Hepatoprotective Effect of Coumarin and Chlorophyll Against Aflatoxicosis in Rat

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ABSTRACT

Aflatoxin contamination of animal diet is still a major problem for breeders since it has adverse effects on animal health and productivity. This study was carried out to investigate the effect of using coumarin and/or chlorophyll in rat diet against aflatoxicosis. Fifty four rats were randomly assigned into 7 groups "6 rats each" and group 5 "12 rats". Group 1 was negative control. Group 2 received water with coumarin 0.5%. Group 3 received water with chlorophyll 0.5%. Group 4 received water with coumarin 0.5% and chlorophyll 0.5%. Group 5-8 received basal diet with aflatoxin B1 1000 ppb. Group 6-8 were administered similar treatments as groups 2-4. The experiment ended after 8 weeks. Water and feed consumption were measured and feed conversion rate was calculated. Random glucose level, total lipid, total cholesterol, triglyceride, total protein, ALT, AST, creatinine and urea were determined in serum. Histopathological examination of liver, kidney and pancreas was performed. The conversion rate was significantly low in group 5 compared to groups 6-8. Water and feed consumption were measured and feed conversion rate were calculated. Random glucose level, total lipid, total cholesterol, triglyceride, total protein, ALT, AST, creatinine and urea were determined in serum. Histopathological examination of liver, kidney and pancreas was performed. The conversion rate was significantly low in group 5 compared to groups 6-8. The serum glucose, cholesterol, ALT and AST were elevated in group 5 compared to groups 6-8. The liver lesions observed in group 5 represented in vacuolation and necrosis were alleviated in groups 6-8. The necrosis and inflammatory cells infiltration in pancreas of group 5 were absent in groups 6-8. In conclusion, the coumarin and or chlorophyll possessed a hepatoprotective effect against aflatoxicosis.

Keywords: Aflatoxin, Coumarin, Chlorophyll, Histopathological Examination

INTRODUCTION

Acute aflatoxicosis causes hepatitis, hemorrhage, immune suppression, genetic damage (carcinogenicity, teratogenicity and mutagenicity) and death. Growth impairment and lowering of reproductive performance are the most sensitive clinical signs of chronic aflatoxicosis (Abdelhamid, 2005 a,b and 2009; Shehata, 2010 and Shouman et al., 2012). Scientific efforts were directed towards using physical, chemical and biological techniques for detoxification or inactivation of aflatoxins (Abdelhamid et al., 2002 and Agag, 2003). These techniques have not been used on a commercial scale due to high costs, the need for special facilities, losses of important nutrients and the questionable safety of chemical degradation products of aflatoxins. One of the effective methods to overcome the toxic and carcinogenic effects of aflatoxins is to enhance aflatoxin metabolism towards its detoxification in humans or animals (Tulayakul et al., 2007).

More than 1300 coumarins have been identified from natural sources especially green plants (Hoult and Paya, 1996). Coumarins are antioxidants, contain the nucleus of benzo-a-pyrene and occur in plants like Tonka beans, Sweet clover, Wood ruff, Cassia leaf (Lake et al., 1989). It is present also in a variety of plants families like Loganiaceae (Bhattacharyya et al., 2008), Orchidaceae, Leguminaceae, Rutaceae, Umbelliferae and Labiatae (Vyas et al., 2009). The synthetic coumarin (4-methyl-7-hydroxycoumarin) derived from resorcinol and ethyl acetate-accepte in presence of concentrated sulphuric acid is structurally close to scopoletin, being a coumarin derivative. Naturally derived and synthetic coumarins have been used in treatment of oedemas (Clasley-Smith et al., 1993), and possessed anti-canceror anti-tumorigenic (Prince et al., 2009 and Battacharyya et al., 2009), anti-mutagenic (Pillai et al., 1999), antibacterial (Devvienne et al., 2005), anticagulants, anti-thrombotic and vasodilatory effects (Hoult and Paya, 1996).

