objective of the present study was to evaluate thyme oil, as natural additive to diets of sheep, on digestibility of nutrients, growth performance, feed utilization, increasing the protozoal activities and its ability to minimize the adverse effects of aflatoxin-contaminated diet fet to Egyptian male sheep at level 10 mg/kg contaminated diet. Thyme oil was offered in encapsulated form to prevent palatability problems reported in previous studies (Cardozo *et al.,* 2006 and Busquet *et al.,* 2003).

2. Materials and Methods

1-The general layout of the study

Eighteen apparently healthy Egyptian male Baladi sheep with a mean body weight of 38.25 ± 2.34 kg, and age from 6 to 8 months; were selected from a local private farm of Kalubia province, Egypt, passed through two main stages, which lasted six weeks as follows: the 1st pre-treatment stage extended for two weeks to make the animal will be adapted for the tested diet, and to avoid any differences in both type and number of ruminal microflora before treatment. During this stage, all experimental animals were fed sound rations (without aflatoxins or thyme oil), checked and observed for any abnormalities. The daily ration contained 70 % concentrated diet (60 % wheat, 9 % soy meal and 1 % mineral/vitamin mixture) and 30 % hay as roughage. The 2nd treatment stage, was extended for further four weeks. The experimental design during this stage is shown in Table (1). Feeds Were offered in two equal portions at 0700 a.m and 1800 p.m. to meet NRC nutritional requirements (NRC, 1985). Berseem hay (BH) was offered once daily at 2100 p.m. Fresh water was freely available to animals. Animals were biweekly weighed in the morning before offering any feed or water. Live body weight changes and feed intakes were recorded at biweekly intervals. Chemical analysis and aflatoxins estimation were conducted at Laboratory of Food toxins and contaminants, National Research Centre, Egypt. The protozoal count were conducted at Regional Center for Food and Feed (RCFF), Agricultural Research Center, Ministry of Agriculture, Egypt.

2- Animals and rations:

At the treatment stage, the tested animals (N =18) divided randomly into six experimental groups in equal numbers (N=3) of animals after approximately similar weight and age (6 to 8 months), and the first three groups served as control groups and fed on sound rations (free from ochratoxins) The 1st one fed on non polluted ration and had no additive to act as "negative control". The 2nd and 3rd groups acted as "positive control", in which the 2nd group fed on non polluted ration + 1 capsule (250 mg thyme oil) from examined thyme oil / head /day; but the 3rd group fed on non polluted ration + 2 capsules (500 mg thyme oil) / head /day. On the other hand, the 4th group fed on AFs-polluted ration only without any additive oil, but 5th group fed on polluted ration + 1 capsule (250 mg thyme oil) from examined oil / head /day. 6th group fed on polluted ration + 2 capsules (500 mg thyme oil) /head /day. The selected dose of AFB1, the selected dose of thyme oil and duration period of AFs-exporure, were literature based (Benchaar et al., 2006b; Cardozo et al., 2006 and Castillejos et al., 2006).

3- Digestibility trial:

At the end of 3rd week of the treatment period, all animals from each group were taken to determine the digestibility and nutritive value of expermintal diets. Samples of rations, refusal, feces and urine were taken daily, for seven days. Refusals were collected daily at 07.30h, weighed, sampled, and then stored. Total daily fecal output for each animal was also collected, weighed, homogenised and 10% of feces samples were dried at 70º C for 24 hr , then blended and kept for aflatoxins analysis according to AOAC methods (1990). Different parameters of digestibility of the different six feeding treatments were measured as: dry matter intake, digestible crude protein intake, percentage of total digestible nutrients (TDN, %), total digestible nutrient intake (TDNI) and percentage digestible crude protein (DCP, %). Nutrient consumed during the digesitibility trail were calculated according to feeds consumed and their chemical composition (AOAC, 1980) as follows:

TDN % = % DCP + % DCF + % DNFE + 2, 25 x % DCEE

With DCP: digestible crude protein
DCF: digestible crude fibre
DNFE: digestible nitrogen-free energy
DCEE: digestible crude ether extract

TDNI = TDN% x DMI
With DMI: Dry Matter Intake (DM eaten)

Digestibility (D)% = (DMI – DMO/DMI) x 100%
With DMO: Dry Matter Output (DM in faeces)
DCP % = (DCP/DMI) x 100%

Table (1) Chemical composition of various ratios (% DM basis), offered to male sheep during the treatment stage of experiment (four weeks).

