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THE PROTECTIVE ROLE OF NATURAL COUMARINS DERIVATIVES AND ANPRO SUPPLEMENT AGAINST AFLATOXIN B1 POLLUTION IN THE QUAILS COTURNIX JAPONICA DIET

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ABSTRACT

This study aimed to investigate the potential of Coumarin C1 and C2 derivatives from apple seeds and Anpro supplementation to neutralize aflatoxin B1 in the quail diet., 3weeks -old quails were distributed into five-treatment each has 10 birds randomly. First treatment, negative control, in which the quails received not contaminated diet. Second treatment positive control the birds were fed on diet contaminated with AFB1 (0.5 mg/kg). Third treatment the diet was contaminated with AFB1 (0.5 mg/kg). And treated with Anpro at 1 g/kg. Fourth treatment diets were contaminated with AFB1 and treated with C1 at 250 mg/kg, fifth treatment diets were contaminated with aflatoxin B1 and treated with C2 at 250 mg/kg. Result showed contamination diet with AFB1 caused reduction in PVC, RBC, Hb, blood glucose, total blood protein, and SOD and causes significant elevations in WBC and uric acid therefore biochemical variables can be boosted by adding Anpro, C1 and C2 to the diet contaminated with AFB1. For that, Anpro, C1, and C2 can serve as beneficial dietary supplements to the quail in order to counteract AFB1's detrimental effects.

INTRODUCTION

In terms of staple foods, after rice and wheat, maize kernels are the most prevalent cereal crop on earth (Gwirtz and Garcia ,2014). On the other hand, this crop is extremely susceptible to Aspergillus flavus infection, which can result in aflatoxin contamination. Aflatoxins have the potential to cause severe health-harmful effects in humans and animals as well as significant financial losses for farmers, making them a global issue (Amirahmadi *et al.*, 2018, Mongalo, *et al.*, 2018 and Sklan ,2003). Due to its capacity to weaken the immune system and causing cancer in many investigated species of animals, including poultry, aflatoxin B1 (AFB1) has been broadly discussed (Shlig,2009)). Additionally, it makes up the majority (75%) of all mycotoxins detected in tainted food products, making it the most prominent and hazardous of all naturally occurring aflatoxins (Manafi *et al.*, 2018). The animal-related toxicity of AFB1 can be manifested as variations in blood biochemical and hematological parameter concentrations (Raju *et al.*, 2000).

The negative environmental and health consequences of most fungicides and preservatives utilized in crop agriculture have promoted humanity to seek natural

alternatives (Abbas *et al.*, 2017). In this regard, natural alternatives to synthetic agrochemicals for controlling pathogenic microorganisms have been proposed, including phytochemicals and bentonites (Zhu and Jiang, 2018). Multipurpose secondary metabolites called coumarin-based products are created through a shikimic acid cascade (Nejres *et al.*, 2020). The diverse variety of biochemical potentials, including antihistamine, anticancer, anticoagulant, anti-inflammatory, antidiabetic, antibacterial, antiviral, and antioxidant characteristics, were established by these compounds' notable substrate specificity (Kovač *et al*., 2017, Mustafa 2021, Mustafa et al., 2021(a) Mustafa *et al.*, 2021 c). Additionally, it has been found that several coumarins, naturally and synthetically, exhibit antifungal properties against various infectious fungi in general and Aspergillus flavus in particular (Pattar *et al.*, 2020). Anpro, the mycotoxin adhesive used in this investigation, is a unique mineral blend of diatomaceous earth (DAE) and sepiolite. Toxin adhesive and anti-caking agent, DAE, was formerly used in chicken feed to decrease the frequency and severity of challenges associated with heterogeneous mycotoxins in the livestock industry (Di Gregorio *et al.*, 2014). On the other hand, sepiolite performs effectively in the adsorption of harmful aflatoxins in animal nutrition (Mustafa *et al.*, 2021 b).

The current study was conducted to assess the effectiveness of these compounds to attenuate the damaging consequences of AFB1 found in the quail diet by examining various hematological and biochemical variables. This was done in consideration of the advantageous properties of natural coumarin-based products extracted from the seeds of Delicious golden apples and also of Anpro.