Clorophyllin (CHL), a water-soluble form of chlorophyll, was recently evaluated as chemopreventive agent in a population at high risk for exposure to aflatoxin and subsequent development of hepatocellular carcinoma (Kensler et al., 2002 and Kumer et al., 2012). CHL, which is used extensively as a food colorant, has numerous medicinal applications. CHL is a safe and effective agent suitable for use in individuals unavoidably exposed to aflatoxins (Enger et al., 2003). Naturally chlorophyll and CHL strongly inhibits aflatoxin B1, prenplasia biomarkers, stomach and liver tumor in rats and rainbow trout (Sudakin, 2003; Simonich et al., 2007 and 2008). Moreover, the CHL was more effective than chlorophyll-A in reducing aflatoxin B1-DNA adduct and liver tumor (Dashwood et al., 1998).

The Goal Of The Research

It is known that aflatoxins are cause serious Effects so that these effects can be mitigated by using some natural compounds.

MATERIALS AND METHODS

Animals

Fifty four Sprague – Dawley male albino rats with average body weight 90±10 g was used. Were obtained from the farm of The Egyptian Organization of Biological products and Vaccines, Egypt. The animals were housed individually in stainless steel cases in a controlled environment 25±5°C, 50-60% relative humidity and 12 hours light-dark cycle, all over the experimental period (8 weeks). Free access to water and maintenance ration twice daily were available. This study was in accordance with Institutional Animal Use and Care Committee (IACUC) guidelines, Cairo University.

Coumarin preparation

Coumarin (4-Methyl-7-hydroxycoumarin) was prepared according to method of Furniss et al. (1989) which is summarized as follow: 1 liter of concentrated sulphuric acid was placed in a 3 liter necked flask. The flask was immersed in an ice bath; a solution of
Precipitated by storing in the freezer at W20 °C for 1 hr. Then it was poured with vigorous stirring into a mixture of crushed ice and water, the crude yield (yield = 155 g, 97% concentration) was collected, recrystallization in ethanol 95% and air dried. The dried coumarin was added to water 0.5%. Coumarin standard from sigma Aldrich.

Chlorophyll preparation

All chlorophyll extractions were performed under dim green light to minimize photo-oxidative reaction (Shio, 2006). Fresh spinach leaves were purchased from local market. Spinach leaves were separated from the stem and then about 100 g were weighed and put into a blender. Cold acetone was added as much as 500 ml. Then the spinach leaves were grinded in a blender for about 3 min. The filtrate of crude chlorophyll was separated using a Buchner funnel with a Whatman filter paper No.1. The residual solids that are not filtered was washed with 100 ml of acetone. Then the filtrate was added to dioxane approximately one seventh of its volume. After that, the mixture was added with deionized water as much as one seventh of the volume of the mixture by drop wise and then stirred using a magnetic stirrer. The mixture was precipitated by storing in the freezer at -20 °C for 1 hr until dark green precipitate was obtained at the bottom of the solution and a yellow liquid on top. The precipitate of crude chlorophyll was separated by filtration using two layers of Whatman filter paper No. 1 with a Buchner funnel. The solution then was added with dioxane approximately one seventh of its volume. After that, the mixture was added with deionized water as much as one seventh of the volume of the mixture by drop wise and then stirred using a magnetic stirrer. The mixture was precipitated by storing in the freezer at -20 °C for 1 hr until dark green precipitate was obtained at the bottom of the solution and a yellow liquid on top. The precipitate of crude chlorophyll was separated by filtration using two layers of Whatman filter paper No.1. The residual solids that are not filtered was washed with 100 ml of acetone. Then the filtrate was added to dioxane approximately one seventh of its volume. After that, the mixture was added with deionized water as much as one seventh of the volume of the mixture by drop wise and then stirred using a magnetic stirrer. The mixture was precipitated by storing in the freezer at -20 °C for 1 hr until dark green precipitate was obtained at the bottom of the solution and a yellow liquid on top. The precipitate of crude chlorophyll was separated by filtration using two layers of Whatman filter paper No.1. The residual solids that are not filtered was washed with 100 ml of acetone. Then the filtrate was added to dioxane approximately one seventh of its volume. After that, the mixture was added with deionized water as much as one seventh of the volume of the solution while slowly stirred. The precipitation was then performed for the second time in the same way at -20 °C for 1 hr. After that the precipitate was filtered using a Buchner funnel and two layers of Whatman paper No.1. The crude chlorophyll solids were filtered and diluted with acetone until colorless paper return. Then a solution of crude chlorophyll is evaporated from the solvent so that only the remaining solid green crude chlorophyll was left. Thus, the chlorophyll was dissolved using the small volume of diethyl ether in order to obtain the chlorophyll solution prior to any further separation using column chromatography according to (Suendo et al., 2010).

