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Determination of Patulin By TLC From Mouldy Apples

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Summary

Analysis of the decayed apples (van. Starking delicious), collected from the local market, for patulin mycotoxin, using column chromotography and TLC Plates showed that the presence of the amount of patulin mycotoxin in the mouldy tissues (1.3 , 2.5 , 3.4 , 4.2, 5.5, and 6.4) cm in diameters were (5.1, 5.8, 6.3, 10.5, 12.3, and 14) µg patulin/g moldy tissues respectively. According to this research also, Patulin mycotoxin could diffused to the surrounding mouldy tissues. It is found that (0.5and 0.2), (1.0 and 0.5), and (1.5 and 0.5) µg patulin/g tissues in the first and second centimeters after mouldy tissues 4.2, 5.5 and 6.4 cm respectively, this phenomenon due to the presence of intracellular spaces in the tissues which allow to patulin to diffuse from mouldy to the healthy tissues there was no patulin found in the other surrounding healthy tissues. Gram positive bacteria found to be more susceptible to patulin, the zone of inhibition against *Staphyllococcus aureus* was 10 to 11 mm in diameter at concentration 10 and 30 mg /ml respectively when compared with 0.3 µg/ml streptomycin and 0.06 µg/ml ciprofloxacin while no inhibitory effect found against Gram negative bacteria *Klebsiella pneumoniae* and *Escherichia coli.*

التحري عن الباتيولين في التفاح المتعفن باستخدام صفائح السليكا جيل الرقيقة

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الخلاصة

تم استخلاص وتشخيص سم البايتولين في ثمار التفاح المتعفن (starkin delicious) , والذي جمع من الاسواق المحلية, وذلك باستخدام التحليل الكروماتوجرافي, وحددت كميات السم المتواجدة في الانسجة المتعفنة بصفائح السيلكا جيل الرقيقة TLC Plates فكانت بتراكيز (5.1, 5.8, 6.3, 10.5, 12.3, 14) مايكروغرام سم الباتيولين/ غرام نسيج متعفن للنماذج ذات الاقطار (1.3, 2.5, 3.4, 4.2, 5.5, 6.4) سم على التوالي وكما اظهرت هذه الدراسة عدم وجود سم الباتيولين في الانسجة المحيطة ببقع التعفن ذات الاقطار (1.3, 2.5, 3.4) سم, في حين وجد سم الباتيولين في الانسجة سليمة المظهر المحيطة بالانسجة المتعفنة وبالكميات (0.5, 0.2) و (1.0, 0.5) و (1.5, 1.0) مايكروغرام باتيولين/ غرام انسجة محيطة في كل من السنتمتر الاول والثاني بعد بقع التعفن ولاقطار التعفن (4.2, 5.5, 6.4) سنتمتر على التوالي. ان انتشار السم الى الانسجة السليمة المظهر والمحيطة للانسجة المتعفنة يمكن تفسيره الى وجود مسافات بينية في انسجة الثمار تسمح الى انتشار سم الباتيولين. ولغرض تقييم الفعالية المضادة لنمو البكتيريا الموجبة لصبغة كرام مثل (*Staphyllococcus aureus*) و (*Bacillus subtilis*) والبكتيريا السالبة لصبغة كرام مثل (*coli*

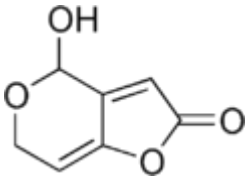
(*Escherichia* و (*Klebsiella pneumoniae*), أظهر الباتيولين (Patulin) فعالية مضادة لنمو البكتيريا الموجبة للصبغة بيبما وجد ان الباتيولين لم يظهر اي تاثير تثبيطي للبكتيريا السالبة للصبغة في الوسط الزرعي المبذور بالبكتيريا السالبة. وجد ايضا ان قطر الهالة الشفافة للباتيولين في الوسط الزرعي المبذور بالبكتيريا الموجبة للصبغة (*Staphyllococcus aureus*) بلغ (10) و (11) ملم عند استعمال تركيز (10) و (30) ملغرام/ مل على التوالي مقارنة مع مضادات حيوية منتخبة مثل الستريتومايسين والسبروفلوكساسين (9) و (12) ملم على التوالي. اما البكتيريا السالبة للصبغة فلم يظهر الباتيولين اي تاثير تثبيطي لهذة البكتيريا .