Item	Groups, DM composition %:					
	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Ingredient %						
Wheat (Aflatoxin free)	60	60	60	57.30	57.30	57.30
Soy meal	9	9	9	9	9	9
[a]AFs- contaminated material (wheat)	0.0	0.0	0.0	2.7	2.7	2.7
Berseem hay	30	30	30	30	30	30
[b]Minerals/vitamin mixture	1	1	1	1	1	1
[c]Thyme oil (as capsule)	0.0	1	2	0	1	2
[d]Chemical composition, %						
Crude protein (CP)	11.82	11.79	11.98	12.05	11.93	11.84
Crude fiber (CF)	27.03	27.09	26.88	27.05	27.11	27.09
Ether Extract (EE)	2.87	2.92	2.98	2.87	2.92	2.98
Nitrogen Free Extract (NFE)	58.28	58.20	58.16	58.03	58.4	58.09
Organic matter (OM)	94.94	94.98	94.97	94.96	94.96	94.95
Ash	5.06	5.02	5.03	5.04	5.04	5.05
DE K cal/kg*	2.93	2.93	2.93	2.93	2.93	2.93
Moisture	8.86	8.64	8.55	7.75	9.71	11.07

N.B. a- 2.70 % AFs- contaminated material equal to 10 mg AFs or 6.48 mg AFB_1/kg concentrated feed on dry matter bases. All ingredients except AFB_1-cont.wheat were AFB_1- free
b- Other ingridients: 1.2% limestone, .24% calcium phosphate, .38% KCl, .4% Co-I salt, .5% trace mineral mix, and 20% vitamin mix. The trace mineral mix was formulated to provide 22 mg of &SO,, .ll mg of Se, 28 mg of ZnO, 28.5 mg of MnO, 750 mg of MgO, 2.0 g of KC1, and 1.6 g of Co-I NaCl per kilogram of mixed diet. The vitamin mix was formulated to provide 7,000 IU of vitamin A, 3,000 IU of vitamin D3, and 6 IU of vitamin E per kilogram of mixed diet.
c- Each one capsule contains 250 mg thyme oil.
d- DM, OM, CP, CF, EE and NFE; are means of dry matter, organic matter, crude protein, crude fiber, ether extract, and nitrogen free extract, respectively.
*DE = 4.36-0.049×NDFDF = 28.924 + 0.657 (CF%) according to Cheeke (1987).

4- Sampling

4.1- Blood samples:

Blood samples were taken weekly from the jugular vein prior to the morning feeding, at the following times: 0, 6, 13, 20 and 27 days of treatment period. Blood samples were placed on ice, allowed to clot and after centrifugation, serum was separated and frozen at -20 °C until it was analyzed for AFB 1, AFB2α and aflatoxicol.

4.2- Sampling of rumen liquor.

Rumen fluid samples were collected from all animals groups using a rubber stomach tube at 3 hrs post feeding and handled according to Nsabimana et al., (2003). For experiment, approximately 250 ml of ruminal contents was collected from sheep by stomach tube, separated into two portions; the first was used for immediate determination of pH using digital pH-meter, while the 2nd portoin was transported to the laboratory at 39°C in anaerobic condition to be used for rumen protozoal count, identification and activity estimation. Tubes and equipments were rinsed with 5% formalin and then saline following each use to prevent artificial inoculation of viable protozoa among animals.

4.3- Sampling of feces and urine

During the 3rd wk of the experiment, total feces and urine of animals were collected twice daily over a 7-days period. Urine was collected from an indwelling cyatic catheter, which was also placed 1-day before the toxin administration. Feces were collected in fecal bags. Following the collection period, total samples of urine and feces from each animal were homogenized, and aliquot samples were stored at -20°C until further analysis.

5- Plant materials

The leaves of Thymus volgaris (5.75 kg) were was purchased from local market,, Cairo, Egypt.

6- Analytical methods

6.1- Preparation of aflatoxin(s)- artificially contaminated ingredient.

Aflatoxins (AFs) which were used in this study were produced by a culture of Aspergillus parasiticus NRRL 2999 (obtained from the Mycotoxin Lab., N.R.C., Dokki, Giza, Egypt.) on wheat which was used as a basal material (Shotwell et al., 1966). Each 500 gm quantity of wheat was potted in sealed 2.8 L-Fernbach flask with sufficient tap water to give 30% water by weight, and then autoclaved at 121 °C for 15 min. Autoclaved samples were cooled at room temperature, and inoculated with 10 ml suspension of Aspergillus parasiticus spores scraped from a 14 day-old potato dextrose agar (PDA) slant. The infected medium was incubated for 17 days at 28 °C.

Qualitative and quantitative assay has been caried out using HPLC (Agilent Technologies, Waldbronn, Germany) as recommended by AOAC (1990). The AFs within the wheat material consisted of 240 mg B1, 30 mg B2, 85 mg G1 and 15 mg G2 /kg. The wheat meal was incorporated into the basal diet in the ratio 2.7. % of the daily ration, to provide the desired level of 10 mg of total AFs or 6.48 mg AFB_1/Kg diet. The diet containing AFs was analyzed and the presence of parent AFs was confirmed by HPLC.

6.2 Extraction of thyme Oil.

Briefly, 250 g fresh plant material of the plant was put in a round bottom flask and 1000 mL distilled water was added before subjecting to hydro distillation (Bankole and Joda, 2004) for 6 hours. The oil was recovered and dried over anhydrous sodium sulphate.