Aflatoxin preparation

Aflatoxin production was carried out according to Davis et al. (1966) using liquid media (2% yeast extract and 20% sucrose) and Aspergillus flavus stain (NRRL 3145). The media which contained detectable amount of aflatoxin was mixed well with the basal diet to get the aflatoxin-contamination diet. The total aflatoxin content in liquid medium was determined according to Roos et al. (1997) and AOAC. (2006) method using monoclonal antibody columns for total aflatoxins (Vicam Science Technology, water town, MA, USA). Aflatoxin identification was performed by a modification of the HPLC – AFLATEST procedure Agilent 1200 series USA. HPLC equipment with two pumps, column C18, Lichrospher 100 RP-18, (5 Nm × 25 cm) was used. The mobile phase consisted of water: methanol: acetonitrile (54: 29:17, v/v/v) at flow rat of 1 mL/min. The excitation and emission wavelengths for all aflatoxins were 362 and 460 nm (Flourcenses detector), respectively.

Experimental design

Fifty four rats were randomly assigned into 7 groups "6 rats each" and group 5 "12 rats". Group 1 received basal diet (negative control) (G1). Group 2 received basal diet and water with coumarin 0.5% (G2). Group 3 received basal diet and water with chlorophyll 0.5% (G3). Group 4 received basal diet, water with coumarin 0.5% and chlorophyll 0.5% (G4). Group 5 received basal diet with aflatoxin B1 1000 ppb (positive control) (G5). Group 6 received basal diet with aflatoxin and water with coumarin 0.5% (G6). Group 7 received basal diet with aflatoxin and water with chlorophyll 0.5% (G7). Group 8 received basal diet with aflatoxin and water with coumarin 0.5% and chlorophyll 0.5% (G8). The ingredients of basal diet are shown in Table 1. Daily fresh water was available the whole time. At the last day of the experiment, the rats were fasted over night. The experiment was terminated after 8 weeks and animals were euthanized by cervical decapitation.

Table 1. Composition of basal diet

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>20.0</td>
</tr>
<tr>
<td>Corn starch</td>
<td>65.0</td>
</tr>
<tr>
<td>Mineral mix</td>
<td>3.5</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>1.0</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>0.3</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>0.2</td>
</tr>
<tr>
<td>Cellulose powder</td>
<td>5.0</td>
</tr>
<tr>
<td>Corn oil</td>
<td>5.0</td>
</tr>
</tbody>
</table>

1-Based on AIN-76 "American Institute of Nutrition"

Vitamins based on AIN-76 | Minerals based on AIN-76
---|---
Vitamin A | 4.00 IU/g Calcium | % 0.52
Vitamin D3 | 1.00 IU/g Phosphorus | % 0.44
Alpha-Tocopherol | 64.24 IU/kg Potassium | % 0.38
Thiamine | 5.90 ppm Sodium | % 0.11
Pantothenic Acid | 15.26 ppm Magnesium | % 0.05
Choline | 104.00 ppm Iron | 34.25 ppm
Pyridoxine | 7.1 ppm Zinc | 36.76 ppm
Folic Acid | 2.10 ppm Manganese | 59.34 ppm
Biotin | 0.21 ppm Copper | 6.73 ppm
Vitamin B12 | 10.10 mcg/kg Cobalt | 0.02 ppm
Vitamin K | 0.50 ppm Iodine | 0.21 ppm

Prepared as Rodent Diet AIN-76A

Serum analysis

Blood samples were collected from each rat from the retroorbital vein and were received into clean dry centrifuge tubes. Serum was separated by centrifugation at 3000 rpm for 15 minutes and kept in deep-freezer at -20 °C until used for estimation of random glucose level, total lipid, total cholesterol, triglyceride, total protein, ALT, AST, creatinine and urea by using Biodiagnostic Kits.

