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MOLECULAR IDENTIFICATION OF *idh* GENE OF THE PATULIN TOXIN PRODUCING FROM *PENICILLIUM EXPANSUM* ISOLATES AND STUDYING OF ITS TOXIC EFFECT IN MALE MICE

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ABSTRACT
Mycotoxins are toxic secondary metabolites produced by fungi, mostly by saprophytic molds growing on a variety of foodstuffs. The aim of this study is to isolate and identification of fungi from apple fruits and studying the toxic effect of Patulin (PAT) and *Penicillium expansum* in some physiological blood criteria, hormonal and biochemical in male mice. Species of Penicillium were isolated and identified in apple fruit samples collected from Baghdad region. *Penicillium expansum* as identified to be the primary species in the population (100%) becoming a potential source of Patulin contamination in the fruits. *P. expansum* isolates were identified depending on the macroscopic characteristics, including colony diameter, colony colors, colony texture, conidial color and reverse color. Results molecular identification by PCR technique has been used to amplify the gene of the *idh* gene from genomic DNA of all *P. expansuin* isolates. Results showed the ability four isolates of *P. expansuin* fungus of producing patulin is carrying the regulatory *idh* gene, and responsible for regulating the Patulin production when 488 bp. The effects of mycotoxin Patulin (PAT) and *P. expansum* mycelium on physiological blood criteria parameters of mice compared with control group. Reached HB, WBC, RBC is the Patulin mycotoxin (1.5 ml/kg) (9.4, 6000, 10.9) respectively, and *P. expansum* mycelium were (11.0, 5205, 8.12) Respectively, compared with control group (12.4, 3800, 5.17) respectively. The effects of Patulin mycotoxin (PAT) and *P. expansum* mycelium Figure (6, 7, 8, 9, 10, 11, 12), on hormonal and biochemical (GOT, GPT, Cholesterol, Blood Sugar, FST, T3 and T4). The Patulin mycotoxin (1.5 ml/kg) (9.3, 11.3, 118, 113, 6.1, 1.1 and 1.8) respectively, and *P. expansum* mycelium were (7.77, 8.30, 121.5, 100, 8.2,0.92 and 2.4) Respectively, compared with control group (6.9, 5.83, 144.5, 85.25, 12.38, 1.41, 5.41) Respectively.

KEY WORDS: *idh* gene, patulin toxin.

INTRODUCTION

Mycotoxins are natural secondary metabolites produced by microorganisms of kingdom fungi, result in health and environmental threat beside economic losses (Richard, 2007). Mycotoxins are highly toxic compounds of small molecular weight and quite stable molecules which are extremely difficult to remove or eradicate, and which enter the food chain while keeping their toxic properties (Reddy *et al*., 2010). Among the most important mycotoxins was Patulin (PAT) is soluble in water and most polar organic solvents – soluble, Patulin is a mycotoxin produced by a number of fungi common to fruit- and vegetable-based products (Magan *et al*., 2004). Patulin is a metabolite produced by *Penicillium expansum*, Patulin has been shown to be genotoxic, cytotoxic, immunotoxic, and neurotoxic in both *in vivo* and *in vitro* studies (Barkai-Golan, 2008). Patulin can harm the immune system and gastrointestinal tract. Also shows carcinogenic, teratogenic, and mutagenic properties toward differentiated mammalian cells, including human cells (Alves *et al*., 2000; Schumacher *et al*., 2005). Exposure to mycotoxins in humans and animals may occur through ingestion, inhalation, and dermal contact, mycotoxicoses in humans or animals that have been characterized as food or feed related, non-contagious, nontransferable, and non-infectious (Zain, 2011). PAT has some health effects (Agitation, convulsions, dispenses, pulmonary congestion edema, hyperemia, GI tract distension, Nausea, intestinal hemorrhage, Ulceration (Matthew *et al*., 2005). The health risks of PAT for humans include acute and chronic symptoms and its effects at a cellular level, in acute toxicity, PAT is toxic primarily through affinity to sulfhydryl groups (SH), which results in inhibition of enzymes. Oral LD_{50} in rodent models have ranged between 20 and 100 mg/kg (Puel *et al*., 2010). Selmanoglu and Kockaya, (2004) measured thyroid and testicular hormones in rats receiving 0.1 mg PAT/kg B.w./day PAT by the oral route for 60 or 90, days a sixty-day exposure increased the plasma level of testosterone and decreased T4 hormone while there was no change in T3, TSH, LH and GH, when the exposure lasted for 90 days, there was an increase in testosterone and in LH without any other clinical signs. Therefore, this study aimed to molecular identification of patulin Producing from *Penicillium expansum* isolates and studying the toxic effect of PAT and *Penicillium expansum* in some Physiological Blood Criteria, Hormonal and Biochemical in male mice.

