Production of Antibody (IgG) Against Aflatoxin B1

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Abstract:

Aflatoxin B1 (AFB1) is a mycotoxin produced mainly by fungus *Aspergillus flavus* in food and feed . It is considered as a carcinogenic toxin for human and animals. The current study was designed for produce antibody (IgG) against aflatoxin B1.It was achieved by immunization of experimental animals (New Zealand White rabbits) with prepared antigen consist of aflatoxin B1-BSA Conjugate (100 and 200 µg) concentrations, and detection of produced antibody using Ouchterlony double immunodiffusion and ELISA techniques,. Ochterlony and ELISA techniques revealed that, high titer of IgG antibody was obtained by rabbit's immunize, and the titer of antibody was increased steadily during the immunization schedule. The highest titer of antibody reached up to 12800.

Key Words: - Aflatoxin B1, Antibody, IgG, Ouchterlony double immunodiffusion, ELISA.

انتاج الاجسام المضادة IgG للأفلاتوكسين B1

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الخلاصة:

يعد الأفلاتوكسين B1 احد انواع السموم الفطرية والذي ينتج بشكل اساسي من الفطر *Aspergillus flavus* في الأغذية والأعلاف ، ويعتبر مادة سامة مسرطنة للأنسان والحيوانات ، صممت الدراسة الحالية لغرض انتاج الأضداد للأفلا توكسين B1 عن طريق تمنيع حيوانات المختبر (الارانب النيوزلندية) بالمستضد المحضر والذي يتألف من المقترن السمي (الأفلاتوكسين B1 المرتبط بألبومين مصل البقر) وبتركيز (100 و 200 مايكروغرام) والكشف عن الأضداد المنتجة بأستخدام تقنية اوختراونى للأنتشار المناعي ا لمزدوج وتقنية ELISA. تم انتاج الأضداد للأفلاتوكسين B1، وقد تم الكشف عنها باستخدام تقنية اوختراونى للأنتشار المناعي المزدوج، واستخدام تقنية ELISA حيث أن معيار الضد كان في ازدياد مضطرد خلال فترة التمنيع وقد وصل اعلى معيار للضد الى 12800 من التمنيع الأولي .

Introduction:

Food can be contaminated by fungi. These molds develop inside or around the seeds, depending on their biological make-up and Eco physiological conditions [1]. Some of these fungi are said to be toxigenic, they can synthesis one or more metabolites which become toxic for man and certain animals

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when ingested in large quantities. These are called mycotoxins [2].Toxigenic fungi do not systematically produce toxins; this depends largely on environmental conditions. The level of toxicity is very variable for different animal species there are more than 300 mycotoxins produced by a variety of common fungi. Include aflatoxins, ochratoxin, Citrinin, Patulin, and *Fusarium* toxins [3]. Aflatoxins are a group of fungal metabolites produced primarily by the fungi *Aspergillus flavus* and *Aspergillus parasiticus*. The four major naturally produced aflatoxins are known as B1, B2, G1, and G2. B and G refer to the blue and green fluorescent colors produced under UV light, while the Subscript numbers 1 and 2 indicate major and minor compounds, respectively. *Aspergillus flavus* and *Aspergillus parasiticus* colonize a wide variety of food commodities including maize, oilseeds, spices, groundnuts, tree nuts, milk, and dried fruit [4]. Whether these fungi produce aflatoxin B1 depends on drought stress and rainfall, suitability of crop genotype for its climate, insect damage, and agricultural practices [5]. These fungi can also produce aflatoxin B1 in "postharvest" conditions: storage, transportation, and food processing. Aflatoxin B1 contamination is a particular problem in maize, oilseeds, spices, peanuts, milk (in the form of aflatoxin B1's metabolite aflatoxin M1, and dried fruit [6]. Maize and peanuts are the main sources of human exposure to aflatoxin because they are so highly consumed worldwide and unfortunately are also the most susceptible crops to aflatoxin contamination [5]. Aflatoxins B1 are low molecular-weight secondary fungal metabolites; they were devoid of antigenicity, so it must be conjugated to suitable carrier (such as bovine serum albumin). Efforts to improve antibody production against aflatoxin B1 were made by immunization of animals with aflatoxin B1 –BSA Conjugates [7]. Vaccination against aflatoxin B1 is able to induce antibodies which reduce the toxic effects against aflatoxin B1 [8, 9]. Therefore, the main goal of this study is: - Production of antibody (IgG) against aflatoxin B1 through immunization of New Zealand white rabbits by prepared antigen consists of aflatoxin B1-BSA Conjugate.