6.3- Mycotoxins analysis:

All standards of Mycotoxins were purchased from sigma company, USA. All Chemicals and solvents used were of ACS grade. Thin layer TLC aluminum plates recoated with 0.25 mm silicagel 60 (Merk). Aflatoxin(s) in feed and feces samples were extracted by B.F. method as described in AOAC (1998). Extracts were dissolved in soul chloform and vortex, 20µl aliquot and 10µ of the standards were stopped on TLC plates and developed in dark room with chloroform : actone (90:10). After drying the spots were examined with U.V at A wave length of 365 nm.

Analysis of aflatoxins.

The AFB_1 and its metabolites $B_{2\alpha}$ and aflatoxicol were analyzed in the feces, urine, and serum samples according to method of Richarda and Lyona (1986).

Visualization and quantitation of aflatoxin B1, aflatoxicol and aflatoxin B2α.

AFB1, aflatoxicol and AFB2α, were located by UV exposure. The plates or the fractions from the purification of the metabolites by high-pressure liquid chromatography were placed at the filter surface of a trans illuminator with an intensity of 6,000 LW/cm2 at a wavelength of 365 nm. For the quantitative determination of the compounds, the silica plates were developed in solvent system (chloroform :actone , 90:10) and scanned in a Vitatron LTD 100 densitometer equipped with a mercury lamp (excitation at 366 nm and emission at

460 mm). The recorded areas of the spots were compared with standards of the respective compounds.

6.4- The chemical composition of the diet and feces:

The chemical analysis of feeds and feces were carried out according to AOAC (1998). Feed samples were collected on days: 0, 14, and 28; and composited. Samples of each group diet were analyzed for determination of dry matter (DM), organic matter (OM), percentage of crude protein (CP), ether extract (EE), crude fibers (CF), and nitrogen-free extract (NFE), (AOAC, 1990). Concentrates, hay, and feces were successively ground in mills with 3- and 1-mm screens. Nitrogen was determined using the standard Kjeldahl procedure with K_2SO4 and CuSO4 as catalysts. The OM was determined by ashing at 550°C overnight. The NDF (cell wall), ADF, and 72% H_2SO4 lignin were analyzed as described by Van Soest et al., (1991), except that NaSO3 was not used in the NDF preparation.

6.5- Rumen protozoal count in rumen content:

The total protozoal count was conducted according to Abou El-Naga (1967). Two 5 ml duplicate liquors of rumen content were separately taken and diluted five times by addition of 15 ml saline solution and 5ml of lugol's iodine solution. Immediately after gentle shaking, one ml liquor was taken up in a one ml wide mouthed graduated pipette. As quickly as possible, exactly 0.1 ml was pour on a dry clean slide which was then carefully covered by a dry clean cover slide with a dimensions of 23 × 51 mm (total area of 1173 mm2). Counting was carried out using the low power; 30 fields in each slide were counted and chosen as representative to the whole area. The average count in 30 fields, which represents the protozoal count per one square mm area of the field, was multiplied by 1173 (the area of the cover slide) to give the protozoal count in 0.1 ml of the diluted sample, which represents 0.02 ml of original sample. Therefore, the total protozoal count /1 ml rumen content = average count in 30 field ×1173×50. Each of the two diluted duplicate was counted and average was calculated.

6.6- Evaluation of protozoal activity and motility:

Survival rate was evaluated according to Nsabimana et al., (2003). The survival rate was estimated by counting the proportion of motile ciliate under a microscope. Motility of ciliates was examined and counts were repeated 5 times per sample and the mean was calculated to be considered as individual reading.

6.7- Determination of ruminal pH:

The pH values of the collected rumen juice samples were estimated by means of an electric pH–meter (Wissens Chaftlish tehcnisch werkstatten D 8/20 weitheim Ph 40) according to Nassar (1971).

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6.8- Determination of several metabolic variables:

Aspartate transaminase (AST), alkaline phosphatase (ALP) and gamma glutamate transferase (γGT) activities; and the concentrations of albumin, uric acid and glucose in serum, were measured colourmetrically using spectrophotometer (Instruction Manual UV-1201, Shimadzu) and commercial kits. AST, ALP and γGT kits were purchased from (Stanbio Laboratory, North Main, Boerne, TX USA). Albumin, uric acid and glucose kits were purchased from Randox Laboratories Co., UK.

7- Statistical analysis:

The differences in feed intake, average daily gain, nutritive values and apparent digestibility coeffecients, were examined using F- Test through the analysis of variance (ANOVA) according to Snedecor and Cochran, (1982). The differences among periods were tested using Duncan' s multiple range test (Duncan, 1955). All data were represented by means ± standard error (SE). All differences were considered statistically significant at ($P < 0.05$).