MATERIALS & METHODS

Culture Medium

Potato Dextrose Agar (PDA)

It was prepared to dissolve 39 g. Of (PDA) powder in1000 ml of distilled water and sterilized the medium in

autoclave (121 and under 15 lbs / In ² pressure for 20 min.), then added 250 mg of chloramphenicol in 1L of sterilized culture medium to eliminate the bacteria. The medium was used for isolation and identification of fungi.

Collection of samples

Fifteen samples of apple fruits collected from local markets in Baghdad in November of 2015. All samples were stored in polyethylene bags and kept at 4ºC before use.

Isolation and identification of fungi

Apple fruits were surface sterilized by using 70% ethanol, the fruits cut into small pieces (0.5 cm), soaked into 1% sodium hypochlorite solution for 3 min. Samples were dried with sterile filter paper, cultured on PDA.Three pieces on each plate incubated at 25°C for 7 days. Pure culture was obtained by sub-culturing many times, then identified on the basis of their morphological characters by observing colony feature (colony and texture) and microscopically and observe under a microscope for the conidia, conidiophores, and arrangement of conidia. Fungi identification was based on the morphological characteristics as described by (Samson and Pitt, 1985; Pitt and Hocking, 2009).

Patulin toxin *concentration*

Patulin toxin was obtained from College of Science – department of biology – Mustansiriyah University, using concentrations 0.5, 1.0 and 1.5 ml/kg.

Molecular identification Primers

The primers which used in PCR for detection *Penicillium expansum* were obtained from Bioneer, Korea, which used ITS1 and using NCBI- Genbank.

TABLE 1: PCR Primer used for *idh* gene involved in patulin Biosynthesis

Gene	Primer	Sequence (5' – 3')		PCR product size Primer
idh	ITS1	Forward	5' primer sequence (5 'AATGTGTACTGACTGGTCGCAG)	488bp
		Reverse	3' primer sequence (5 'CAACCAACATATTCGTGCCTGAC)	

DNA extraction

Fungal mycelium was obtained from *Penicillium expansum* isolates grown on PDA for 7 days at 25°C. One g mycelia in liquid nitrogen using a mortar and pestle. The sample was transferred to a 15 ml tube with 4 ml of extraction buffer (200 metros, 250 mMNaCl, 25 mm EDTA, pH 8.5, 0.5% SDS), and mixed thoroughly by vortexing and then incubated at 56°C for 30-60 min, 100 µluniversal. Buffer PF was added, then mixed by inverting, and incubated at -20°C for 5 min. Mixture tube centrifuged at 12,000 for 5 minutes at 25C°. The supernatant was transferred to a sterile tube and 1.5 ml of chloroform was carefully added. Tubes were centrifuged 9,000 x g (12,000 RPM) for 5 min. The top layer was removed to a new tube and 10 µlofRNAse A (100 mg/ml) was added; tubes were incubated at 25°C for 10 min., 2 ml of 5 M LiCl was added to precipitate the RNA and tubes were placed on ice for 15 min and then centrifuged for 1 min at 9,000 x g (12,000 RPM) to elute the DNA. Total DNA was precipitated with isopropanol and resuspended in 30 µl of TE buffer (10mM Tris pH 8 and 1 mm EDTA) and stored at -20˚C until use.

Detection of patulin producing from isolated *Penicillium expansum* by Molecular identification

For molecular identification of patulin Producing from isolates *Penicillium expansum*, PCR Technique was carried out four of the isoepoxydon dehydrogenase gene (*idh*) gene. The name of the gene, their primer sequences and their product size is shown in Table 1. All PCR reagents were supplied by Bioneer (Korea), primers designed from Thermo scientific and all PCR attempts were carried out in PCR Thermal Cycler. Thermal cycle conditions were carried as mentioned (Cardoso *et al.*, 2007). PCR amplified products were checked on 1.8 % agarose by gel electrophoresis and visualized under Gel documentation system and detecting the gene *idh*.

