Evaluate the Antifungal and detoxification activity of silver nanoparticles prepared with the Curcuma plant extract against Aflatoxin B1 in broiler feed.

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Abstract. The research was carried out in the laboratories of the Ministry of Science and Technology/Iraq. Green nanoparticles were prepared using turmeric (Curcuma plant) extracted by using biological methods and were characterized by using some techniques including Scanning Electron Microscopy (SEM), X-ray Diffractometer (XRD) and zeta potential Analyzer. The results showed that the spherical particles were shown individually or in the aggregate, crystalline, and the value zeta voltage at -33.22 mV. The inhibition activity of green nanoparticles was tested against Aspergillus flavus growth and compared with Basten fungicide as a comparison treatment and negative control sample. The results of nanoparticles showed high inhibitor activity in the 590.33 ppm concentration compared to negative control and comparative treatment in concentration 750 ppm. Feeds contaminated with A. flavus isolate were treated with cur. AgNPs at 590.33 ppm for a four-week (incubation period) and Aflatoxin B1. The results showed no Aflatoxin in feed samples were treated with nanoparticles while showing Aflatoxin B1 at a concentration of 38 ppb in control samples. To study the effect of cur. AgNPs against the fungal toxicity in meat broilers feeding to feeding contaminated with Aflatoxin B1 by using some blood and histopathological signs of bird’s liver. The results showed the nanoparticles treatment samples significant superiority compared with the positive control (contaminated with Aflatoxin B1) (p ≤0.01), and no significant differences with control treatment (feed free from any addition). We conclude that the use of green nanoparticles (Curcuma longa L.) produced by biological method as good alternatives to fungicides that harmful to public health and inhibitory activity of A. flavus and prevent the production of Aflatoxin B1 and improve the blood and histopathological signs of birds. 

Keyword: nanoparticles, Antifungal, detoxification, broilers.

1. Introduction:
Aspergillus flavus considered one of the important contaminated mould crops before and after harvest and plant pathogenic, a number of them produce large quantities of toxins known as aflatoxin, which cause poisoning of mammals. Usually, excessive moisture conditions with high temperatures for storage pulses and legumes raise the range of Aspergillus flavus production. A. flavus are opportunistic moulds for humans and are considered to be a pathogen of animals, and also cause aspergillosis in individuals with weakened immunity [1]. Aflatoxin B1 is the most effective toxin and most dangerous carcinogen in different types of aflatoxin. A human may be exposed to aflatoxin B1 immediately after eating contaminated food or consuming foods of animal origin previously fed with contaminated feed [2]. Leong et al. (2012) [3] reported the aflatoxin B1 has toxic effects on liver cells as it can cause damage to nucleic acids, genetic mutations, cancer, miscarriage and birth defects, and a relapse of the immune system. It can be exposed to many health-related conditions including liver cancer, jaundice, chronic hepatitis, enlarged and cirrhosis. Curcuma longa L. is a rich source of biologically active compounds such as antioxidants, polyphenols and flavonoids, which can be a good alternative to antimicrobials used in food and food products, and curcumin is a yellow crystalline substance that is insoluble in water-soluble orange [4]. In general, nanoparticles can be produced in two ways, from top
to bottom or from bottom to top. There are several methods, including a physical, chemical, and biological method, by using to prepare green products such as bacteria, filamentous fungi, yeast and plants [5]. The biological nanoparticle synthesis certainly has a unique ability to produce a precise and controlled form and structure. In addition, it does not require sophisticated devices, but is simple, efficient, environmentally friendly, inexpensive, safe, the plants have a wide range of metabolites that can help reduce silver ions and faster than microbes, during the assumption [6]. Therefore, it was considered to produce green nanoparticles using turmeric plant and to test its anti-fungal efficacy of aflatoxin and its application in feeds fed by broiler chickens and to observe its positive or negative effect.

2. Material and Methods

2.1. Preparation of plant extract.
Ethanolic extract of *Curcuma longa* L. rhizome plant was prepared according to Wong et al., (2014) [7] by using a Soxhlet apparatus.