3. Results and Discussion

1- Animal performance

Sheep data for daily feed intake, daily gain, and feed efficiency are presented in Table (2). When examined during the entire 28-d feeding period, sheep fed aflatoxins-contaminated rations with or without thyme oil had significantly lower ($P< 0.05$) daily feed intake, TDN, DCP and body weight gain, the greatest decrease was seen in sheep fed AFs-contaminated diet only. However, Thyme oil treatment significantly ($P< 0.05$) imoproved the recorded numbers of those parameters especially at high dose of thyme oil. The average daily body weight gain values were 0.103, 0.113, 0.132, 0.039, 0.076 and 0.081 kg for groups, 1, 2, 3, 4, 5 and 6, respectively. Also, DM intake increased significantly ($P< 0.05$) by thyme oil treatment, especially at high dose treatment. In this respect, The use of thyme oil, as feed supplement to sheep diet in this study, had no adverse effect on DM intake or average daily gain where aflatoxins may disrupt growth and function of ruminal microorganisms and together with changes in volatile fetty acids (VFA) production may be responsible, in part, for the decreased growth and performance seen in ruminants fed aflatoxins Mertens (1979). On the other hand, supplemented oil

to growing heifers fed 10: 90 forage to concentrate diet had a trend to increase DM intake (Cardozo *et al.,* 2006).

Feed conversion expressed as kg DM /kg gain indicated that addition of thyme oil to sheep diets improved DM conversion by 11.96, 25.08, -59.97, -24.58 and -22.26% for groups, 2, 3, 5 and 6; respectively ; over the negative control group. The same trend was observed with TDN and DCP conversion, however group 3 tended to get better feed conversion with DM, TDN and DCP conversion. There is no a clear reason for thes observations, But we suggest that thyme oil may increased rumen microbial fermentation in the direction which total VFA and ammonia-N concentrations increased. The results of Molero, *et al.,* (2004) may support this hypothesis. Our results were not in agreement with those obtained in vitro by Castillejos *et al.*, (2006), who found that thymol at higher doses (500 mg/L) decreased total VFA and ammonia-N concentrations, and increased the acetate to propionate ratio. Differences in those results than our results may attributed to other factors (Oil ingredients and dosing level, Composition of the diet and /or others). Several in vitro studies suggested that the effects of thymol are diet and pH dependent (Castillejos *et al.,* 2006; Cardozo *et al.,* 2005).

Decreased growth rate is considered the most common effect of chronic aflatoxicosis in young livestock. Additional signs of poisoning include loss of appetite, decreased feed efficiency, and immunosuppression (Raisbeck *et al.,* 1991; Pier, 1992). In this study, feeding AFs markedly decreased feed intake and daily gain remained lower in sheep fed AFs, particularly in sheep fed dietary aflatoxins alone. The exact mechanism by which AFs impairs growth is unknown, but it is probably multifactorial, involving disturbances in carbohydrate, lipid, and protein metabolism, nutrient interactions, and disturbances in hormonal metabolism (Raisbeck *et al.,* 1991). Additionally, poor appetite and reduced feed intake, common in chronic aflatoxicosis, may account (at least partially) for reduced performance.

Table 2: The efficacy of thyme oil and / or aflatoxin(s)-contaminated diet on sheep performance (Nutrients intake, nutritive values, body weight gain and feed conversion) during the study.

Parameters	Expermental groups (Means ±SE)						LSD (P<0.05)
	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	
DailyDM intake, gm	1715 ± 23.2^{b}	1685 ± 31.5^{b}	1755 ± 18.6^{c}	1630± 26.5^{a}	1690 ±34.1^{b}	1740 ± 22.6^{c}	36.5
Body weight							
Initial B.W.	39.17±0.48^{a}	38.67 ±0.69^{a}	38.52 ±1.12^{a}	37.88±1.23^{a}	38.45 ±0.62^{a}	39.45 ±0.93^{a}	1.64
Final B.W.	42.06 ±0.86^{b}	41.85±0.89^{b}	42.22±0.63^{b}	38.98±0.92^{a}	40.60 ±1.23^{b}	41.73 ±2.03^{b}	1.51
Total B.W. gain	2.89 ±0.23^{c}	3.18 ±0.34^{c}	3.70±0.19^{c}	1.10±0.09^{a}	2.15 ±0.19^{b}	2.28 ±0.66^{b}	0.43
B.W. gain (gm/day)	103.21 ±2.13^{b}	113.57 ±3.60^{b}	132.14±1.65^{c}	39.28±1.14^{a}	76.78 ±3.11^{b}	81.43 ±2.25^{b}	18.40
Nutritive values:							
TDN intake (gm/day)	958.17 ±16.7^{d}	966.63±28.40^{d}	1063.42±14.8^{c}	641.57±22.5^{a}	823.87 ±19.48^{b}	899.76±26.1^{c}	23.15
DCP intake (gm/day)	203.06 ±11.5^{d}	200.34±16.9^{c}	213.23±14.^{d}	131.05±25.5^{a}	178.29 ±13.45^{b}	191.05 ±16.8^{c}	12.59
TDN%	55.87	56.18		39.36	48.75	48.90	-
DCP%	11.84	11.89	56.89 12.15	8.04	10.55	10.98	-
% Feed conversion ,as							
B.W. gain / DMI, kg	6.02±0.13^{b}	6.74±0.09^{b}	7.53±0.17^{c}	2.41±0.14^{a}	4.54±0.12^{d}	4.68±0.10^{d}	0.83
TDN/ DMI, kg	55.87±4.8^{c}	57.37±7.8^{c}	60.59±6.5^{d}	39.36±8.5^{a}	48.75±5.9^{b}	51.71±8.3^{b}	3.0
DCP/ DMI, kg	11.84±0.11^{c}	11.89±0.15^{c}	12.15±0.4^{c}	8.04±0.6^{a}	10.55±0.16^{b}	10.98±0.4^{b}	1.1

a,b,c,d Means in the same row having different superscripts are significantly different at (p<0.05)