Amplification of the *idh* gene

PCR Technique was used to amplify the DNA encoded gene *idh* According to (Dehghan *et al.*, 2008) and depending on bulletin from Bioneer, (Korea).

Biochemical and histological studies

Experimental animals

One month old male mice put at the animal house Laboratory, Biotechnology Research Center, Al-Nahrain University, Baghdad-Iraq. Figure1, which were housed under constant environmental conditions (25 °C and 65 ± 5% relative humidity; 12-h light/dark). After an acclimatization period of 1 week, the animals were divided into three groups (3 mice/ group). They were allowed to freely consume tap water and were fed according to the indicated experimental diets. The animals were adapted to the environmental conditions for 7 days prior to the start of the experiment.

Experimental design

Mice were assigned to three groups of three animals each and received a single oral dose of 1.5, 1.0 and 0.5 ml/Kg, using Patulin toxin as a drink, animal group two, treated oral (2.5 ml/Kg) with apple juice extracted from the infected apple fruits with *Penicillium expansum* mycellim (as a drink) and group three, untreated control which received normal water. The animals were observed daily for signs of toxicity. Body weights were recorded during the experimental period. The experimental determinations were made in mice fed on diets for two weeks with the same basic according to Madrigal et *al.*, (2006).

Blood samples

The blood samples were collected from the retro-orbital venous plexus for biochemical analysis in 15 ml-polypropylene tubes containing heparin as anticoagulant and centrifuged at 3000 RPM for 10 min to sepamice the plasma and used for biochemical serum analysis of kidney and liver function. Also, Blood draws (150-200 µl) were made using 1 ml syringes connected to the jugular

cannula. Blood samples were collected into anticoagulant tubes that containedethylene-diamine-tetra-acetic acid (EDTA) and were stored frozen at -20°C. Blood samples obtained from treated and untreated rats were analysis (Mangano *et al*., 2001; Awni *et al.*, 2015) and were used for the measurement of parameter physiological blood criteria (HB, WBC, RBC) and parameter hormonal and biochemical (GOT, GPT, Chol., Blood Sugar, FST, T3 and T4.)

FIGURE 1: Male mice in the animal house Laboratory

RESULTS & DISCUSSION

Isolation and Identification of Fungi

From 15 samples of apple fruits, a total of four isolates of *Penicillium* spp *were* identified into *Penicillium expansum* (p1, p1, p1 and p1). Were identified based on cultural and microscopic characteristics. Using identification keys by (Samson and Pitt, 1985; Pitt and Hocking, 2009) (Table2).

TABLE 2: *Penicillium expansum* isolated from rice samples

Isolate number	Fungal isolates	Isolates
4	*Penicillium expansum*	p1, p1, p1, p1

P. expansum is growing on stored foods and fruits (Pitt *et al.*, 2000). *Penicillium* species spotted in the air and dust of indoor environments, such as homes (Larous *et al*., 2007). Blue mold is one of the economically most important problems worldwide. It is caused by *Penicillium* spp., among which *P. expansum*. Blue mold production of the carcinogenic mycotoxin patulin (Barkai-Golan, 2008). Polygalacturonase enzyme plays a significant role in *P. expansum* virulence (Jurick *et al*., 2010). *Penicillin* identification is not easy. It is primarily based on morphological features incorporating the use of different media and standardized laboratory conditions *P. expansum* that they did not produce cyclopiazonic acid, but produced other alkaloid (Marek *et al.*, 2003)

Molecular identification of isoepoxydon dehydrogenase gene

Results molecular identification by PCR technique has been used to amplify the gene of the *idh* gene from genomic DNA of all *P. expansuin* isolates. Results showed the ability four isolates of *P. expansuin* fungus of producing patulin is carrying the regulatory *idh* gene, and responsible for regulating the Patulin production when 488 bp (Figure 2). The results of this study agree with several studies (Paterson, 2004; White *et al.*, 2006). The present study showed that most of isolates of *P. expansuin* produces *idh* gene. Show Dombrink-Kurtzman, (2005) Purified DNA from isolates of *Penicillium griseofulvum* and *P. expansum* was used as a template to amplify a 600 bp fragment of theisoepoxydon dehydrogenase (*idh*) gene of the patulin biosynthetic pathway. Dombrink-Kurtzman and McGovern (2007) found that Penicilliumexpansum *is* the most commonly detected species linked to the presence of patulin in apple juice. At least 10 different enzymes are involved in the patulin biosynthetic pathway, including the isoepoxydondehy-drogenase *(idh)* gene.