MATERIALS AND METHODS

1-Sampling of corn kernels

There was a total of 18 specimens collected from three distinct silos in Iraq, including those of Al-Hawija (S1), Tikrit (S2), and Al-Hamdanya (S3), during the course of two months in March and April 2021. Six specimens were harvested from each location at a rate of 5 kg each, stored in sterile plastic containers that were adequately sealed, and preserved at 5°C. The specimens were all taken at random, with a handy tagging code being given for each one.

2-Identification of A. flavus by PCR (polymerase chain reaction)

Three fungal isolates were documented and validated based on the specific fungal PCR primer pairs for A. flavus, including FW (5'AACCTCCCACCCGTGTTTA 3') and RV (55' GGAAAAAGATTGATTTGCG 3'). The PCR experiments involved five sequential steps and were programmed to operate under specific conditions. These included preliminary denaturation for 6 minutes at 95°C, subsequent denaturation for 45 seconds at 95°C, annealing for 1 minute at 54°C, extension for 1 minute at 72°C, and final extension for 5 minutes at 72°C.

3-Determination of AfB1 level in polluted maize samples

The Elabscience Biotechnology Inc USA, ELISA kit., was used to quantify the AfB1 content in the polluted corn, and the specimen was prepared in accordance to the package instructions. In this regard, the maximum visible wavelengths of the prepared specimens were specified at 450 nm using an ELISA reader manufactured by Biotek Company.

4-Abstraction of the natural coumarin-based products

The natural coumarin-based products, namely C1 and C2, were extracted from the seeds of Delicious golden apples according previous work by same authors, which included extraction, phytochemical inspection, isolation, identification, and characterization (Majoumouo et al., 2019; Montero-Calderon et al., 2019; Mahrose *et al., 2021*; Mustafa and Abdulaziz ,2021andAhmed *et al.*, 2022).

5-Preparation of the Anpro bio-agent

The mycotoxin adhesive utilized in this study was prepared in a concentration of 1 g/kg from a standard Anpro stock from Anpario PLC, UK .

6-Animals under study

Quails weighing 150–200 g were evacuated from the animal house at the University of Mosul, College of Agriculture, Department of Animal Production, where they had been provided with the appropriate air circulation, sunlight, feeding, and ambient temperature. The quails (50 birds) aged 21 days were employed in the experiment, distributed into 5 DT groups each 10 birds randomly. They were offered diets with the following ingredients: maize (54.5%), soybeans (37.5%), protein (5%), vegetable fat (2%), limestone (0.7%), and salt (0.3%) (Mustafa *et al.*, 2021 d). Concerning these DT groups, the quails of DT1 received uncontaminated feed as a negative control group. The birds in the DT2 positive control group were fed a diet contaminated with AFB1 (0.5 mg/kg). For DT3, the diet was contaminated with AFB1 and medicated with 1 g/kg of Anpro. DT4 and DT5 diets were polluted with AFB1 and medicated with C1 and C2 at 250 mg/kg concentrations, respectively.

7-Assessment of the hematological markers

7-1 Enumerating the RBC

To estimate RBC, the blood specimen was concentrationally lowered with a Natt and Herricks solution, achieving a 1:200 ratio. A drop of the dilute was inserted in the scoring compartment developed for this function after completely blending the blood with the attenuating solution. The cells were given two minutes to calm down after removing the transparent cover and quantifying the count of RBC in the hemocytometer's central square at (40X) amplification by applying the following mathematical formula (Olupot-Olupot *et al.*, 2018): RBC count (cell/mm3) = n×10000.

7-2 Scoring the WBC

The same methodology employed to estimate the RBC enumerate was followed to computed the WBC score, except applying the following calculating law (Olupot-Olupot et al., 2018): WBC score= (n+10%) ×200.

7-3Quantifying the Hb concentration

By dipping a drop of blood into a calibrated tube containing 0.1N hydrochloric acid, Sahli's methodology was applied to quantify Hb concentration. In which, the blood was converted into acid haematin, which was then mixed with distilled water until it suited the hue of the colored comparative glass. The Hb was determined in g/dl by

simply taking a reading score from the scale on Sahli's tube based on the final solution's altitude (Bull et al.,2003).