2.2. Synthesis of green silver nanoparticles
Five ml of plant extract was sprayed into 95 ml of 10 mM silver nitrate AgNO₃ solution (which prepared by dissolving 1.69g AgNO₃ into 1L deionised water) separately dropwise with a flow rate of 0.2 ml/min under ultrasonic conditions, after sonication for 20 min, the solutions were stirred at 800 rpm in 25°C for 30 min, then kept in dark bottles at 25°C for 24 h. After 24 h the reaction mixture was purified by centrifugation for 10 min at 10000 rpm to get clear supernatant. The final colloid samples were stored in dark bottles at 25°C. During 5 days the colour of the solutions was changed from clear yellowish to dark greenish yellow for Curcuma silver nanoparticles (Cur. AgNPs), this change in colour indicates the formation of silver nanoparticles (AgNPs) were done according to Ojha et al., (2017) [8].

2.3. Characterization of the synthesized nanoparticles.
The characterization of the synthesized nanoparticles was performed using different instruments example: Scanning electron microscopy (SEM) was carried according to Dimitrijevic et al. (2013) [9], and the X-ray Diffractometer (XRD) was carried according to the Ojha et al. (2017) [8]. The result obtained from the XRD pattern was interpreted with standard reference of the Joint Committee on Powder Diffraction Standards (JCPDS card number 04-0783) for the characterization of AgNPs [10]. The particle size of the prepared samples was determined by using Debye–Scherrer equation as follows:

\[
D = \frac{0.9\lambda}{\beta \cos \theta}
\]

Where *D* is the crystal size, *λ* is the wavelength of x-ray, *θ* is the diffraction angle (Braggs angle) in radians and *β* is the full width at half maximum (FWHM) of the peak in radians [11].

The synthesized nanoparticles stability was assessed in terms of zeta potential using the zeta potential analyzer was carried according to the Aljabali et al. (2018) [12].

The concentration of silver ions for each of prepared green silver nanoparticles solutions cur. AgNPs were estimated using atomic absorption spectrophotometer (AAS) after stabilizing the colour change of green silver nanoparticles solutions [13]. Different concentrations of silver nitrate AgNO₃ (1, 2, 3, 4, 5, 6) mM was prepared in deionized water and used as a standard by which a calibration curve was obtained, Figure 1, the concentrations were 787.1 ppm for cur. AgNPs. Then different concentrations of Cur. AgNPs (590.33) ppm.
Figure 1: Standard curve of AgNO\textsubscript{3} concentrations for a concentration of silver ions determination.

2.4. Detection of inhibition activities of synthesized green silver nanoparticles using Curcuma (In-vitro)

This experiment was conducted for the detection of inhibitory activities of Curcuma silver nanoparticles (cur. AgNPs) against the growth of aflatoxin producing fungus A. flavus (In vitro). The inhibitory activity of cur. NPs. At 590.33 ppm compared to the bestene fungicides in the 750-ppm concentration was evaluated against A. flavus depending on the agar diffusion method described by da Silva [14]. These experiments were carried with three replicates. The feed was contaminated by the addition of the A. flavus producer the Aflatoxin B1 toxin. After that, adding the cur. AgNPs at a concentration of 590.33 ppm. The feed was incubated for four weeks.

2.5. Detection of aflatoxin B1 (AFB1)

2.5.1. Extraction of aflatoxin B1 (AFB1). Aflatoxin B\textsubscript{1} was extracted from the feed samples under the study according to [15].

2.5.2. Aflatoxin B1 (AFB1) standard curve. The standard aflatoxin B\textsubscript{1} solution was prepared according to Liu, et al. [16] with some modification in acetonitrile at a concentration of 25 mg/ ml to prepare a stock solution and kept on -20\textdegree C. The standard curve drawn with concentrations (20, 40, 60 and 80) μg/ ml of Aflatoxin B1 apposite area by using high-performance liquid chromatography (HPLC) technique was appeared by Figure 2.