Histopathological examination

Liver, kidneys and pancreas samples were collected at the end of the experiment and were fixed in 10% neutral buffered formalin solution for at least 48 hrs. Tissue specimens were processed using routine paraffin embedded technique in which tissues was dehydrated in ascending concentration of alcohol and cleared in xylene. Sections 3-5μ thick were made using microtome (Leica 2135) and stained with Haematoxylin and Eosin (H&E).
(Suvarna et al., 2012). The histopathological alterations in liver, kidney and Pancreas were examined by light microscopy and photographed using camera Olympus XC30 (Tokyo, Japan).

RESULTS AND DISCUSSION
Weight gain and water consumption
Aflatoxin contaminated diet decreased total and daily body weight gain, daily feed intake and water consumption (Table 2). These results agree with the finding of Shehata (2002 and 2010), Meshrey et al. (2007) on rabbits and Shehata et al. (2009 a,b) and Mehrim et al. (2016) on fish. The reduction in body weight gain by aflatoxin is not only due to depression of feed intake, but may also be due to the reduction in metabolism of protein, lipids, carbohydrate and dissolved vitamin in lipid (Maria and Askar, 2008). Also, it might be due to detoxification process in the body utilizing glutathione enzymes. Glutathione is the intracellular antioxidant (Deng et al., 2010) and partly composed of methionine and cysteine, hence this detoxification process depletes the metabolic availability of methionine leading to poor growth and feed conversion. Adding coumarin to diet improved growth performance, the beneficial effect of coumarin may be due to: (1) reduction AFB1-DNA adducts formation by both liver and intestinal microsomes. Coumarin enhanced aflatoxicol formation; therefore, decrease AFB1-DNA adduct, because direct interaction of aflatoxicol epoxide with DNA is major compared with AFB1 epoxide, (2) enhancement of glutathione -s- transferase (GST) activity in the intestine to conjugate AFB1, (3) suppression of P450 enzyme activity in the lives and the enhancement of GST in the intestine (Tulayakul et al., 2007), and (4) improving liver function and body health.

The beneficial effect of CHL may be due to: (1) CHL form molecules complexes with carcinogens material and block the bioavailability therefore reduce the hazard effects on organs body functions,(2) CHL modify the genotoxic effects of aflatoxin B1 by inhibiting bioactivation pathways and stimulating detoxification pathways (Sudakin, 2003), (3) CHL enhance the activities of antioxidant enzymes such as glutathione peroxidase, superoxide dismutase catalase and glutathione -s- transferase, indicating that CHL possess a potent protective effect against of aflatoxin B1 (kumar et al., 2012 ),(4) CHL reduce multi organs aflatoxin B1-DNA adducts (Simonich et al., 2007).

Table 2. Body weight gain, food intake and Consumer water of different experimental group for 8 week

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Initial weight (g)</th>
<th>Final weight (g)</th>
<th>Body weight gain (g)</th>
<th>Bwg / Initial weight (%)</th>
<th>Weekly body weight gain (g)</th>
<th>Average daily Feed Intake (g)</th>
<th>Average daily Feed/gain</th>
<th>Water consumption (ml)/day</th>
<th>Weight gain from the control%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>117.58±</td>
<td>241.25±</td>
<td>123.67±</td>
<td>105.18</td>
<td>15.46</td>
<td>21.00±</td>
<td>1.36</td>
<td>14.9±</td>
<td>100</td>
</tr>
<tr>
<td>Coumarin</td>
<td>121.78±</td>
<td>280.5±</td>
<td>168.73±</td>
<td>150.07</td>
<td>21.10</td>
<td>25.60±</td>
<td>1.21</td>
<td>17.6±</td>
<td>142.68</td>
</tr>
<tr>
<td>Chlorophyll</td>
<td>118.67±</td>
<td>230.33±</td>
<td>111.67±</td>
<td>94.09</td>
<td>13.96</td>
<td>1.21±</td>
<td>1.56</td>
<td>15.1±</td>
<td>106.48</td>
</tr>
<tr>
<td>Coumarin +</td>
<td>117.67±</td>
<td>260±</td>
<td>142.33±</td>
<td>121</td>
<td>17.80</td>
<td>41.77±</td>
<td>2.35</td>
<td>16.9±</td>
<td>115.04</td>
</tr>
<tr>
<td>Chlorophyll</td>
<td>2.14±</td>
<td>9.06±</td>
<td>0.50±</td>
<td>1.80</td>
<td>1.22</td>
<td>0.50±</td>
<td>0.96±</td>
<td>115.04</td>
<td></td>
</tr>
<tr>
<td>Aflatoxin</td>
<td>114.33±</td>
<td>221.67±</td>
<td>107.33±</td>
<td>93.88</td>
<td>17.89</td>
<td>21.37±</td>
<td>1.20</td>
<td>13.5±</td>
<td>89.26</td>
</tr>
<tr>
<td>Aflatoxin +</td>
<td>127.17±</td>
<td>248.33±</td>
<td>121.17±</td>
<td>95.28</td>
<td>15.15</td>
<td>33.90±</td>
<td>2.24</td>
<td>16.5±</td>
<td>90.59</td>
</tr>
<tr>
<td>Coumarin</td>
<td>1.65±</td>
<td>10.55±</td>
<td>8.90±</td>
<td>88.6</td>
<td>13.29</td>
<td>29.47±</td>
<td>2.22</td>
<td>15.7±</td>
<td>84.24</td>
</tr>
<tr>
<td>Chlorophyll</td>
<td>1.32±</td>
<td>12.25±</td>
<td>10.93±</td>
<td>2.86±</td>
<td>0.52±</td>
<td>2.86±</td>
<td>0.52±</td>
<td>84.24</td>
<td></td>
</tr>
<tr>
<td>Aflatoxin + Coumarin</td>
<td>121.5±</td>
<td>256.83±</td>
<td>135.33±</td>
<td>111.38</td>
<td>16.92</td>
<td>28.42±</td>
<td>1.68</td>
<td>16.6±</td>
<td>105.89</td>
</tr>
</tbody>
</table>