Materials and Methods:

Preparation of Aflatoxin B1 Carboxymethyl oxime:

Aflatoxin B1 Carboxymethyl oxime was prepared according to the method described by [10]

Preparation of Aflatoxin B1 – BSA Conjugate:

The conjugate was prepared according to the method described by, [7]

Antibody production against Aflatoxin B1:

Preparation of injecting solutions:

A- The first injecting solution was consisted of 1mg of BSA dissolved in 1 ml NaCl (0.9%) which mixed with complete Freund adjuvant (1:1) ratio, and the final result was very dense emulsion.

B- The second injecting solution was made by adding of 100 µg of Aflatoxin B1- BSA Conjugate to 1 ml NaCl (0.9%) which mixed with complete Freund adjuvant (1:1) ratio.

C- The third injecting solution was made by adding of 200 µg of Aflatoxin B1- BSA Conjugate to 1 ml NaCl (0.9%) which mixed with complete Freund adjuvant (1:1) ratio.

D- The fourth injecting solution was made by adding of 100 µg of Aflatoxin B1- BSA Conjugate to 1 ml NaCl (0.9%) which mixed with incomplete Freund adjuvant (1:1) ratio.

E- The fifth injecting solution was made by adding of 200 µg of Aflatoxin B1- BSA Conjugate to 1 ml NaCl (0.9%) which mixed with incomplete Freund adjuvant (1:1) ratio.

Immunization schedule:

For Immunization the multiple – site intradermal method [11] was followed with some modification, New Zealand White rabbits were immunized in their back using the following schedule (Two groups of New Zealand White rabbits ((3 rabbit for each concentration)) were used):

• Week (Zero): Two milliliter of solution (A) was injected as priming dose for the both groups of rabbits.

• Week (1): Two milliliter of solution (B) and (C) was injected to each group of rabbit separately.

• Week (3): Five milliliter of blood was collected by bleeding the rabbits using cardiac puncture.

• Week (4): Booster injection, two ml of solution (D) and (E) were injected to each group of rabbit separately

• Week (5 , 7 , 9 , 11) : Five milliliter of blood was collected by bleeding the rabbits using cardiac puncture .

After blood collection, blood was left at room temperature for 5 minutes, then centrifuged at 800 rpm for 10 minutes, serum was transferred to 1 ml eppendrof tube using micropipette, then kept in deep freezer until use.

Detection of antibodies:

Ouchterlony double immunodiffusion:

Ouchterlony double immunodiffusion technique was used for antibodies detection according to method described by [12].

Enzyme Linked Immune Sorbent Assay (ELISA):

This test was used for detection of Antibody titer (IgG) which produced against Aflatoxin B1; it was performed using Rabbit IgG Titer ELISA Kit which supplied by General Bioscience Company.

Results and discussion:

Production of antibody (IgG) against aflatoxin B1:

The antibody (IgG) was produced against aflatoxin B1 through injection of New Zealand White Rabbits by already prepared antigen consisted of aflatoxin B1-BSA Conjugate (100 µg and 200 µg) using suitable immunization schedule. As is well known that aflatoxin B1 was low molecular-weight toxic fungal metabolites and thus were devoid of antigenicity. The toxin also lacks a reactive group for the coupling of the toxin to a macromolecule carrier for antibody production. However, through derivation, a free carboxylic group is introduced to the toxin molecule, the result of this process was formation of yellow residue, and the residue was chromatographed on TLC plate. TLC plate was observed under UV light at 365 nm , the formation of aflatoxin B1 carboxymethyl oxime detected as a fluorescent spot were determined by visual comparison with standard aflatoxin B1, That's where the spot of aflatoxin B1 standard appeared higher than the spot of aflatoxin B1 carboxymethyl oxime , because of the free carboxylic group which made the derivative heavier due to the increasing of molecular weight , Figure1, thus permitting the molecule to react covalently with protein (BSA). The preparation of aflatoxin B1-BSA-Conjugate showed in Figure 2. The Produced antibodies were detected using Ouchterlony double immunodiffusion technique and Enzyme Linked Immune Sorbent assay (ELISA) technique.