Figure 2 Standard curve of AFB\textsubscript{1} concentrations determined by HPLC.

To study the effect of adding turmeric silver nanoparticles on meat broilers contaminated with fungus A. flavus and its effect on some blood and histopathological signs of (liver birds) to reached into the optimal treatment in, where 135 chickens of Ross-308 broiler chickens were used, one day old, randomly distributed by 3 replicates for each treatment and each repeater consists of 15 birds. It was fed on two balanced diets of energy and protein.
2.6. Physiological study.

2.6.1. Blood tests. Blood samples were collected after 42 days of incubation. Randomly, Brachial Vein was withdrawn and blood was collected in anticoagulant tubes (Potassium – Ethylene Diamine Tetra Acetic Acid-K-EDTA). Was carried according to Archer [17], the method of estimating haemoglobin in blood was based on the method reported by Samou [18], while the ratio of H/L heterophylia and lymphocytes was estimated according to the method adopted by Cotter [19]. Then the ratio of heterophilic cells to cells was calculated for lymphocyte H/L.

2.6.2. Liver enzymatic assay. Measuring the activity of the enzymatic Aspartate Amino Transaminase and Alanine Amino Transaminase: The assay kit used to measure the efficacy of AST enzyme imported from Biomeriux used a test tube for each sample. The first contains a blank reagent and the other contains a sample to measure the activity of the enzyme in them (Table 1).

|                | ALT | AST |
|----------------|-----|-----|
| Indicator 1    | 1ml | ----|
| Indicator 2    | ----| 1ml |

Incubate for 5 minutes at 37 ° C

|                   | ALT | AST |
|-------------------|-----|-----|
| Sample serum      | 0.2 ml | 0.2 ml |
| Shake the tubes and incubate at 37°C | 1 h | 30 min |
| Indicator 3       | 1 ml | 1 ml |
| Shake the tubes, and leave them at room temperature for 20 min | MI | MI 10 |
|                   | Na OH 0.4 N |

Shake the tubes, and read the absorbance after 5-10 minutes along the wavelength of 520 - 520 nm

2.7. Histological study.

Slides of liver samples were prepared according to Palipoch and Punsawad [20].

2.7.1. Statistical analysis. Statistical analysis was conducted using SAS/STAT software (2012) [38] in the data analysis to study the effect of different treatment on the studied signs according to a complete random design (CRD). Significant differences between the averages were compared with the polynomial test.

3. Result and Desiccation.

3.1. Synthesis of nanoparticles

3.1.1. The colour change of nanoparticle solutions. The colours of green silver nanoparticles solutions were changed from clear yellowish-orange to dark greenish-yellow for Curcuma silver nanoparticles (cur. AgNPs) where adding Curcuma ethanolic extracts to silver nitrate solution Figure 3. The colour change indicates the formation of green silver nanoparticles (AgNPs) due to the reduction of silver metal ions Ag⁺ into silver nanoparticles Ag⁰ via the active molecules present in the Curcuma extract.
Plants possess different metabolites like phenols, terpenoids, alkaloids, flavonoids; proteins, carbohydrates etc. play a key role in the stabilization and reduction of metallic silver into AgNPs [21]. The reduction rate and the formation of nanoparticles can be increased further by increase the incubation time, so the intensity of the colour increased with increasing the time of reaction [22]. Metal nanoparticles show different colours in solution due to their optical properties [23]. The change in colour is referred to the excitation of surface plasmon vibrations in metal nanoparticles. Silver nanoparticles exhibit interesting optical properties directly associated with localized surface plasmon resonance [24]. This result is agreed with [25].

4. Characterization of Nanoparticles.

4.1. Scanning electron microscope (SEM)

The results of SEM analysis showed the particles are spherical in shape with nanometre in sized for cur. AgNPs, the large nanoparticles were seen due to aggregation Figure 4, this aggregation took place due to the presence of cell components on the surface of nanoparticles and acts as capping agent. This result agrees with Surega [26] who observed the morphology of the synthesized AgNPs revealed that the AgNPs were spherical in their shape and agglomerated.