7-4Assessing the PCV%

The percentage of this blood marker was calculated via a Hematocrit reader in a related capillary tube after centrifuging the blood at 500rpm for 5 minutes (Ramasarma, *et al.,* 2015).

7-5Assessment of the biochemical markers

Specifying the blood glucose concentration

The blood glucose concentration was measured as a value using a glucometer from Accu-Chek, Germany. The familial technique entails placing a blood drop into the green center of the device's test strip, which directly displays the glucose measurement in mg/dl.

7-6 Determining blood uric acid concentration

With the use of a standard kit supplied by SPINREACT, Spain, the blood level of uric acid was measured. The blend of 20 µl of blood and 1 ml of a pH 7.4 buffered solution was added to a tiny tube along with the uricase enzyme solution. For 5 minutes, the tube was placed in the oscillator, which was set to 37°C. The maximum light absorption at 510 nm was measured using a UV/Vis spectrophotometer, and the concentration of this marker was computed through the incoming formula (Manafi *et al.,* 2014): Blood uric acid concentration (mg/dl) = (absorbance of the sample/absorbance of the standard solution) ×6(standard solution concentration) .

7-7 Marking the total blood protein concentration

This concentration was marked using a standard kit provided by Biolabo, Frenc and the Biuret methodology. Through which, three test tubes each with 1 ml of working solution were functionalized with 0.02 ml of the standard solution as a positive control, 0.02 ml of blood plasma, and 0.02 ml of distilled water as a negative control, respectively. After the incubation period of 15 minutes at room temperature, a spectrophotometer calibrated at a wavelength of 570 nm was used to afford the values that can be applied in the incoming formula (Manafi *et al., 2014*): Total protein concentration (g/dl) = (absorbance of the sample/absorbance of the standard solution) ×6(standard solution concentration) .

7-7 Determination of SOD activity

The enzymatic activity of SOD was estimated according to Elijah Marklund and Marklund (Chang *et al.,* 2020).

8-Statistical analysis

The purely random design system (RCD) in SAS, 2012, was utilized to quantitatively investigate the data, with the Duncan multiple-range test being employed to establish the significance of the distinctions between the averages of the variables influencing the researched characteristics at the probability level of 0.05.

RESULTS AND DISCUSSION

1-Molecular characterization of *A. flavors*

The presence of *A. flavus* was verified by establishing a 550-base pair (bp) DNA band in the S1, S2, and S3 specimens after molecular analysis using PCR amplification and agarose gel-electrophoresis, as shown in Figure 1.

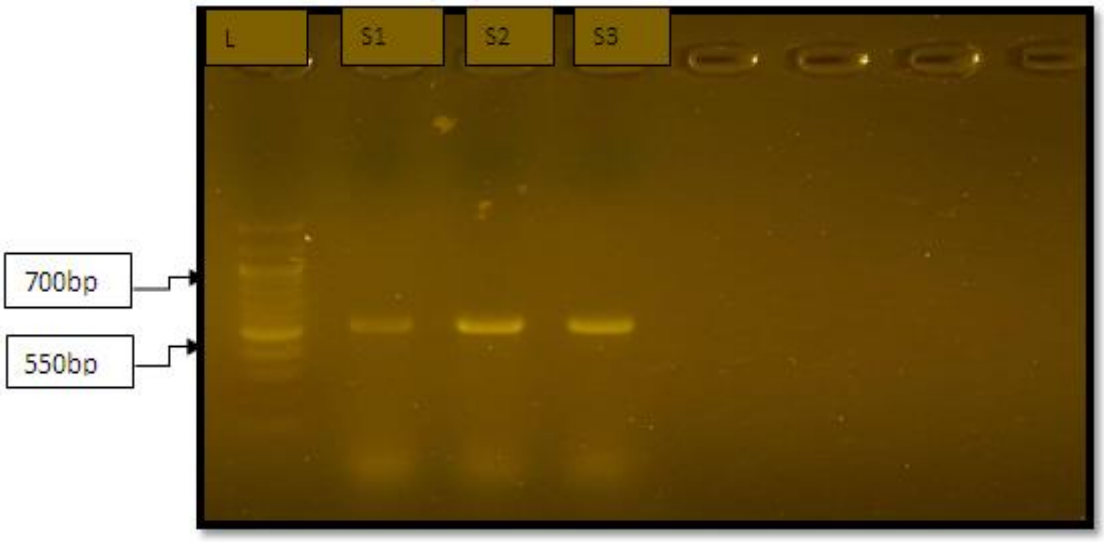

Figure 1: PCR product for the ITS specific region of *A. flavus* and 550 bp reaction yield in 1.5% lactation gel. L is the DNA ladder of the investigated fungus