2- The efficacy of thyme oil and / or aflatoxin(s)-contaminated diet on some rumen liquor parameters:

Results of pH values, protozoal count, motility and activity of rumen protozoa are presented in Tables (3, 4, and 5). Aflatoxin(s)-treated groups recorded higher pH values, decreased protozoal count and reduced protozoal motility and activity at three hours post feeding; compared with the control groups. Adding thyme oil, improved the negative effects of aflatoxin treatment, and these effects of oil increased as their level increased (Tables, 3,4 and 5). These results are in agreement with those found by Mahmoud, (1994) and Evans and Martin (2000). These differences in pH values may be related to fermentation process of both non-structural and structural carbohydrates and production of volatile fatty acids (VFAs) as which affected the pH to same limit until they were proportionally and relatively absorbed from the rumen wall. This assumption is in agreement with the conclusion of Reddy and Reddy (1985), who stated that the pH values were inversely related to VFAs.

Table (3) Mean pH values after three hours post feeding during the study.

groups	Stages of experiment			
	Two weeks before treatment	Four week treatment stage		
		Zero time	After two weeks	After four weeks
Group 1	$6.08\pm.001^{Aa}$	$5.41\pm.012^{Aa}$	$5.99\pm.003^{Ba}$	$5.68\pm.009^{Aa}$
Group 2	$6.30\pm .006^{Ab}$	$5.85\pm.017^{Ab}$	$5.12\pm .028^{Aa}$	$5.23\pm.035^{Aa}$
Group 3	$6.38\pm.053^{Ab}$	$6.02\pm.007^{Ab}$	$5.35\pm.016^{Aa}$	$5.18\pm.017^{Aa}$
Group 4	$5.82\pm .003^{Aa}$	$5.46\pm.008^{Aa}$	$6.56\pm .031^{Bb}$	$6.68\pm.012^{Bb}$
Group 5	$5.96\pm .008^{Ab}$	$5.90\pm.016^{Ab}$	$5.20\pm .016^{Aa}$	$5.15\pm.015^{Aa}$
Group 6	$5.70\pm .006^{Ab}$	$5.88\pm.043^{Ab}$	$5.07\pm .004^{Aa}$	$5.13\pm.026^{Aa}$
LSD	0.61			

N.B: Different capital letters in columns between means denote significant difference between treatment in the same period at ($p<0.05$) and vice versa. But means in the same row having different small superscripts denote significant change between periods in the same treatment and vice versa.

The results of protozoal count, motility and activity showed that animals fed aflatoxin(s)-contaminated diets with or without natural oil, were also affected with supplementation compared with the control groups (groups: 1, 2 and 3).

Our results were contrast with those obtained by Busquet *et al.,* (2005a), who reported that in vitro, the main thyme oil componants, Carvacrol and thymol at higher doses (300 mg/L); increased pH values and butyrate proportion, and decreased acetate and propionate proportions, and total VFA concentration. Benchaar *et al.,* (2006b and 2007) reported that feeding essential oils to dairy cattle increased ruminal pH and ADF digestion, but had no effects on protozoal counts or animal performance. Benchaar *et al.,* (2006a) also reported no effects of the addition of CRINA (A blend of essential oils containing thymol, eugenol, vanillin and limonene, among other compounds; patent EP 0646321 B1; Rossi, 1999) on DM intake and growth rate of beef cattle. The differences in results may attributed to differences in the experimental conditions (Feeding and animal type, treatment period, the tested material, etc).

Ruminal motility may be affected by single acutely toxic doses of AFs (Cook *et al.,* 1986); however, effects of lower, more environmentally prevalent concentrations have not been determined. Such effects on ruminal motility would potentially contribute to the decreased gain seen in chronic intoxication.

3- Some biochemical analysis of sheep serum (liver and kidney function tests) as affected by dietary aflatoxin and / or thyme oil treatment.

Metabolic indicators did not show a consistent pattern as influenced by the mycotoxin or the two levels of thyme oil. Data for selected serum constituents are presented, from the beginning and the end of the treatment period, in Table (6). In the present study, serum activities of AST and γGT were elevated in sheep fed AFs-contaminated diets. Serum activities of aspartate amino transferase (AST) of animals fed dietary AFs only, had elevated ($p \leq 0.05$) compared with those fed sound rations with or without thyme oil. Similarly, γGT activity was higher ($p \leq 0.05$) at the end of the treatment period for group which fed AFs-contaminated diet only (group 4).