All values are represented as mean ±S.E.
Means with different letters are significantly different (p<0.05).
H (Higher than normal rang), L (Lower than normal rang)

Biochemical findings
Results in Table 3 revealed that positive controls (group receiving basal diet with aflatoxin) gave a significant increase in serum liver function enzymes, alanine amino transferase (ALT) and aspartate amino transferase (AST). Furthermore, this group showed significantly higher level of serum urea, creatinine, cholesterol and triglycerides as compared to healthy group fed the basal diet. Increasing AST and ALT activities may be due to hepatocellular necrosis or increasing the permeability of cell membrane (Zaky et al., 2000). On the other hand, the increased concentrations of urea and creatinine indicate kidney injury caused by aflatoxins toxicity. These results coincide with those reported by Zohair. (1996) in treated rats and those of Matri (2001) who reported that Japanese quail birds receiving contaminated feed with aflatoxin showed significantly higher serum total cholesterol, creatinine and urea. The total protein was significantly decreased in the group fed the aflatoxin contaminated diet. These finding were in accordance with previous studies (Abd El Baki et al., 2002 and Shehata, 2002 and 2010). Decreasing of serum protein may be attributed to degeneration of endoplasmic reticulum and inhibition of protein synthesis (Srivastava, 1984). Addition of coumarin CHL alone and coumarin with CHL improved blood parameters measured. These results may be due to the effect of coumarin and CHL on detoxification of aflatoxin (Kelly et al., 2000 and Tulayakul et al., 2007) and improvement of body health (Prince et al., 2009).
Table 3. Serum AST, ALT, urea, creatinine, total protein, glucose, total cholesterol and total triglycerides levels in the different experimental groups.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Treatment</th>
<th>AST (IU/L)</th>
<th>ALT (IU/L)</th>
<th>Urea (mg/dl)</th>
<th>Creatinine (mg/dl)</th>
<th>Total protein (g/dl)</th>
<th>Glucose (mg/dl)</th>
<th>Total cholesterol (mg/dl)</th>
<th>Total triglycerides (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>36.6± 32</td>
<td>4.0± 1</td>
<td>27.0± 2</td>
<td>1.76± 1</td>
<td>8.6± 1</td>
<td>108.0± 8</td>
<td>79.3± 7</td>
<td>40.6± 7</td>
</tr>
<tr>
<td>Coumarin</td>
<td></td>
<td>1.33± 4</td>
<td>2.0± 3</td>
<td>1.76± 1</td>
<td>0.03± 1</td>
<td>0.73± 1</td>
<td>5.6± 1</td>
<td>1.45± 1</td>
<td>1.42± 1</td>
</tr>
<tr>
<td>Chlorophyll</td>
<td></td>
<td>38.67± 1</td>
<td>34.00± 1</td>
<td>37.33± 0.75</td>
<td>0.75± 0.03</td>
<td>8.67± 1</td>
<td>149.67± 7</td>
<td>73.00± 7</td>
<td>67.67± 7</td>
</tr>