2-The AFB1 concentration in the gathered corn samples

The outcomes illustrated in Figure 2 indicated that the AFB1 concentrations in maize samples from S1, S2, and S3 silos were 195, 282, and 310 PPb (parts per billion, dilution factor is 5), respectively. The greatest concentration of this polluting agent was explored in the silo of S2.

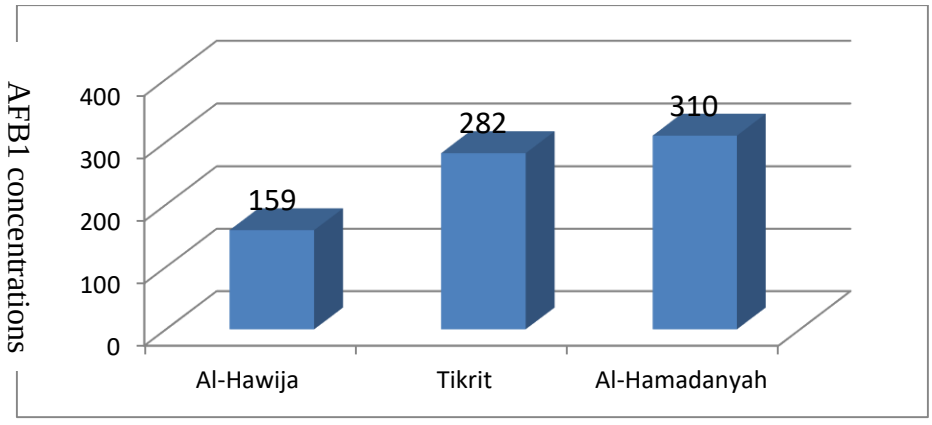

Figure 2: Graph depicting the mean AFB1 concentrations (PPb) in samples from Al-Hawija (S1), Tikrit (S2), and Al-Hamdanya(S3)

3-Effect of Anpro, C1, and C2 additions on the toxicity of AFB1 in the quail diet

Table 1 shows the effect of AFB1 alone and in combination with Anpro, C1, or C2 on the hematological markers of quails. With the exception of WBC counts, which increased significantly, quails fed a diet containing 0.5 mg of AFB1/kg had a

significant drop (P<0.05) in hematological markers when compared to the control group (DT1). Treatments with Anpro, C1, and C2 were successful in lowering the detrimental effects of AFB1 on the examined hematological markers when compared to birds given an AFB1-only diet, although they stayed considerably lower than the DT1 group.

Table 1: Effect of C1, C2, and Anpro additions on hematological values of quails fed diets containing 0.5 mg AFB1/kg diet

Investigated groups	**RBC×10^6/mm^3**	**WBC×10^3/mm^3**	**PCV%**	**Hb (g/dl)**
DT1	3.402 A	29.025 D	42.333 A	13.333 A
DT2	2.295 C	52.575 A	30.333 C	10.333 D
DT3	3.600 B	47.513 B	39.667 B	12.000 B
DT4	2.967 B	42.352 C	32.000 B	11.667 C
DT5	3.030 B	41.621 C	40.333 B	12.667 B

*At the 0.05 probability level, different letters within a single column indicate a significant difference.

Table 2 displays the impact of AFB1 either alone or in association with Anpro, C1, or C2 on quail blood biochemical markers. Quails fed a diet containing 0.5 mg AFB1/kg exhibited a substantial (p<0.05) reduction in SOD and total blood protein relative to the DT1 group, with the exception of blood uric acid, which significantly increased.