4.2. X-ray diffractometer (XRD)

The green synthesis of AgNPs was supported by X-ray diffraction (XRD). Figure 5 recorded four obvious diffraction peaks at 20 values 37.42, 45.61, 63.11, 75.89 for cur. AgNPs which were corresponded to 111, 200, 220 and 311 planes of silver. The XRD pattern clearly showed that the AgNPs formed by the reduction of Ag⁺ ions using Curcuma extracts are crystalline in nature. Some unassigned peaks were observed, it may be due to the bio-organic phase/ metalloproteins occurs on the surface of silver nanoparticles or it may be due to the
fewer biomolecules of stabilizing agents such as enzymes or proteins in the plant extract [27]. The stronger planes indicate silver as a major constituent in biosynthesis.

![Figure 5: XRD test curve of cur. AgNPs.](image)

4.3. **Zeta potential analysis.**

The results of zeta potential values of the synthesized nanoparticles were -33.22 mV for cur. AgNPs, Figure 6. Zeta potential values are a key indicator of the stability of colloidal dispersions. Zeta potential values indicate the extent of electrostatic repulsion between adjacent particles similarly charged in dispersion. For molecules and particles small enough, the high zeta potential will provide stability, that is, the solution or dispersion will resist agglomeration. When the voltage values are small, the attractive forces may exceed this repulsion and the dispersion may be broken and sintered, and therefore, the high potential colloids of zeta (negative or positive) are fixed electrically while colloids with lower potentials tend to coagulate or flocculate. Generally, the zeta potential of the nanoparticles should be either higher than +30 mV or lower than -30 mV [28].

![Figure 6: The zeta potential value of cur. AgNPs.](image)

4.4. **Estimation of silver ion concentration of green silver nanoparticles solutions**

The results of an atomic absorption spectrophotometer (AAS) analysis indicated that the concentrations of Curcuma silver nanoparticles (cur. AgNPs) were 590.33 ppm.

4.5. **Evaluate the inhibitory activities of synthesized green silver nanoparticles using Curcuma extract (In vitro).**

The results in Table 2, Figure 7 confirmed that cur. silver nanoparticles (cur. AgNPs) displayed antifungal activity against *A. flavus* 590.33 ppm concentration was completely inhibited mycelia growth and inhibition percentage was 100%. The inhibition percentages 100% to fungus for synthetic fungicidal bastene 750 ppm, while there was no effect for AgNO$_3$ (10 mM) as a control (+ve) on fungal growth.

The mechanism of green synthesized AgNPs was disturbed transport systems, including ion efflux and interrupt cellular processes such as metabolism and respiration of target organisms. Further, it is speculated that nanoparticles penetrate the cell wall, affect the DNA and its ability to inactivate the expression of ribosomal subunit proteins as well as certain cellular proteins and enzymes essential for ATP production [29].
A. flavus (control -ve)  
A. flavus (AgNO$_3$ 10 mM)  
A. flavus (cur. AgNPs 590.33 ppm)  

**Figure 7** Effect of cur. AgNPs on *A. flavus* compared with control and AgNO$_3$.

**Table 2** Effect of cur. silver nanoparticles (cur. AgNPs) on fungal growth.

| Cur. AgNPs Concentration (ppm) | *A. flavus* isolate | Colony diameter (mm) | Inhibition percentage |
|---------------------------------|---------------------|----------------------|----------------------|
| 590.33                          | Control (-ve)       | 27.1 ± 1.42          | -                    |
| Bastene (750) ppm               |                     | 0 ± 0                | 100                  |
| AgNO$_3$ 10Mm                   |                     | 27.08 ± 1.33         | 0.07                 |
| Control (-ve)                   | LSD value           | 2.97*                | 8.38*                |

* (P ≤ 0.01)

4.6. AflatoxinB1 detection using *High-performance liquid chromatography technique* (HPLC).

The high-performance liquid chromatography (HPLC) technique that used to detect the Aflatoxin B$_1$ exhibited that feed samples which treated with cur. AgNPs avoid the Aflatoxin B$_1$, compared with the control sample (feed samples without treated with nanoparticles) which gave the Aflatoxin B$_1$ 38 ppm concentration this producing from *A. flavus*. Where levels of Aflatoxin B$_1$ Disappeared after feed treatment with nanoparticles Figure 8. This is due to the presence of effective antioxidant compounds in addition to the inhibitory activity of many fungi in these plants. Note that the nanoparticles compete with aflatoxin to bind to the active sites in the feeder cells, which prevents the aflatoxin from binding to the substrate in the feed.