<tr>
<td>Cholorphyll</td>
<td></td>
<td>3.18± 4</td>
<td>2.31± 1</td>
<td>1.76± 1</td>
<td>0.03± 1</td>
<td>0.33± 1</td>
<td>2.73± 1</td>
<td>2.52± 1</td>
<td>2.03± 1</td>
</tr>
<tr>
<td>Coumarin + Chlorophyll</td>
<td></td>
<td>27.00± 1</td>
<td>34.00± 1</td>
<td>45.33± 0.81</td>
<td>0.81± 0.03</td>
<td>8.33± 1</td>
<td>109.00± 7</td>
<td>73.67± 7</td>
<td>87.00± 7</td>
</tr>
<tr>
<td>Aflatoxin</td>
<td></td>
<td>2.52± 4</td>
<td>2.31± 1</td>
<td>3.38± 0.04</td>
<td>0.04± 0.02</td>
<td>0.67± 1</td>
<td>4.05± 1</td>
<td>2.01± 1</td>
<td>3.11± 1</td>
</tr>
<tr>
<td>Coumarin + Aflatoxin</td>
<td></td>
<td>35.00± 4</td>
<td>32.67± 1</td>
<td>45.33± 0.77</td>
<td>0.77± 0.03</td>
<td>8.50± 1</td>
<td>105.67± 7</td>
<td>76.33± 7</td>
<td>60.83± 7</td>
</tr>
<tr>
<td>Cholorphyll</td>
<td></td>
<td>4.04± 4</td>
<td>2.31± 1</td>
<td>3.38± 0.03</td>
<td>0.03± 0.02</td>
<td>0.29± 1</td>
<td>2.33± 1</td>
<td>2.03± 1</td>
<td>1.36± 1</td>
</tr>
<tr>
<td>Aflatoxin</td>
<td></td>
<td>15.23± 4</td>
<td>14.53± 1</td>
<td>83.00± 1.86</td>
<td>1.86± 0.1</td>
<td>3.60± 1</td>
<td>180.33± 7</td>
<td>212.33± 7</td>
<td>295.00± 7</td>
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<tr>
<td>Aflatoxin + Coumarin</td>
<td></td>
<td>4.41± H</td>
<td>4.63± H</td>
<td>3.21± 0.5 H</td>
<td>0.05± 0.02</td>
<td>0.28± L</td>
<td>4.84± H</td>
<td>2.73± H</td>
<td>33.6± H</td>
</tr>
<tr>
<td>Cholorphyll</td>
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<td>57.00± 4</td>
<td>35.00± 1</td>
<td>52.33± 0.76</td>
<td>0.76± 0.03</td>
<td>8.50± 1</td>
<td>78.00± 7</td>
<td>76.33± 7</td>
<td>76.33± 7</td>
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<tr>
<td>Aflatoxin + Chlorophyll</td>
<td></td>
<td>2.08± 3</td>
<td>2.08± 3</td>
<td>2.90± 0.02</td>
<td>0.02± 0.02</td>
<td>0.29± 1</td>
<td>1.73± 1</td>
<td>2.03± 1</td>
<td>2.03± 1</td>
</tr>
<tr>
<td>Aflatoxin + Coumarin + Cholorphyll</td>
<td></td>
<td>66.33± 4</td>
<td>39.00± 1</td>
<td>51.33± 0.75</td>
<td>0.75± 0.03</td>
<td>6.17± 1</td>
<td>86.00± 7</td>
<td>81.33± 7</td>
<td>87.67± 7</td>
</tr>
<tr>
<td>Aflatoxin + Chlorophyll</td>
<td></td>
<td>2.90± H</td>
<td>2.31± 1</td>
<td>2.84± 0.35</td>
<td>0.35± 0.1</td>
<td>0.17± 1</td>
<td>2.31± 1</td>
<td>1.85± 1</td>
<td>1.76± 1</td>
</tr>
<tr>
<td>Aflatoxin + Coumarin + Chlorophyll</td>
<td></td>
<td>52.33± 4</td>
<td>40.67± 4</td>
<td>48.67± 0.87</td>
<td>0.87± 0.03</td>
<td>7.67± 1</td>
<td>80.33± 7</td>
<td>79.67± 7</td>
<td>101.33± 7</td>
</tr>
<tr>
<td>All values are represented as mean ±S.E.</td>
<td></td>
<td>H (Higher than normal range), L (Lower than normal range).</td>
<td></td>
<td></td>
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Histopathological Findings