Table 2: Effect of C1, C2, and Anpro additions on blood biochemical values of quails fed diets containing 0.5 mg AFB1/kg diet

Investigated groups	**Blood uric acid (mg/dl)**	**SOD (µmol^{-l})**	**Total blood proteins (g/dl)**	**Blood glucose (mg/dl)**
DT1	2.067 D	2.04 A	3.750 A	181.667 A
DT2	9.600 A	1.190 D	2.800 C	101.667 D
DT3	6.033 C	2.030 A	3.330 B	165.000 B
DT4	8.400 A	1.670 C	3.560 A	144.000 C
DT5	7.300 B	1.800 B	3.250 B	155.333 B

*At the 0.05 probability level, different letters within a single column indicate a significant difference.

The scientific studies that the research team has analyzed indicate that mycotoxins have contaminated around 25% of field crops globally (Shareef, 2007). In 450 samples of broiler mixed feed collected over a four-year period in the Mosul province of Iraq, 66% screened positive for one or more of the aflatoxins B1, B2, G1, and G2 (Shareef *et al.*, 1998)**.**

Previous research demonstrated that in comparison to the control group, birds exposed to the aflatoxin phenotype had lower values for Hb, WBC, PCV, and blood glucose. Numerous studies have shown that aflatoxin alone in the food significantly decreased SOD, total blood proteins, and blood uric acid in comparison to birds in

the control group. (Guo *et al.*, 2021 Sakhare *et al.*, 2007). The results of this study on the impact of AFB1 on the quail diet are consistent with those of other studies. The author proposed that the increased WBC counts are very certainly connected to the inflammation brought on by the irritating effects of aflatoxins on the gastrointestinal mucosa (Yildirim *et al.*, 2011). According to similar results (Mustafa and Mohammed, 2021), which were used to support the current inquiry on elevated blood uric acid levels in aflatoxicosis, a rise in blood creatinine and uric acid may be a sign of inflammatory or progressive abnormalities in the kidney (Di Gregorio *et al.*, 2014)**.**

From their spectral information and the data gathered from the literature, the isolated coumarin-based products, C1 and C2, whose chemical structure is shown in Figure 3, were confirmed to correspond to the furanocoumarin group (Kubrak, et al., 2019, Ozek *et al.*, 2019; Mustafa and Mohammed ,2021). By preventing the synthesis of AFB1, these coumarins, in particular C2, at a dosage of 250 mg/kg, can improve the hematological and biochemical parameters in the DT4 and DT5 groups. In this line, Ali *et al.* showed in 2021 that the 40 mg/ml dosage of the coumarin derivative 50-hydroxyaurapten can block the production of AFB1 (Ali *et al.*, 2021). Furthermore, at 10 mg/ml, the synthetic coumarin derivative N-(4-chlorophenyl)-2-((4-methyl-2-oxo-2H-chromen-7-yl) oxy) acetyl) hydrazine-1-carbothioamide may completely inhibit aflatoxin formation (Kovač *et al.*, 2017).

Anpro (1 g/kg) in the AFB1-contaminated feed for quails in this study dramatically reduced the negative effects of AFB1 on the hematological and biochemical markers shown in the DT3 group. Anpro (sepiolite Plus DAE) is used as a mycotoxin adhesive and adsorbent. Similar findings were published in several scientific papers (El-Katcha *et al.*, 2017 and Ibrahim *et al.*, 2020) which found that significantly increasing the total blood proteins, WBC, RBC, and Hb values in broiler chicken helped to mitigate the deleterious effects of mycotoxin **.**

The betterment of hepatic and renal functions following the administration of mycotoxin adhesive supplementation is consistent with the report of Suksombat et al. (Suksombat *et al.*, 2011). In which, the addition of such an adhesive type was significantly effective in decreasing the blood levels of urea, uric acid, and liver-released enzyme levels, such as glutamate oxaloacetate, glutamate pyruvate transaminase, and glutamate pyruvate transaminase **.**

Earlier research conducted by Hedayati in 2014 revealed that the adverse effects of aflatoxins can be significantly modified by the addition of a mycotoxin adhesive (clay containing DAE) and that when chicks were given the adhesive alone, their performance and nutrient content were better than in the control group ((Hedayati *et al.*, 2014). According to in vitro models of the gastro-intestinal tract (Di Gregorio *et al.*, 2014) bentonite, diatomite, sepiolite, and zeolite may all have a comparable modification impact**.**