Introduction

Patulin is a mycotoxin produced by a variety of molds, particularly *Aspergillus* species and *Penicillium expansum* specie(1)It was commonly found in rotting apples and the amount of patulin in the apples products is generally viewed as a measure of the quality of the apples used in the production. It is not a particularly potent toxin ,but a number of studies have shown that it is genotoxic(2and3)which has led to some theories that it may be a carcinogen, though animal studies have remained inconclusive patulin is also an antibiotic(4,5 and6)..Several countries have instituted patulin restrictions in apple products. The World Health Organization recommends a maximum concentration of (50) µg /L in apple juice.
In European Union the limit is set to 50 µg/ kg in both apple juice and cider, and to half of that concentration, namely 25 mcg/kg in solid apple products and 10 µg /kg in products for infants and young children which recorded in European countries these limits came on 1 November 2003.

properties	
Molecular structure	$C_7H_6O_4$
Molar mass	154.12 g mol^{-1}
Appearance	Compact prisms
Melting point	110 °C, 383 K, 230 °F

Chemical structure 2-Hydroxy-3,7-dioxabicyclo[4.3.0]nona-5,9-dien-8-one[7-10] .



The evaluation of patulin was studied as a potential antibacterial activity.

The aim of this research is to detect the amount of patulin mycotoxin in the mouldy parts of apples and in the healthy tissues surrounding mouldy parts, and to study the antibiotic activity of it against Gram positive bacteria like *Staphyllcoccus aureus, Escherichia Coli* and *Klebsiella pneumoniae.*

Material and Methods

This research was carried out at laboratories of college of Dentistry Al-mustansiriya university, from the period beginning of 15/1/2009 to 20/ 8/2009.

1- Extraction of patulin mycotoxin from rotting apples :

Samples of rotting apples (Pyrus malus var sharking delicious) collected from the local markets, rotting diameters were determined, the rotting parts and the healthy tissues surrounding the decayed tissues (1cm, 2cm and 3cm after the decayed tissues) were removed and weight individually, both above tissues were placed, in beakers to measure the presence of patulin mycotoxin.

Each kind of tissues above placed individually in mixer for (5) minutes then (50) ml of ethyl acetate was used three times for extact, to remove the water, 250 ml separatory funnel was used, the acetate passed to anhydrate sodium sulphate to remove the remaining water ,then sulphate was used to wash two times by adding (25) ml acetate.(11).

Ethyl acetate evaporated by water bath (60 C^0) and passing current of nitrogen gas until the volume of acetate became nearly a half, then added (10 ml) of mixture (25 ml ethyl acetate and 75 ml benzene). (11).

Column chromatography (2.5 × 30 cm was used to purified the extraction, then evaporated at (60 C^0) by water bath until drying, pass current of nitrogen then dissolve the precipitate by (2ml) of chloroform.

TLC (thin layer chromatography) plates (E MARK, Germany) was used to determine the amount of patulin mycotoxin, by putting the spots of extraction and standard solution of patulin mycotoxin (Adimistration poland A E Division of food chemistry and technology food Drug Washington. D. C 2004) on TLC Plates, then the TLC plates were put in developing tank containing Toluene, 90% Ethyl acetate and Formic acid 1:2:3 respectively used as a developing solution.

Patulin mycotoxin which was present in extraction measured by using U.V spectrophotometer (wave length 2537-3000Å) and compare the patulin mycotoxin which present in extraction with standard solution of patulin mycotoxin, this procedure carried out according to Christopher[11].

2- Evaluation of antibacterial activity:

Local strain of bacteria obtained from Microbiology laboratory-college of Dentistry, Al-mustansiriyia University , was used in this study.

3- Drugs:

Pure substances of antibacterial drug Ciproploxacin – Streptomycin obtained from Samarra drug Industries (SDI) were used in this work.

4- Bacterial cultures:

The bacterial strains were cultured on Trypton Soya agar (TSA) for (24) hours at $37C^0$ then suspended in saline solution (85%) NaCL and adjusted to yield 25% approximately 1.0×1^8-1.0×10^9 colony forming unit/ ml (CFU) by using Spectrophotometer (transmittance at 530 nm).

In vitro antibacterial activity of different concentrations of patulin evaluated by using agar well diffusion assay (12). 0.1 ml of adjusted culture was mixed with 100 ml of Muller Hinton agar (MHA) and poured 15 ml each into sterile petridishes (90mm) at $45C^0$ (12) and this was allowed to solidify and then individual plates were marked for organism inoculated, after solidification, plates were punched to make well (6mm) in diameter with the help of Sterile cork bore, (50) micro liter of patulin were pipettes In the well, and the antibiotic in other well (13) . Plates were incubated overnight at

($37C^0$) and all plates were observed for the zone of inhibition these zones were measured in millimeters (All experiment were performed under Sterile conditions).