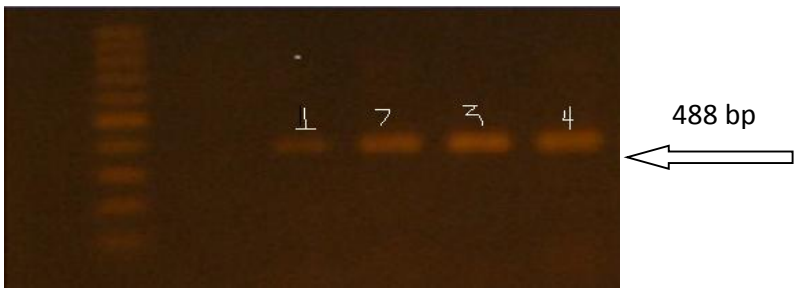

FIGURE 2. *idh* gene of *P. expansum* isolates

Regions (ITS and β-tubulin) were amplified and sequenced. The internal transcribed spacer rDNA area (ITS) is used for molecular identification of *Penicillium* species and is useful for placing isolates into species complex or one of the 25 sections. Sometimes this region provides species identification. That is why another

region – the β-tubulin gene region can be successfully used for accurate identification of *Penicillium* species (Visagie *et al.*, 2014, 2016).

Effect of *Pencillium expansum* mycellim and their mycotoxin Patulin on Physiological Blood Criteria, Hormonal and Biochemical test in male Mice

Selected isolation P2 of *P. expansum* because of its growth rapid therefore used in subsequent experiments. Blood specimens were taken to studying the toxic effect of PAT and Pencillium *expansum* mycellim in some physiological blood criteria, hormonal and biochemical in mice.

Physiological Blood Criteria and Biochemical studied

Changes in physiological blood criteria (HB, WBC and RBC) and Hormonal and Biochemical (GOT, GPT, Chol., Blood Sugar, FST, T3 and T4) parameters caused by the Patulinmycotoxin (PAT) and *Pencillium expansum* mycelium were recorded in Figure (3, 4, 5). The effects of mycotoxin Patulin (PAT) and *Pencillium expansum* mycelium on physiological blood criteria parameters of mice compared with control group. Reached HB, WBC, RBC is the Patulin mycotoxin (1.5 ml/kg) (9.4, 6000, 10.9) Respectively, and *Pencillium expansum* mycelium were (11.0, 5205, 8.12) Respectively, compared with control group (12.4, 3800, 5.17) Respectively.

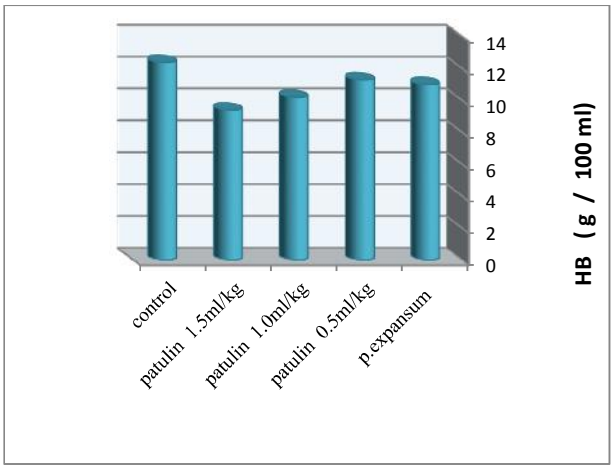

FIGURE 4: Effect of Patulin and *Pencillium expansum* on HB of Male mice

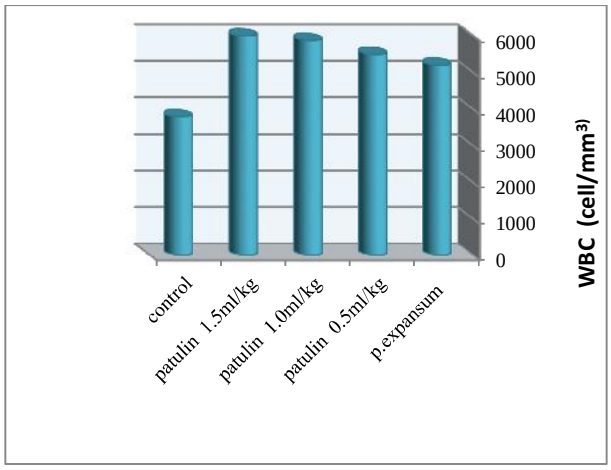

FIGURE 3: Effect of Patulin and *Pencillium expansum* mycelium on WBC of Male mice