Because toxin adhesives may connect toxins and prevent them from being absorbed via the gastro-intestinal tract, they have less of an impact on animals' health and are less likely to be found in animal organs and diets (Ramos *et al.*,1996 and Kana *et al.*, 2011). The electronegativity of the adhesive can be effectively related to

its potential to counteract the myotoxin adverse effects. The mycotoxin is trapped and subsequently eliminated from the animal's body as a result of its electrical coupling with the positive charge of the adsorbent. Priorly, a lot of research was conducted to explore the mycotoxin-neutralizing effects of diatomite, aluminum silicate, activated charcoal, and yeast, and the outcomes indicated that they had a positive impact against the harmful effects of some mycotoxin types (Girish and Devogowda, 2006).

CONCLUSIONS

Our result showed ,the employment of natural products counteract the deleterious potential of A. flavus. Such Anpro, coumarin1(C1), and coumarin 2(C2), can be applied to the diet of quails to neutralize the toxic effects of the fungal-released AFB1. Quail hematological markers RBC, WBC, PCV%, Hb had a significant drop when compared to the control group (DT1). Treatments with Anpro, C1, and C2 were successful in lowering the detrimental effects of AFB1 on the examined hematological markers when compared to birds given an AFB1-only diet, although they stayed considerably lower than the DT1 group .With the exception of WBC counts, which increased significantly, Treatments with Anpro, C1, and C2 were successful in lowering the detrimental effects of AFB1 on the examined hematological markers ,including Blood uric acid ,SOD ,Total blood proteins ,Blood glucose ,when compared to birds given an AFB1-only diet, although they stayed considerably lower than the DT1 group, reduction in SOD and total blood protein relative to the DT1 group where observed , while blood uric acid, significantly increased.

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CONFLICT OF INTEREST

The authors state that there are no conflicts of interest with the publication of this work.

الدور الوقائي لمشتقات الكومارين الطبيعية و Anpro ضد التلوث بالأفلاتوكسين B1 في عليقة طيور السمان Coturnix Japonica

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الخلاصة

هدفت هذه الدراسة إلى التحقق من إمكانات مشتقات الكومارين الطبيعية C2,C1المستخرجة من بذور التفاح ومنتج Anpro على تحييد اضرار الأفلاتوكسين B1 في عليقة طيور السمان. استخدمت طيور السمان بعمر ثلاثة أسابيع وتم توزيعها بشكل عشوائي على خمس معاملات بواقع 10 طيور كل منها. المعاملة الاولى

مقارنة سلبية، حيث تلقت طيور السمان عليقة غير ملوثة، المعاملة الثانية مقارنة ايجابية تم تغذية الطيورعلى عليقة ملوثة بـ AFB1 (0.5 ملغم / كغم)، المعاملة الثالثة عليقة ملوثة بـ AFB1 (0.5 ملغم / كغم) ومعالجته بـ Anpro بتركيز 1 غم / كغم، المعاملة الرابعة عليقة ملوثة AFB1 (0.5 ملغم / كغم) مع كومارين C1 بتركيز 250 ملغم / كغم، المعاملة الخامسة عليقة ملوثة AFB1 (0.5 ملغم / كغم) مع كومارين C2 بتركيز 250 ملغم / كغم بينت النتائج ان العلائق الملوثة بـ AFB1 ادت الى انخفاض كبير في حجم وعدد خلايا الدم الحمراء والهيموكلوبين وكلوكوز الدم وإجمالي بروتين الدم ومستويات انزيم ديسموتيز الفائق(SOD). مع ارتفاع كبير في عدد خلايا الدم البيضاء الكلية وحامض البوليك. لذا يمكن تعزيز صفات الدم الكيميائية والحيوية ايجابيا عن طريق إضافة Anpro والكومارينات C1 وC2 إلى العلائق الملوثة بالأفلاتوكسين B1 كذلك يمكن ان تعزز اضافات Anpro والكومارينات C1 وC2 مواجهة الضرر الذي يسببه AFB1 لطيور السمان.

الكلمات المفتاحية: A.spergillus flavus ، Coturnix japonica ، افلاتوكسين ، ذرة ، كومارين.

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