Result

Figure (1) and table (1) showed that patulin mycotoxin was present in all the decayed tissues of all apples, collected from the local markets, at the level (5.1 – 14) µg patulin / gram moldy tissues. The presence of the amount of patulin mycotoxin in the following mouldy tissues (1.3 , 2.5 , 3.4 , 4.2, 5.5, and 6.4) cm in diameters were (5.1, 5.8, 6.3, 10.5, 12.3, and 14) µg patulin/ g mouldy tissues respectively. According to this research also, Patulin mycotoxin could diffused to the surrounding mouldy tissues. It is found that (0.5and 0.2), (1.0 and 0.5), and (1.5 and 0.5) µg patulin/g tissues in the first and second centimeters after mouldy tissues 4.2, 5.5 and 6.4 cm respectively.

The results of antibacterial activity of the patulin showed that 10 mg/ ml revealed 10 mm zone of inhibition while patulin 30 mg /ml revealed 11 mm zone of inhibition against *Staphyllococcus aureus* (Figure 2), also it was found that no inhibitory effect against *Klebsiella pneumoniae* and *Escherichia coli.* The comparison with other selective antibiotics like streptomycin at concentration 0.3 µg/ ml and ciprofloxacin 0.06 µg/ ml revealed 9 and 12 mm zone of inhibition respectively.

Discussion

There are many studies supporting our results, (14and16) got (1- 16) and (1- 18) µg Patulin /g mouldy tissues respectively, this difference in the amount of patulin mycotoxin due to presence of different types of moulds or to the different temperatures during the time of collection and test the moldy apples (decreasing the temperatures leads to increase the production of amount of patulin mycotoxin)(14) Patulin mycotoxin could diffused from the healthy to the surrounding mouldy tissues, this phenomenon due to the presence of intracellular spaces in the tissues which allow to patulin to diffuse from mouldy to the healthy tissues there was no patulin found in the other surrounding healthy tissues. Buchanan (15and 16) found that 99% of patulin mycotoxin can remove by removing the mouldy tissues and no more than (2%) of total amount of the patulin in mouldy tissues can diffuse to the healthy tissues.

Wallen (17) tested patulin at a concentration of 100 µg/ paper disc for activity against *Bacillus subtilis*, Micrococcus luteus, *Escherichia coli*, *Saccharomyces cerevisiae*, *Candida albicans*, and *Mucor ramannianus*, respectively, two Gram positive bacteria, one Gram negative bacteria, two yeasts and a mold. Found that the patulin has ability to inhibit the above organisms.

Table (1) : Amount of patulin mg/ g at healthy tissues surrounding mouldy tissues and the amount of patulin in moldy tissues and the diameter (cm) of moldy tissues.

			Amount of patulin in mouldy tissues (mg/ g)	Diameter of mouldy tissues (cm)
3 cm after mouldy tissues	2 cm after mouldy tissues	1 cm after mouldy tissues		
-	-	-	5.1	1.3
-	-	-	5.8	2.5
-	-	-	6.3	3.4
-	0.2	0.5	10.5	4.2
-	0.5	1.0	12.3	5.5
-	1.0	1.5	14.0	6.4