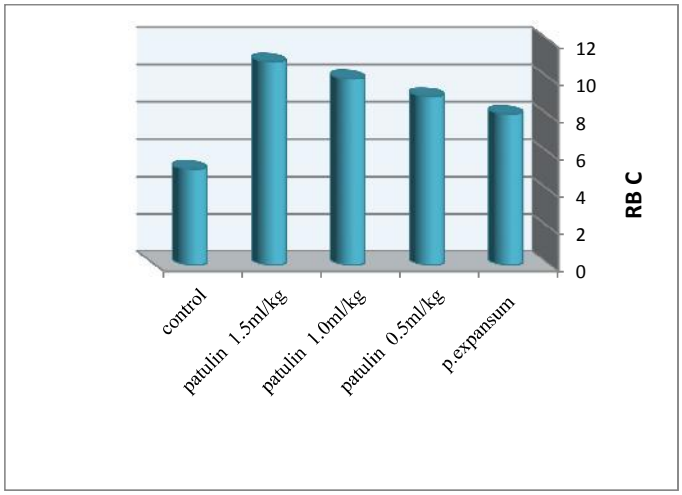

FIGURE 5: Effect of Patulin and *Pencillium expansum* mycelium on RBC of Male mice

The effects of Patulin mycotoxin (PAT) and *Pencillium expansum* mycelium Figure (6, 7, 8, 9, 10, 11, 12), on hormonal and biochemical (GOT , GPT, Chol., Blood Sugar, FST, T3 and T4.) is the Patulin mycotoxin (1.5 ml/kg) (9.3, 11.3, 118, 113, 6.1, 1.1 and 1.8) Respectively, and *Pencillium expansum* mycelium were (7.77, 8.30, 121.5, 100, 8.2, 0.92 and 2.4) Respectively, compared with control group (6.9, 5.83, 144.5, 85.25, 12.38, 1.41, 5.41) Respectively.

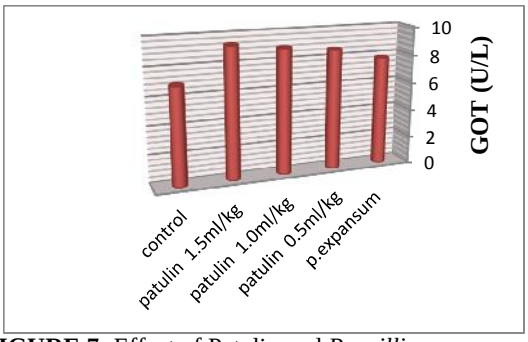

FIGURE 7: Effect of Patulin and *Pencillium expansum* mycelium on GOT of Male mice

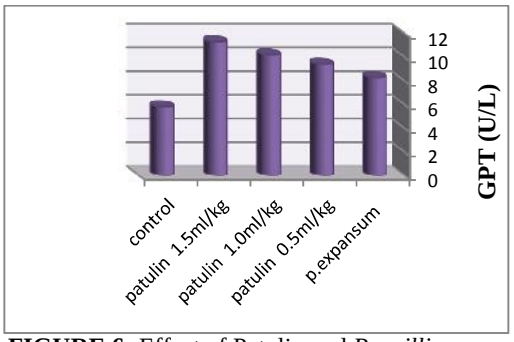

FIGURE 6: Effect of Patulin and *Pencillium expansum* mycelium on GPT of Male mice.