In the current study, there was vacuolation of hepatocytes, karyomegaly and binucleation of hepatocytic nuclei in the liver of rats administrated aflatoxin (Figure 1a) coinciding with the degenerative changes induced by aflatoxin administration in the hepatic tissues of rats (Abdel-Wahhab et al., 2002). Moreover, portal mononuclear inflammatory cells infiltration and focal areas of hepatocyte necrosis accompanied with mononuclear cells infiltration in the liver were demonstrated in the present study similar to histopathological changes recorded in previous studies (Naidu et al., 1991; Salem et al., 2001 and El-Shewy and Ebrahim, 2004) which showed that the main lesions in liver of rats exposed to aflatoxin were progressive hepatic degeneration, multifocal hepatic necrosis, bile ductular proliferation and areas of altered hepatocytes.

The liver histopathology of rats in the groups administered aflatoxin and receiving coumarin and/or chlorophyll exhibited an apparently normal hepatic structure in which the hepatocyte vacuolation and necrosis was almost diminished (Figure 1b, c, d). This improvement was more obvious in the group receiving coumarin and the group receiving coumarin and chlorophyll. Subsequently, the coumarin and chlorophyll is believed to have exerted a protective effect against aflatoxin toxicity. Similarly, in a previous study, the chemoprotective effect of coumarin was evident against aflatoxin B1 induced mutations and cytotoxicity in the presence of chick embryo liver S9 but not with rat liver S9. It was said that coumarin reduced the invitro formation of aflatoxin B1 8, 9- epoxide involved in aflatoxin toxicity (Goeger et al., 1998). Furthermore, adding coumarin (2.5 g coumarin/ Kg diet) to aflatoxin B1 contaminated rabbit diet was found to be safe and practical method to minimize aflatoxin B1 toxicity (Halel et al., 2014).

Perivascular mononuclear inflammatory cells and interlobular edema and mononuclear inflammatory cells infiltration were demonstrated in the pancreas of rat administered aflatoxin in accordance with previous studies (Figure 1c) (Beger et al., 2000, Şimşek et al., 2007, and Abd El-Haleem and Mohamed, 2011). Aflatoxin ingestion leads to reactive oxygen species production that regulates chemokine receptor expression and also facilitates the recruitment and localization of polymorphonuclear leukocyte to the site of infection and inflammation (Shephard, 2003). Additionally, in this study, perivascular fibrosis in the pancreas was observed in the rats administered aflatoxin. Similarly, the pancreas revealed many collagen fibers, especially around ducts and blood vessels in previous studies (Abd El-Haleem and Mohamed, 2011). Stellate cells observed in both rat and human pancreas was thought to play a role in the development of pancreatic fibrosis (Bachem et al., 1998). They were stimulated to synthesize collagen due to oxidative stress (Casini et al., 2000). In such case the oxides generated during the metabolic processing of aflatoxin B1 results in oxidative stress that in turn induce stellate cells to produce collagen. Additionally, Perivascular mononuclear inflammatory cells and interlobular edema and mononuclear inflammatory cells infiltration were demonstrated in the pancreas of rat administered aflatoxin in accordance with previous studies (Beger et al., 2000; Şimşek et al., 2007 and Abd El-Haleem and Mohamed, 2011). Aflatoxin ingestion leads to reactive oxygen species production that regulates chemokine receptor expression and also facilitates the recruitment and localization of polymorphonuclear leukocyte to the site of infection and inflammation (Shephard, 2003).