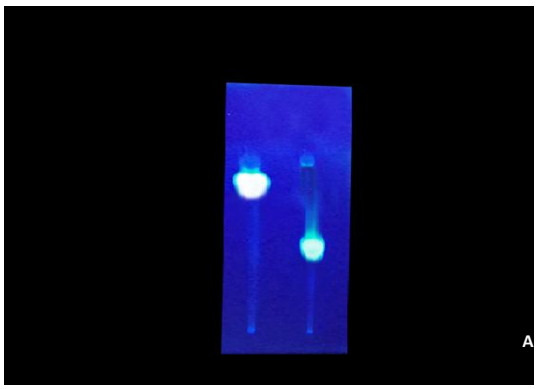

Figure 1- Detection the formation of Aflatoxin B1 Carboxymethyl Oxime using TLC technique (A: Aflatoxin B1 standard, B: aflatoxin B1 carboxymethyl oxime).

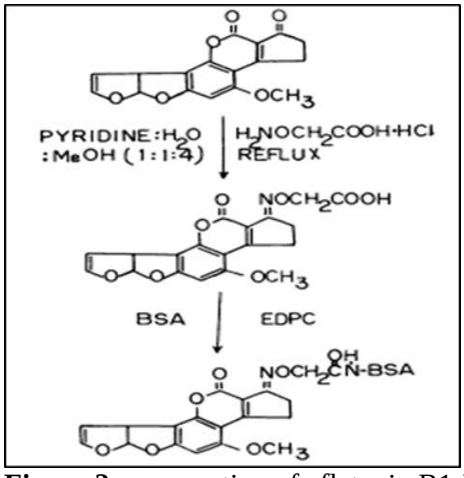

Figure 2 - preparation of aflatoxin B1-BSA-Conjugate (Chu and Ueno, 1977).

Detection of antibodies by Ouchterlony double immunodiffusion technique:

Antibody have been detected using Petri plates containing agarose by making four peripheral wells contain (antigens) and central well contain (test serum) .

The results showed the formation of four precipitate lines between the peripheral wells which contain antigens (aflatoxin B1- BSA Conjugate in ((two opposite wells)) and BSA only in ((other two opposite wells)) and the central well which contain test serum, figure 3.

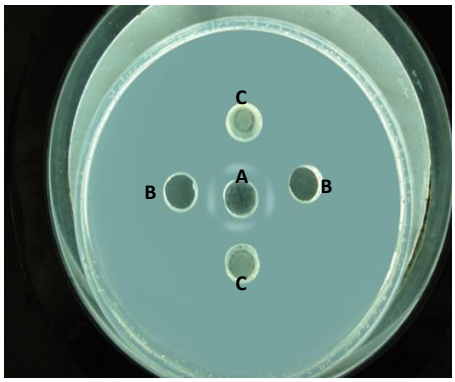

Figure 3 - Ouchterlony double immunodiffusion technique showed the four perceptation lines / A: well contain test serum collected in the ninth week of the first immunization /B: wells contain antigen (BSA only, 200 µg) / C: wells contain antigen (aflatoxin B1-BSA Conjugate, 200 µg).

This test was used for detection of antibody in rabbits´s sera which immunized by aflatoxin (B1-BSA Conjugate 100 µg and 200 µg) and that has been conducted through the weeks (3 , 5 , 7 , 9 ,11) , It has been shown the absence of antibody from immunized rabbits´s sera in the third week , while obtained sera were contained antibody which reacted with antigen and formed a precipitate lines in the week (5 , 7 , 9 , 11) .

The formation of precipitate lines which antigen intersected with antibody resulted from the reaction between antigen and antibody in immunized rabbits sera , (immunized with aflatoxin B1 – BSA and BSA only) , Ouchterlony double immunodiffusion technique was used for detection of high level of antibody [13, 14]., This explain the lack of precipitate lines between antigen and antibody in the third week of experiment., While the precipitate lines were formed starting from the fifth week until the eleventh week .

Our results showed that the pattern of immune diffusion was Identity, where the antibodies in the antiserum react with both the antigens resulting in a smooth line of precipitate. The antibodies cannot distinguish between the two antigens and the two antigens are immunologically identical [15].