However, no significant differences were noted for groups fed AFs-contaminated diets plus thyme oil either at low or high levels (groups, 5 and 6).
Serum enzyme activities of AST, γGT, and ALP are generally elevated in aflatoxicosis and indicate hepatocyte damage. It is of interest to mention that in animals fed diets contaminated with toxicants, the serum levels of these enzymes increased after liver damage because of increased membrane permeability or because of liver cell necrosis and cytosol leakage into the serum (Abdel-Wahhab, *et al.,* 2002 ; Saad and Abdel-Fattah, 2008 and Ozer *et al.,* 2008).

Table (4) Mean protozoal count ± SD (cell / mL rumen liquer) after three hours post feeding dietary aflatoxins and / or thyme oil during the study.

groups	Stages of experiment			
	Two weeks before treatment	Four week treatment stage		
		Zero Zero time	After two weeks	After four weeks
Group 1	328225± 430^{Aa}	356125 ±.553^{Aa}	352800±287^{Aa}	339675±603^{Ba}
Group 2	316225± 908^{Aa}	328125 ±629^{Aa}	342025± 153^{Ab}	313475±484^{Bb}
Group 3	303450±759^{Aa}	331225±415 ^{Ac}	365050±560^{Aa}	381800±512^{Cb}
Group 4	313225± 503^{Ac}	356350.±758^{Ac}	263925± 830 ^{Bb}	215050±314^{Aa}
Group 5	321225± 712 ^{Ab}	351900 ±.189^{Ab}	244375± 129^{Ba}	234600±940^{Aa}
Group 6	297725± 558^{Ab}	305235±348^{Ab}	297355±550^{Ca}	271450±940^{Ca}
LSD	28715			

N.B: Different capital letters in columns between means denote significant difference between treatment in the same period at ($p<0.05$) and vice versa. But means in the same row having different small superscripts denote significant change between periods in the same treatment and vice versa.

Table (5) : Evaluation of protozoal activity and motility.

groups	Stages of experiment			
	Two weeks before treatment	Four week treatment stage		
		Zero time	After two weeks	After four weeks
Group 1	+ + + +	+ + + +	+ + + +	+ + + +
Group 2	+ + + +	+ + + +	+ + + +	+ + +
Group 3	+ + + +	+ + + +	+ + + +	+ + + +
Group 4	+ + + +	+ + + +	+ +	+ +
Group 5	+ + + +	+ + + +	+ + +	+ + +
Group 6	+ + + +	+ + + +	+ + +	+ + +

The semi-quantitative determination of both motility and activity of rumen protozoa classified the obtained results into four degrees with certain symbols. The 1st (+), 2nd (++), 3rd (+++) and 4th (++++) degrees refer to weak, moderate, good and extremely of protozoal motility and activity, respectively.

The effects of AFs on liver function can vary with the amount and duration of intoxication. In dairy sheep, the ingestion of pure AFB1 did not alter liver enzymatic activity when the daily intake ranged between and 128 µg/d for an exposure period of 1 wk (Battacone *et al.,* 2005). In contrast, when sheep

were fed 128 µg/d of AFB1 for a longer period of intoxication (2 wk), glutamic pyruvic transaminase (GPT) activity increased significantly (Battacone *et al.,* 2003). In lambs fed 2.5 mg of AF/kg of diet for 35 to 67 d, serum contents of AST and γGT increased, indicating a transient alteration of liver enzymatic activities (Edrington *et al.,* 1994). Our results were similar with those observed by Harvey *et al.,* (1991), who reported that a dose of 2.6 mg of AF/kg of diet significantly increased serum enzyme activities as indicative of liver damage. Similar findings have been reported in sheep (Harvey *et al.,* 1991), goats (Clark *et al.,* 1984), cattle (Helferich *et al.,* 1986), pigs (Lindemann *et al.,* 1993), and in rats (Abdel-Wahhab, *et al.,* 2002; Saad and Abdel-Fattah, 2008).

Alkaline phosphatase (ALP) activity exhibited a different pattern of change. The enzyme activity increased significantly ($p \leq 0.05$) with AF exposure, this suggests that the level of AFs used in this study caused hepatocyte damage and such increase was reduced significantly ($p \leq 0.05$) with thyme oil treatment (Table 3). Finding of the present study could be attributed to previous reports (Schell *et al.,* 1993a; Harvey *et al.,* 1994; Rastogi *et al.,* 2001; and Karakilcik *et al.,* 2004).

Serum albumin concentration was decreased by feeding AFs-contaminated diet. However, the uric acid concentration was higher. Lower serum albumin concentration support hypothesis that dietary AFs impaired protein synthesis. In this respect, Harvey *et al.,* (1988) and Lindemann *et al.,* (1993) reported lower serum albumin, total protein, and urea nitrogen concentrations in swine, suggesting that AFB1 impaired protein synthesis.