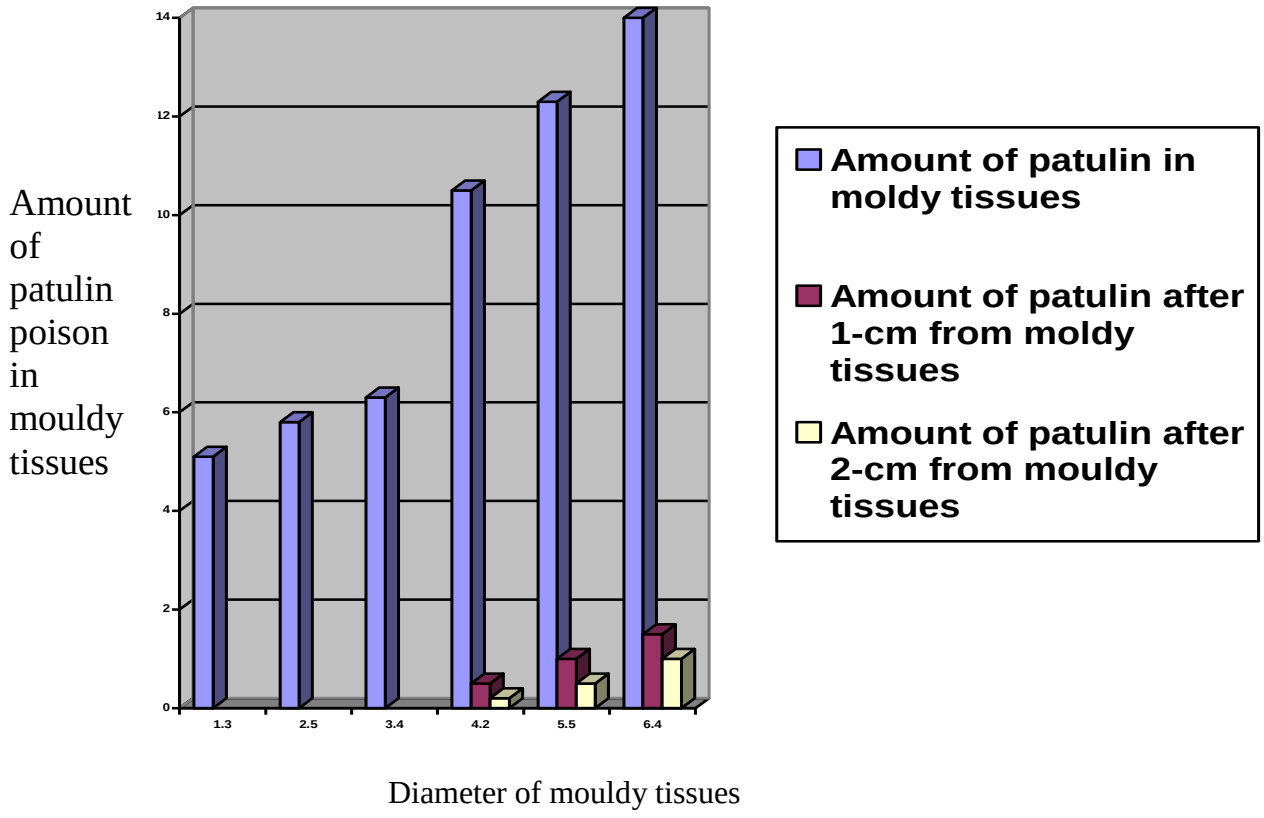


Figure (1). Show the amount of patulin in mouldy and healthy tissues

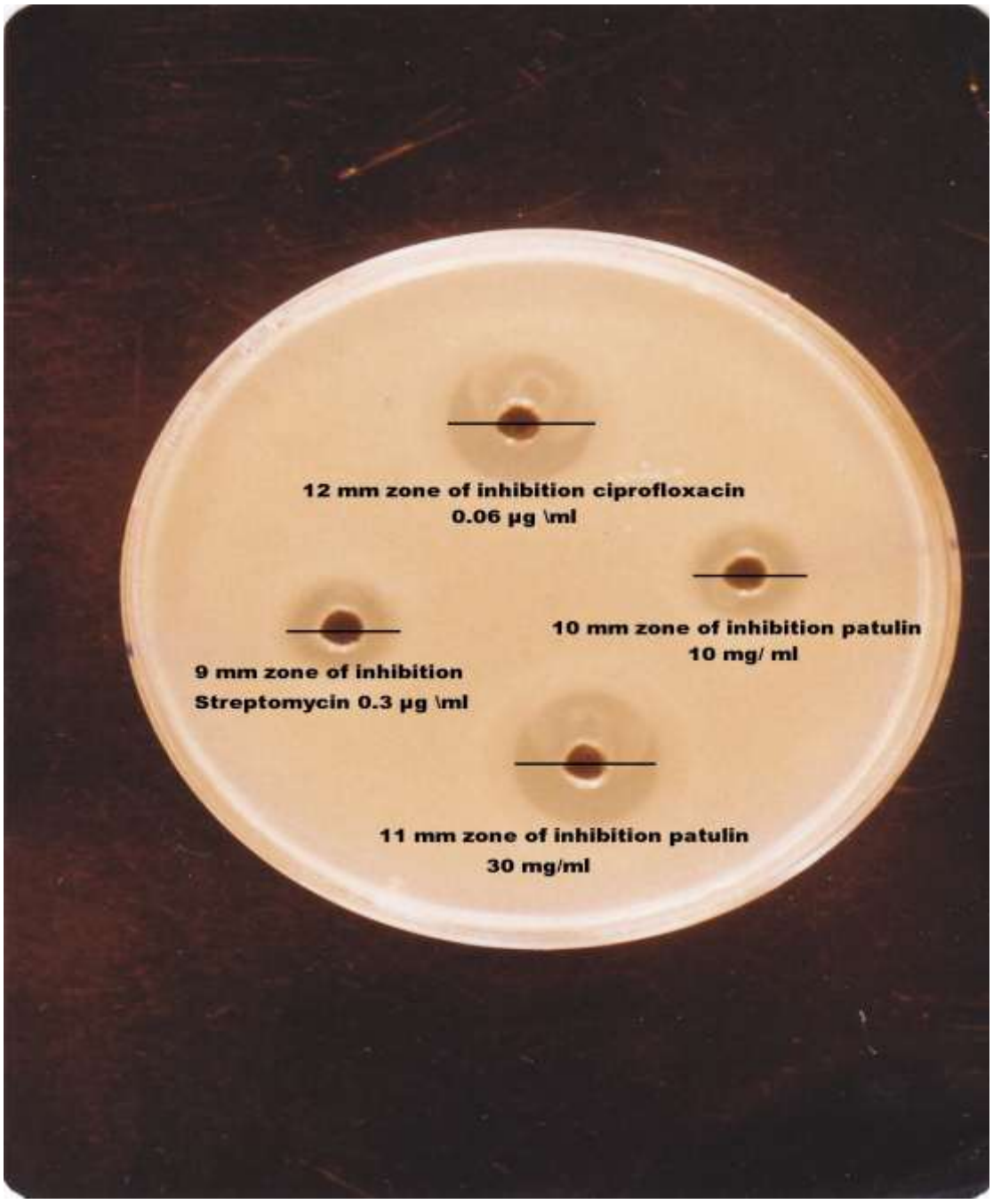


Figure (2): Demonstrate the zone of inhibition for Patulin extracted, and Streptomycin, Ciprofloxacin from standared against *Staphyllococcus aureus* inoculated in Muller Hinton agar media.

References

1- Gashlan H M (2009). High performance liquid chromatographic determination of patulin in apple juice: Investigation of its contamination levels in Saudi Arabia. Sci Res Essay. 2: 69–72.
2- Reddy K R N Salleh B Saad B Abbas H K Abel C A and Shier W T (2010). An overview of mycotoxin contamination in foods and its implications for human . Toxin Reviews. 29: 1, 3-26(24)
3. Liu B Yu F Wu T Li S Su M Wang M and Shih S (2003). Evaluation of genotoxic risk and oxidative DNA damage in mammalian cells exposed to mycotoxins, patulin and citrinin. Toxicol Appl Pharmacol. 191: 255–263.
4- Mims CA Playfair J HL Roitt I M Wakelin D and Williams R (1993). Medical Microbiology, Mosby, London.
5- Ellen CH . (1999). Patulin: A mycotoxin In Apples. Perishables Handling Quarterly. Issue No. 91 P 5.
6. Carcinogenic Lactones. In Microbial Toxin .VIb ed. By Ciegler AKadis and Ajil S J (2008) pp. 409-434 .Academic Press, NY London.
7- Spadaro D Garibaldi A and Gullino M L (2008) .Occurrence of patulin and its dietary intake through pear, peach, and apricot juices in Italy. Food Add Contam. 1: 134–139.