Detection of antibody (IgG) by indirect ELISA technique:

This technique was considered as a sensitive, precise and specific technique so it used for detection of antibody (IgG) in immunized rabbit´s sera. Rabbit IgG Titer ELISA Kit was used for this purpose. The results measured optically using micro plate reader at 450 nm for yellow color or at 650 nm for unstopped blue color. The absorbance values obtained were read in terms of the level of antibody in the test sera. The antibody (IgG) were detected by taking of serum samples from rabbits that immunized according to immunization schedule used in this experiment, the results showed that the level of antibody (IgG) was increased steadily starting from the third week until the end of the ninth week using 100 µg of antigen as shown in figures 4, 5, 6, 7, 8 and 200 µg as shown in figures 9, 10, 11, 12, 13.

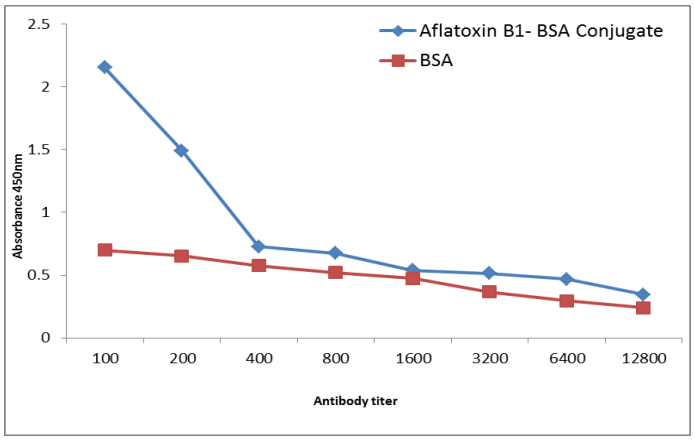

Figure 4- Antibodies (IgG) titers in rabbit's sera immunized with 100 µg of aflatoxin B1-BSA Conjugate at the third week of immunization using ELISA technique.
Each value of the curve represents the average of three replicates.In all cases, the standard error not to exceed (7%) of the average value.

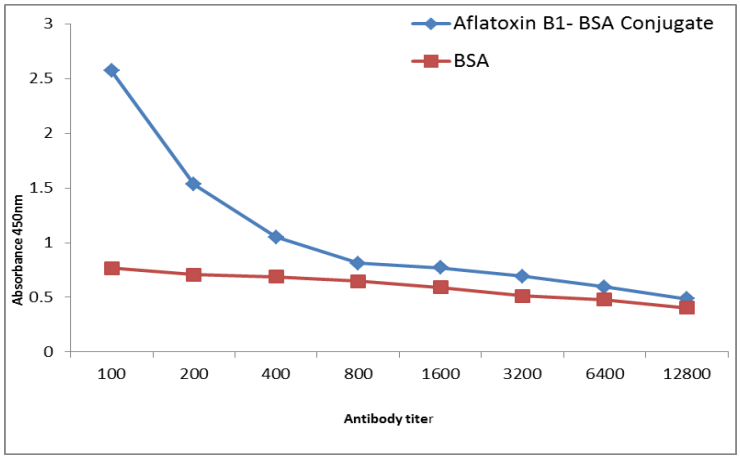

Figure 5- Antibodies (IgG) titers in rabbit's sera immunized with 100 µg of aflatoxin B1- BSA Conjugate at the fifth week of immunization using ELISA technique.
Each value of the curve represents the average of three replicates. In all cases, the standard error not to exceed (6%) of the average value.

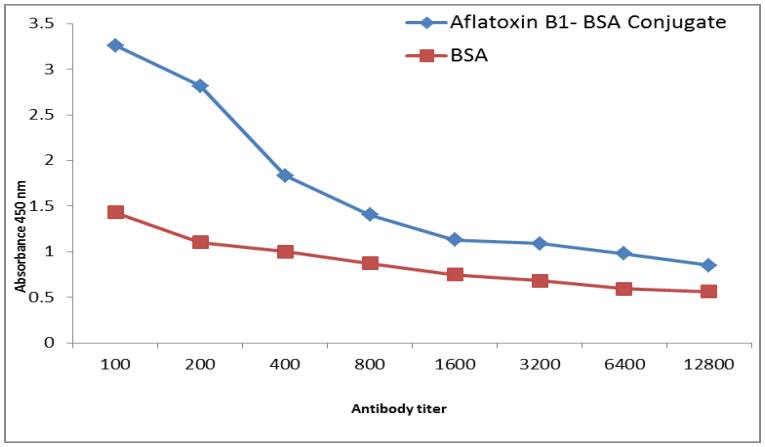

Figure 6- Antibodies (IgG) titers in rabbit's sera immunized with 100 µg of aflatoxin B1- BSA Conjugate at the seventh week of imm Each value of the curve represents the average of three replicates.
In all cases, the standard error not to exceed (4.7%) of the average value.unization using ELISA technique.