Data represented in Table (3), also indicate that Dietary AFs increased the levels of serum uric acid. As mentioned by (Farombi *et al.,* 2005), the levels of serum uric acid may not change significantly until 50% of renal function is impared. The increased levels of uric acid in our study may us could suggest that the level of AFB_1 used in this study may have impaired normal protein synthesis and/or kidney dysfunction and consistent with those reported of aflatoxicosis (Abdel-Wahhab *et al.,* 2002; Abdel-Wahhab and Aly 2003; and Farombi *et al.,* 2005), and thyme oil could protect sheep from the adverse effects of aflatoxins.

Serum glucose concentrations were decreased by dietary AFs, lower than normal levels in all groups (Table, 3) and thyme oil treatment improved the negative effects of aflatoxin treatment. Decreased serum glucose concentration probably reflected a lower nutritional state in sheep fed AFs that was probably related to lowered feed intake (Table, 2). Similar results for glucose were observed by many researchers who have reported that glucose and its metabolite concentrations were affected by aflatoxicosis (Edrington *et al.,* 1994 and Rastogi *et al.,* 2001).

Our results may lead us to suggest that there is a significant liver dysfunction in these groups, 10 mg of AFs/kg of diet was sufficient to impair performance and cause liver damage in male sheep, but thyme oil could protect sheep from hepato-toxicities which induced by AFs-contaminated diet when used as a sole diet for sheep.

Items / groups	[a]GGT (U/L)		AST (U/L)		Albumin (gm/dL)		AL-P (U/L)		Glucose, mg/dL		Uric acid (U/L)	
	Day (0)	Day (27)	Day (0)	Day (27)	Day (0)	Day (27)	Day (0)	Day (27)	Day (0)	Day (27)	Day (0)	Day (27)
Group 1	62 ± 3.03^{A}	69± 4.1^{A}	54± 3.7^{A}	61± 6.1^{A}	2.87± 0.70^{A}	2.91± 0.08^{C}	605± 43.5^{A}	620± 35.5^{A}	115± 9.2^{A}	109± 8.4^{C}	0.48± 0.03^{A}	
Group 2	54 ± 3.0^{A}	48 ± 1.08^{A}	57± 2.8^{A}	65± 4.5^{A}	2.63± 0.70^{A}	2.70± 0.42^{C}	690± 39.7^{A}	723± 27.1^{B}	100± 7.5^{A}	103± 3.5^{B}	0.41± 0.03^{A}	0.55± 0.03^{A}
Group 3	68± 5.1^{A}	71± 2.7^{A}	51± 7.1^{A}	48± 5.2^{A}	2.91± 0.50^{A}	2.85± 0.53^{C}	701± 42.0^{B}	705± 22.3^{B}	98± 4.6^{A}	101± 7.4^{B}	0.46± 0.02^{A}	0.51± 0.01^{A}
Group 4	69± 2.09^{A}	158± 13.7^{C}	67± 4.4^{A}	139± 12.5^{C}	2.77± 0.09^{A}	1.49± 0.82^{A}	568± 36.5^{A}	985± 55.4^{C}	108± 4.3^{A}	69± 6.1^{A}	0.39± 0.01^{A}	0.46± 0.02^{A}
Group 5	63± 4.2^{A}	112± 17.5^{B}	58± 3.2^{A}	87± 10.5^{B}	2.67± 0.13^{A}	1.78± 0.14^{A}	680± 29.4^{B}	690± 13.5^{B}	113± 10.1^{A}	89± 5.8^{B}	0.43± 0.03^{A}	1.54± 0.05^{C}
Group 6	74± 12.9^{A}	97± 8.2^{B}	60± 6.4^{A}	89± 7.5^{B}	2.38± 0.19^{A}	1.96± 0.45^{B}	653± 47.5^{B}	587± 28.9^{A}	105± 3.9^{A}	89± 4.1^{B}	0.52± 0.01^{A}	1.05± 0.07^{B} 0.89± 0.03^{B}
LSD p≤0.05	21.03	25.3	15.5	19.8	0.61	0.53	60	35.5	17.5	15.7	0.14	0.17

N.B.: 1- The same capital litters in columns denotes no significant difference between treatments in the same period at ($p \leq 0.05$) and vise versa.
2- a GGT = gamma-glutamyl transferase; AST = aspartate aminotransferase; and AL-P = alkaline phosphatase.
Day (0): Beginning of the experiment Day (28): End of the experiment

4- Proportional urinary and fecal excretion of aflatoxib B_1, and its corresponding metabolites ($AFB_{2\alpha}$, aflatoxicol), for sheep fed AFs-contaminated diets.