In this study, there was paucity and cytoplasmic vacuolation in the langerhan’s cells in compliance to previous researchers’ observations who recorded vacuolation of few islet cells and showed that most of the β cells had rounded euchromatic nuclei, whereas some cells had small condensed heterochromatic nuclei (Abd El-Haleem and Mohamed, 2011). Aflatoxin as described previously induces oxidative stress due to generation of oxides and β cells are particularly vulnerable to oxidative stress because of their low levels of antioxidant enzyme expression (Kaneto et al., 2005). Ensuing the damage to B cells, the serum glucose concentration is increased due to a decrease in serum insulin as evidenced in this study by increased random blood sugar in the group administered aflatoxins. On the other hand, the pancreatic lesions were remarkably alleviated in the groups administered aflatoxin and treated with coumarin and/or chlorophyll.
suggesting a protective effect of these treatments against oxidative stress induced by aflatoxin (Figure 1 f, g, h).

The most predominant lesions observed in the kidneys of rats administered aflatoxin in the present study were congestion of intertubular blood vessels, hemorrhage, vacuolar degeneration and necrosis of tubular epithelium, presence of hyaline droplets in the lumen of renal tubules and focal areas of peritubular mononuclear inflammatory cells infiltration (Figure 1+1). Likewise to previous studies which recorded the dilatation and engorgement of renal blood vessels and intertubular capillaries with blood, focal areas of cloudy swelling of the lining epithelium of convoluted tubules together with presence of some eosinophilic debris in some lumen of other tubules and leucocytic cellular infiltration of renal interstitial tissues particularly lymphocytes with shrinkage of the glomerular tufts (El-Shewy and Ebrahim, 2004). The groups that were administered coumarin and chlorophyll showed a remarkable improvement of the kidney lesions caused by aflatoxin (Figure 1j, k, l).

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Figure 1. (a-d) Liver, rat. (a) vacuolation and karyomegaly of hepatocytes in G5 receiving aflatoxin 1000 ppb in diet. (b,c,d) apparently normal hepatocytes in G6 receiving aflatoxin in diet and coumarin in water, in G7 receiving aflatoxin in diet and chlorophyll in water and in G8 receiving aflatoxin in diet, coumarin and chlorophyll in water, respectively. (X 200). (e-h) Pancreas, rat. (e) Interlobular edema and mononuclear cells infiltration in G5 receiving aflatoxin in diet. (f, g, h) showing apparently normal pancreatic structure (normal islets of langerhans and normal acini tissues G6 receiving aflatoxin in diet and coumarin in water, in G7 receiving aflatoxin in diet and chlorophyll in water and in G8 receiving aflatoxin in diet, coumarin and chlorophyll in water respectively (X 400). (i-l) Kidney, rat. (i) Focal area of peritubular mononuclear inflammatory cells infiltration in G5 receiving aflatoxin in diet. (j) Apparently normal renal structure with few intratubular casts in G6 receiving aflatoxin in diet and coumarin in water (k) congestion of intertubular blood vessels and slight vacuolation of renal tubular epithelium in G7 receiving aflatoxin in diet and chlorophyll in water. (l) Apparently normal renal structure with vacuolation of few renal tubular epithelium in G8 receiving aflatoxin in diet, coumarin and chlorophyll in water (X 200). Hematoxylin and eosin stain.
The beneficial effect of coumarin may be due to: 1- Reduction of aflatoxin B$_1$-DNA adducts formation by both liver and intestinal microsomes (coumarin enhanced aflatoxicol formation therefore decrease aflatoxin B$_1$-DNA adducts, because direct interaction of aflatoxicol-epoxide with DNA is minor compared with aflatoxin B$_1$-epoxide 

1987), 2- Enhancement of glutathione S transferase (GST) activity in the intestine to conjugate aflatoxin B$_1$, 3- Suppression of p 450 enzyme activity in the liver and enhancement of GST in the intestine (Tulayakul et al., 2007); 4- improving liver function (Gilan and Janbaz, 1993) and body health (Maucher et al., 1994; Houl and Paya, 1996; Pillai et al., 1999; Devienne et al., 2005) and 5- increasing the digestibility of crude protein and ether extract (Ko et al., 2006).

**CONCLUSION**

Results indicated that addition of coumarin and / or chlorophyll to rat aflatoxin contaminated diet was safe and practical meth.

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Tأثير الكومارين والكلوروفيل على حماية الكبد ضد التسمم الألفا-توتوكسيني في الفئران

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Abdel-Latif, M. S. et al. 490