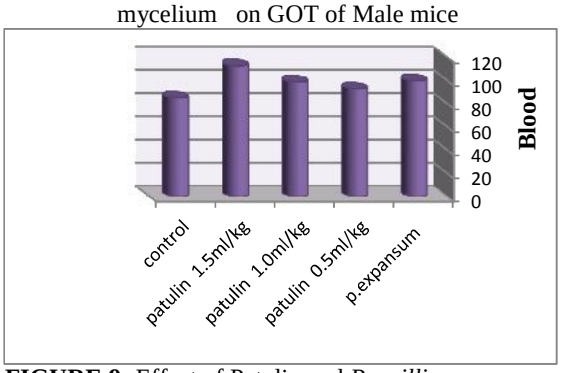

FIGURE 9: Effect of Patulin and *Pencillium expansum* mycelium on Blood sugar of Male mice

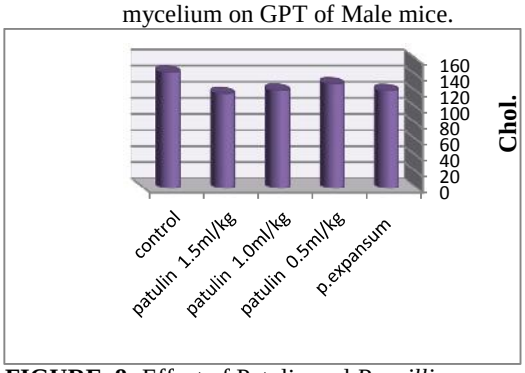

FIGURE 8: Effect of Patulin and *Pencillium expansum* mycelium on Chol. of Male mice

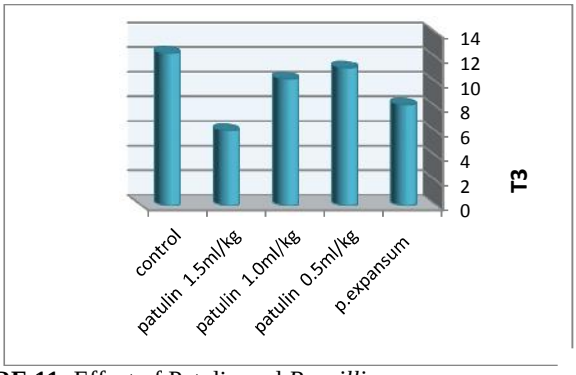

FIGURE 11: Effect of Patulin and *Pencillium expansum* mycelium on FST of Male mice

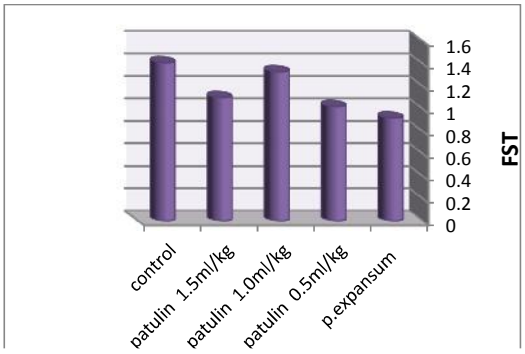

FIGURE 10: Effect of Patulin and *Pencillium expansum* mycelium on T3 of Male mice.

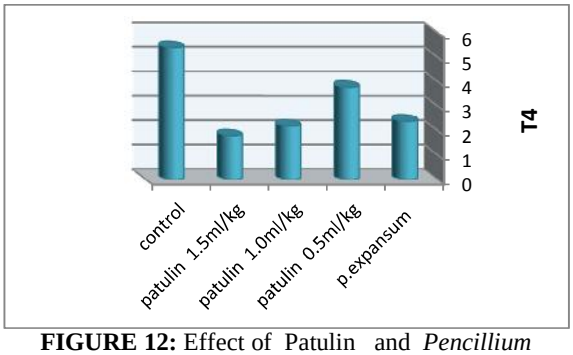

FIGURE 12: Effect of Patulin and *Pencillium expansum* mycelium on T4 of Male mice

All animals received *Pencillium expansum* with the mycotoxin Patulin were similar results with Mézes (2008) showed that mycotoxins may cause blood abnormalities. There are some clinical signs, which may appear in rabbit, such as high urea and creatinine levels, calcium-phosphorus imbalance, abnormal levels of liver enzymes (AST, ALT and GGT) weight loss. Exposure to PAT caused a decrease in the cell antioxidant capacity (El-Sawi *et al*., 2012). Results agreement with AL-Anati *et al.* (2005) findings indicated that several mycotoxins are able to modulate the production of cytokines. Reported Awni *et al.*, (2015). That Patulin mycotoxin with *Penicillium expansum* fungus showed non-significant decreased all body weight gain of rats during first week period were decreased significantly all body weight gain of rats during the second week period and increased significant all tested biochemical parameters as urea, GOT and GPT.

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