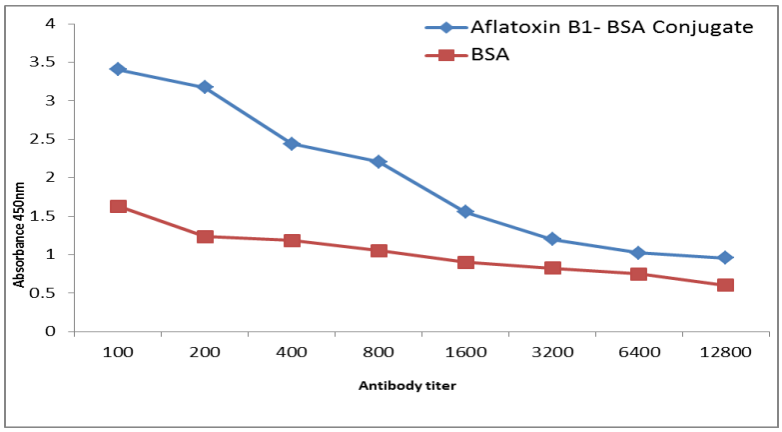

Figure 7- Antibodies (IgG) titers in rabbits sera immunized with 100 µg of aflatoxin B1-BSA Conjugate at the ninth week of immunization using ELISA technique.
Each value of the curve represents the average of three replicates.In all cases, the standard error not to exceed (5.4%) of the average value.

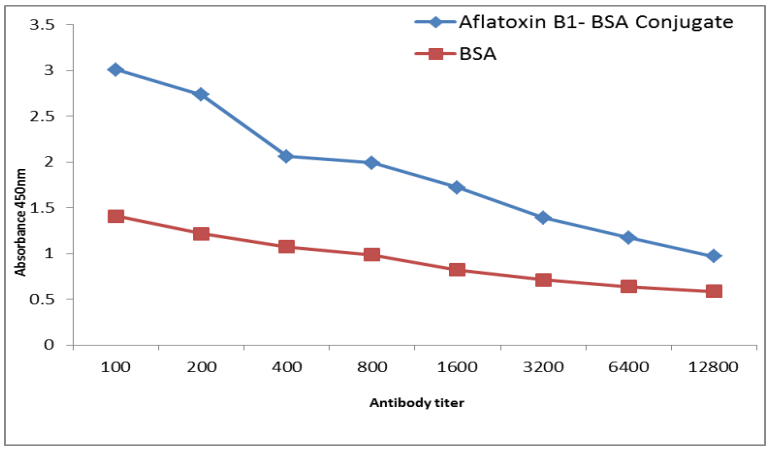

Figure 8- Antibodies (IgG) titers in rabbit's sera immunized with 100 µg of aflatoxin B1- BSA Conjugate at the eleventh week of immunization using ELISA technique.
Each value of the curve represents the average of three replicates. In all cases, the standard error not to exceed (5.2%) of the average value.

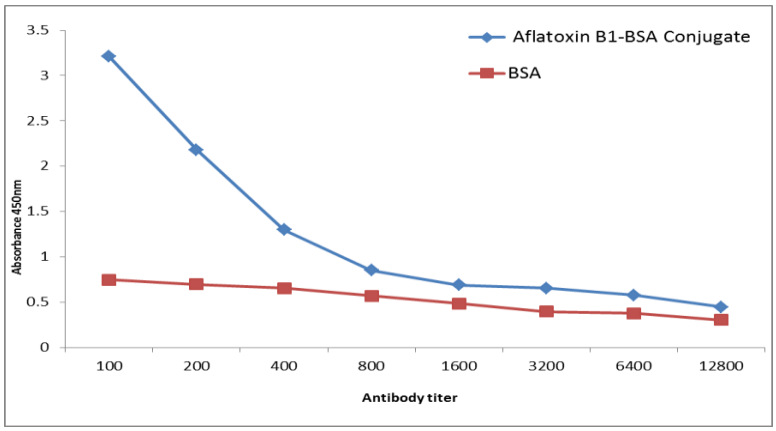

Figure 9- Antibodies (IgG) titers in rabbit's sera immunized with 200 µg of aflatoxin B1- BSA Conjugate at the third week of immunization using ELISA technique.
Each value of the curve represents the average of three replicates. In all cases, the standard error not to exceed (5%) of the average value.