The proportional excretions of AFB_1 and its metabolites ($AFB_{2\alpha}$ and aflatoxicol) via feces and urine are shown in Table (7). The concentrations of AFB1 and $AFB_{2\alpha}$ in feces and urine varied according to dietary treatment. The cumulative excretion of $AFB_{2\alpha}$ is expressed in terms of AFB_1 equivalents. Data represented in Table (7) indicate that in groups treated with dietary aflatoxin plus thyme oil, most of the excreted AFB_1 was found as the metabolite $AFB_{2\alpha}$ in the urine (59.04 to 62.88%), whereas approximately 5.71 to 8.82% was found as $AFB_{2\alpha}$ in feces. Relatively small amounts of intact AFB_1 were excreted in the urine (5.44 to 6.00% of AFs intake) and feces (0.82 to 1.52% of AFs intake). In contrast, fecal and urinary excretion of $AFB_{2\alpha}$ in the group fed dietary aflatoxin only was very low (2.51 and 8.82%, respectively). No aflatoxicol amount was detected neither in feces nor urine samples tested. These results indicate that the major excretory route was found to be the urine (accounting for 39.71% to 68.32% of the tatal AFs-excretion forms, whereas less than 10% of these forms were excreted in the feces. Only about 52% of the dose was recovered in the feces and urine in identifiable forms, mainly $AFB_{2\alpha}$ and unchanged AFB_1 without any detectable amount of aflatoxicol. In this respect, our results were in contrast with those observed by Richarda and Lyona (1986) in pigs.

Thyme oil-treatment significantly reduced fecal excretion of $AFB_{2\alpha}$, this might be explained by a more pronounced renal elimination, which in turn might result in lower biliary secretion of $AFB_{2\alpha}$ in these groups. The highly percentages of $AFB_{2\alpha}$ in blood serum for groups fed dietary aflatoxin plus thyme oil, may explained these results (Table, 6). Our reslts were in the same trend with those observed by (Bennett *et al.,* 1981), who found that the lower toxicities of AFB_1 and $AFB_{2\alpha}$ in mammals are mainly as a result of a faster rate of clearance via urine and feces compared with that of AFB_1. The half-life of aflatoxins in the blood is directly related, in part, to the ability of plasma proteins to bind aflatoxins. Our results indicated that AFB1 metabolites are cleared at a much faster rate than AFB1. Hence, the rate of AFB1 biotransformation represents the main mechanism through which detoxification occurs. Therefore, processes that enhance the conversion of AFB_1 to AFB2α will tend to reduce the general toxicity of AFB_1 in the animal itself or the toxic potential of AF-contaminated feed (e.g., when dietary aflatoxin is administered to ruminants rather than to non ruminants).

Table (7) Proportional urinary (ng/mL) and fecal excretions (ng/gm) of aflatoxin B_1, and its corresponding metabolites ($AFB_{2\alpha}$, aflatoxicol), of sheep fed aflatoxin(s)-contaminated diets.

Item		Groups												LSD (p≤0.05)
		Group 1		Group 2		Group 3		Group 4		Group 5		Group 6		
		M+SD	%	M+SD	%	M+SD	%	M+SD	%	M+SD	%	M+SD	%	
Feces	AFB1	0 ± 0^{A}	0.0	0 ± 0^{A}	0.0	0 ± 0^{A}	0.0	885 ± 23.1^{C}	0.60	1290 ± 24.8^{D}	.82	208 ± 13.6^{B}	1. 52	24.5
	AFB2α	0 ± 0^{A}	0.0	0 ± 0^{A}	0.0	0 ± 0^{A}	0.0	1005 ± 17.5^{B}	8.82	3120 ± 13.5^{C}	5..92	3395 ± 25.8^{D}	5.71	37.4
	Aflatoxicol	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-
Urine	AFB1	0 ± 0^{A}	0.0	0 ± 0^{A}	0.0	0 ± 0^{A}	0.0	1615 ± 28.3^{C}	37.20	80 ± 4.1^{B}	5.44	76 ± 4.7^{B}	6.00	18.5
	AFB2α	0 ± 0^{A}	0.0	0 ± 0^{A}	0.0	0 ± 0^{A}	0.0	23582 ± 36.5^{D}	2 ..51	1365 ± 19.5^{C}	62.88	1297 ± 24.5^{B}	59.04	49.5
	Aflatoxicol	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-
Total recovery, %			0.0		0.0		0.0		49.13		75.06		72.27	-

N.B: 1- The same capital litters in columns denotes no significant difference between treatments in the same raw at ($p \leq 0.05$) and vice versa.

2- Excretion of $AFB_{2\alpha}$ expressed in terms of AFB_1 equivalents as calculated from the molecular weight of AFB_1 /molecular weight of $AFB_{2\alpha}$ (412/430) x mg of $AFB_{2\alpha}$.

4. Conclusions

In conclusion, Aflatoxins contaminated rations induced significant decrease in daily feed intake, TDN, DCP and body weight gain. Additionally, serum constituents and ruminal measurements indicated impaired liver function and digestive disturbances in sheep fed aflatoxin. An addition of thyme oil at doses between 250 and 500 mg/head/day, for exactlly 28 days, were able to modify rumen fermentation by changing protozoal activity and motility and could approximately normalized the adverse effects of aflatoxin, perhaps attributed to its effect on ruminal pH and improving digestibility and animal performance. The exact mode of antitoxic action may need further clarification and there is an urgent need to conduct further in vivo studies with thyme oil, which may

provide a useful tool to improve efficiency of nutrient utilization in the rumen and could be recommended to sheep diet with possibility of aflatoxin contamination

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