![Figure 8: High-performance liquid chromatography curve of the contaminated feed sample appears the Aflatoxin B1 detection.](image)

4.7. Green silver nanoparticle activity in meat broiler chicken (in vivo).

4.7.1. Some physiological signs in the broiler blood serum. Table 3 shows the effect of treatments on some physiological signs in the broiler blood serum at the age of 42 days, showed significant differences in the effect of treatment positive control (S2) compared with treatment negative control (S1) and treatment of the addition of silver nanoparticles to Curcuma plant extract (S3) in the concentration of liver enzymes ALT and AST and showed no significant differences between treatment S1, S3. The toxic effects of aflatoxin lie in chickens as a result of liver damage and inhibition of protein synthesis, the damage is usually through the hypertrophy, necrosis, cirrhosis of the liver, as it has effects inhibition of protein...
synthesis, leading to the decreased serum concentration of TP and ALB, and increased serum \( \gamma \)-glutamyl activities transferase. AST and ALP are indicators of liver and kidney toxicity, the changes in the concentration of blood metabolites are often associated with corresponding increases in enzyme activity and liver function including ALP, AST [20].

**Table 3:** measurement the liver infection enzyme in broiler blood serum at the age of 42 days.

| Sample | Means ± SE |
|--------|------------|
|        | AST (U/L)  | ALT (U/L) |
| S1     | b 118.83 ± 3.00 | b 6.23±0.35 |
| S2     | a 172.21 ± 3.00 | a 10.62 ± 0.30 |
| S3     | b 121.27 ±1.11 | b 7.35 ± 0.33 |
| P      | **         | **         |

Aromatic medicinal plant extracts and essential oils can be used to increase the activity of the enzymes ALT and AST which ultimately increases the readiness of the chicks during their exposure to aflatoxin poisoning and the possible explanation for the effect of the plant extract is that regardless of the concentration of poison Increase or decrease with pH and note that with pH 6.8 [30].

Table 4 shows that the proportion of hemoglobin, the number of Red blood cells, leukocytes and platelet were 5.23 g /100 ml, 2.08 ×10^6 /ml blood, 14.53 ×10^3, 18% and 0.86% when fed samples on feed contaminated with fungus the product of aflatoxin B1 (S2) and a significant difference compared to the control samples (S1), which in turn did not differ significantly with the treatment model nanoparticles (S3). The results did not differ between S1 samples. These findings are consistent with Gholami- Ahangaran and Ziajahromi, [31] that nanoparticles stimulate erythrocytes. Many researchers also pointed out that nanomaterials have given with aflatoxin-contaminated feed to improve the content of hemoglobin and increase hematocrit in chicken blood, reduce the negative effects of the toxin. The improvement of most of the studied signs in the experiment for broiler meat, represented by physiological characteristics and blood in treatments of aflatoxin B1 contaminated feed treated with silver nanoparticles of curcumin extract, maybe due to the plant extract of various active compounds such as alkaloids, phenols, Coumarins, glycosides and alkaloids that inhibit the growth of many pathological microorganisms. These include bacteria and fungi as well as the widespread use of antioxidants as food additives to provide protection against oxidative decomposition of foods [32].