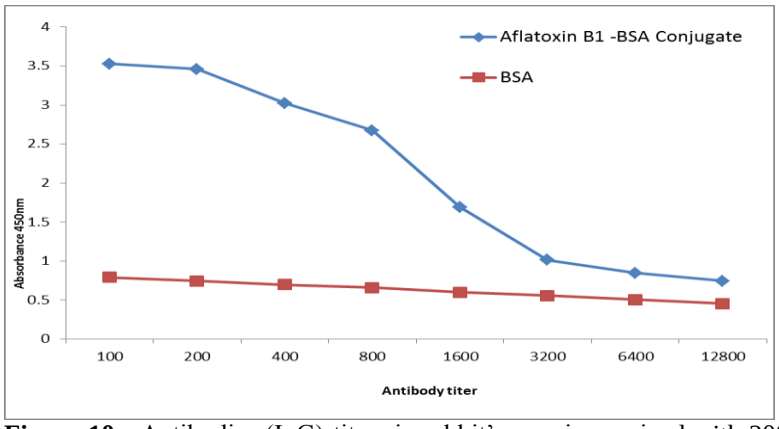

Figure 10 - Antibodies (IgG) titers in rabbit's sera immunized with 200 µg of aflatoxin B1- BSA Conjugate at the fifth week of immunization using ELISA technique.
Each value of the curve represents the average of three replicates. In all cases, the standard error not to exceed (4%) of the average value.

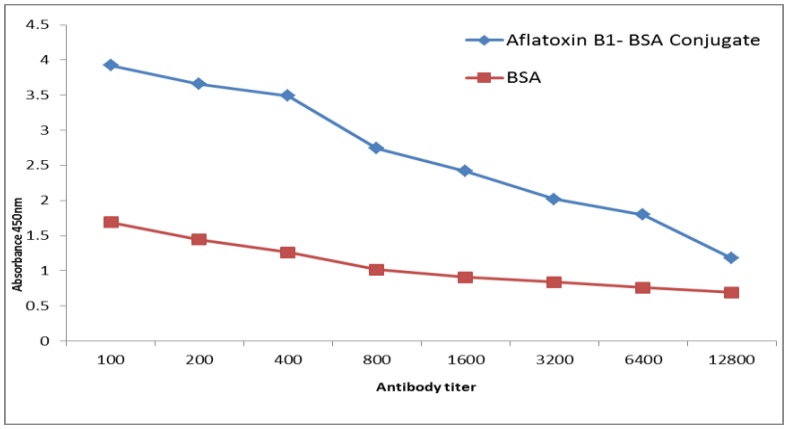

Figure 11 - Antibodies (IgG) titers in rabbit's sera immunized with 200 µg of aflatoxin B1- BSA Conjugate at the seventh week of immunization using ELISA technique.
Each value of the curve represents the average of three replicates. In all cases, the standard error not to exceed (6%) of the average value.

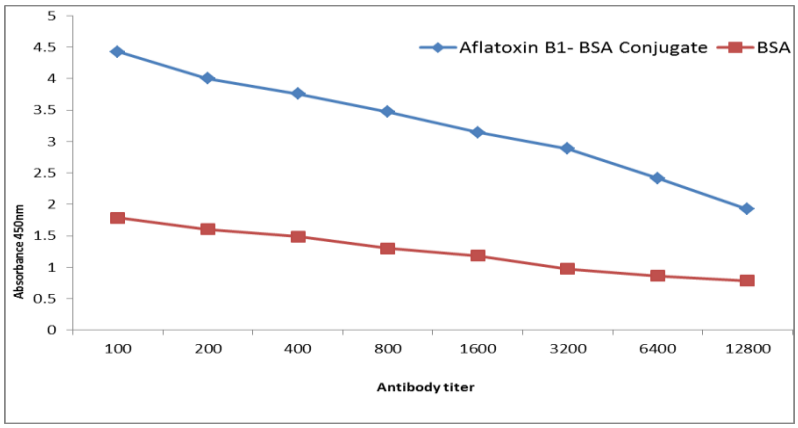

Figure 12 - Antibodies (IgG) titers in rabbit's sera immunized with 200 µg of aflatoxin B1- BSA Conjugate at the ninth week of immunization using ELISA technique.
Each value of the curve represents the average of three replicates. In all cases, the standard error not to exceed (7.6%) of the average value.