8- Reddy K R N Abbas H K Abel C A Shier W T and Salleh B (2010). Mycotoxin contamination of beverages: Occurrence of patulin in apple juice and ochratoxin A in coffee, beer and wine and their control methods. Toxins. 2:229-261.
9- Karimi G Hassanzadeh M Yazdanpanah H Nazari F Iranshahi, M and Nili A (2008). Contamination of patulin in clear apple juice in Mashhad, Iran. J Food Safety. 28: 413–421.
10- Rychlik M Schieberle P (2010). Model studies on the diffusion behavior of the mycotoxin patulin in apples, tomatoes, and wheat bread. Toxins. 2: 274–278
11- Christopher YJ (1979). Fluorescence detection and determination of patulin by TLC of its amiline . J Environ Sci Health B14. (1) : 15.
12- Perez C Pauli M and Bazerque P (1990). An antibiotic assay by agar Acta. Biologiae et Medicine Experimentalis. 15: 113-115.
13- Kaushik P and Goyal P (2008). In vitro evaluation of Datura innoxia antibacterial activity. Indian J Microb. DOI. 10:1007.
14- Khilbas J S (1998). Effect of temperature on the production of patulin mycotoxin by *Penicillium expansum* link on apple fruits Sirt Univ. Libya.
15- Buchanan J R Sommer N F Fortlage R J Maxi E C Mitchell FG and Tlsieh DPH (1975). Patulin from *Penicillium expansum* in store fruits and pears. J Am Soc Hort Sci. 99: 262.
16- Lovett J Thompson R G and Boutin B K (1975). Trimming as means of removing patulin from Fungus - rotted apples. JAOAC. 58 (5): 909.
17- Wallen L L Lyons A J and Prdham TJ (1980). Antimicrobial activity of patulin derivatives: Apreliminary report. The J Antibiotics. 33: 767-769.