**Table 4:** measurement of the blood film of broiler blood serum at the age of 42 days.

| Samples | (%) HL Ratio | lymphocyte (%) | Heterophyle (%) | PCV (mg/dl) | WBC (x10^9) | RBC (x10^6 / ml) | Hb (g/dl) |
|---------|--------------|----------------|-----------------|-------------|-------------|-----------------|-----------|
|         | SE           |                |                 |             |             |                 |           |
| S1      | 0.65±0.05    | 51.48±2.78     | 33.66±2.10      | 34.00±1.36  | 23.36±1.42  | 3.80±0.12       | 11.69±1.58|
|         | B            | B              | B               | A           | A           | A               | A         |
| S2      | 0.86±0.001   | 68.23±1.53     | 58.50 ± 1.11    | 18.00±3.57  | 14.53±2.47  | 2.08±0.20       | 5.23±1.19 |
|         | A            | A              | A               | B           | B           | B               | B         |
| S3      | 0.7±0.002    | 49.63±1.05     | 34.63 ± 0.74    | 33.17±0.79  | 22.73±0.52  | 4.00±0.18       | 12.75±0.25|
|         | B            | B              | B               | A           | A           | A               | A         |
| P       | **           | **             | **              | **          | **          | **              | **        |

The superiority of nanoparticle treatment may be due to the fact that the use of silver nanoparticles and their addition to plants are considered as protective additives for animal feed [33]. Studies indicate that there has been often a positive effect of nanosilver on beneficial bacteria in the digestive system of poultry as well as their ability to prevent the development of pathogenic bacteria, as well as their effect on immune response and lipid oxidation in chicken blood [34]. This may be due to the many properties of nanoparticles, particularly their antibacterial activity, without any toxicity to animal cells [35]. This is confirmed by studies of laboratory animals given nanoparticles where the stability of body cells and the immune system is observed, depending on the dose and size of nanoparticles and the method and duration of treatment, as well as the method of production [36].
4.7.2. **Some Histopathological signs in the meat broiler liver samples.** When examining sections of liver tissue for different treatments at the end of the 42 days, it was found that there are normal hepatic lobes and hepatic plaques are not swollen and not broken as we note the absence of inflammatory exudation or haemorrhagic spots around or inside the centre. Also, the hepatic vein with the appearance of the bile duct was natural and no appearance of manifestations of inflammation or damage in hepatocytes and the absence of any accumulation of amyloid protein or fatty spots or bacterial or parasitic concentrations in the two treatments (16,72). However, in the tissue sections of the liver samples of treatment 62, the presence of fatty hepatitis index in a number of hepatocytes later turned into fibroblast tissue instead of healthy tissue. This process leads to high blood pressure in the hepatic veins and thus lead to the explosion of those vessels and the occurrence of hemorrhagic spots on perivascular tissue. Moreover, we notice the occurrence of damage to the walls of hepatocytes and the presence of infiltrations of inflammatory cells. This change leads to the loss of liver function due to loss of tissue function. Between the columns of cells and this indicates, the presence of inflammation and is bound to be a fibrous layer to break the walls of hepatic cells in place of inflammation Figure 9, which affects the function of the cell, tissue, organ and note the presence of exudate inflammatory and points bleeding from blood vessels and congestion in the veins and the presence of images Inflammatory cells.

![Figure 9: Sections of broiler liver tissue samples of 16 and 72 indicating the safety and regularity of hepatic platelets around the hepatic vein as well as bile duct integrity, while 62 samples showed fatty hepatitis in the cells and fibrosis of the tissue (hematoxylin and eosin) X200.](image)

The morphological and histological changes caused by aflatoxin B1 in the liver can lead to functional changes in the liver. Increased abnormal liver size may be associated with impaired liver function due to aflatoxin B1 metabolism of fat and thus increased fat deposition in the liver. The harmful effects of aflatoxinB1 on liver cells occur as a result of increased concentrations of AST and APT enzymes in chicken blood after feeding on diets containing aflatoxin B1 as they participate in the metabolism of the hepatic protein through which cell safety can be determined [37].

5. **Conclusion**
Possibility of synthesis green nanoparticles with a high stability and high storage period, in addition, Increasing the efficiency against *Aspergillus flavus* that produce aflatoxin (*In vitro* and *In vivo*).

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