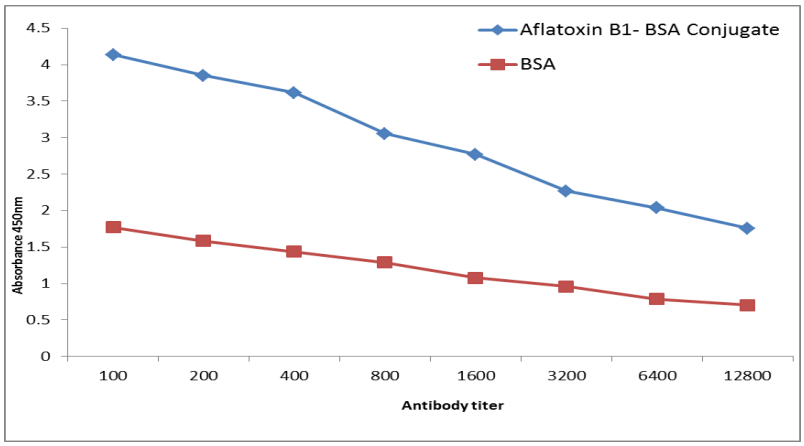

Figure 13 - Antibodies (IgG) titers in rabbit's sera immunized with 200 µg of aflatoxin B1- BSA Conjugate at the eleventh week of immunization using ELISA technique.
Each value of the curve represents the average of three replicates. In all cases, the standard error not to exceed (2.6%) of the average value.

It can conclude from the results which obtained., that the absorbance values were decreased whenever the dilutions levels increased and the absorbance of flatoxin B1 – BSA Conjugate were highe than the absorbance of BSA, only due to the formed antibody against aflatoxin B1 – BSA. Conjugate were consisted of two type, the first against aflatoxin B1 and the other against BSA , This mean that there are other antibody were formed against aflatoxin B1 molecule led to increase of absorbance values of aflatoxin B1 -BAS Conjugate .

Many factors which effects on the titers of antibody including: type of carriers using in coupling, concentration of antigen, type of animals and using of booster injection.

Antibody titer affected by type of carriers using in coupling, [7] showed that BSA-Afla B1-oxime was a good antigen, while PL-Afla B1-oxime was not a good antigen. This may be due to the molecular weight of poly-lysine (PL) (30,000), it was consider smaller than Bovin Serum Albumin (BSA))(66000) . The titer of antibody increase when the concentration of antigen increases [7]. Type of animals also have an effects on titer of antibody, [18] showed that the titer of antibody were 20 time higher in the rabbits than in the goat. Using booster injection also has an effect on titer of antibody by maintaining the immune response at an appropriate level [16].

Antibody titer was constantly increasing since the third week until the end of the ninth week with a slight decrease in antibody titer at the end of the eleventh week of experiment., While [7] were used the same experimental animals , antigens concentration , immunization schedule , except not using booster injection in the fourth week and the blood was collected every week starting from the third week. They reached to results that antibody titers were demonstrated 3 weeks after immunization , and maximum production occurred in 5 to 8 weeks , but the results of [16] has shown antibody production against aflatoxin B1 , was compared between rabbits and goat and titer obtained were 20 times higher in the rabbits than in the goat, [17] were immunized rabbits (Chinchilla Bastards) with aflatoxin B1-BSA Conjugate., the rabbits immunized intradermally with an emulsion of distilled water (0.5 ml) and complete Freund's adjuvant (1.5 ml) containing 400 µg afatoxin B1-BSA Conjugate., Booster injections with the same amount and composition of immunogen were given 11 weeks (intramuscularly) and 32 weeks (subcutaneously) after the initial injection. High titers of antibodies for aflatoxin B1 were obtained 15 and 4 weeks after the initial immunization and the first booster immunization respectively , [10] was used immunization approach including intradermal and intramuscular injections of aflatoxin B1-BSA Conjugates into New Zealand white rabbits, The immunogen was diluted in 0.9% saline and emulsified in Freund's complete (for first immunization) or incomplete adjuvant (for subsequent immunization) to give 0.5-1 mg/ml (for first immunization) or 0.25-0.5 mg/ ml (for subsequent immunization). After three initial injections at two week intervals, booster injections were given monthly. Each immunization was given in a total volume of 1 ml. Blood was collected from the marginal ear vein 7-10 days after each booster injection.

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