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# Isolation and Characterization of Patulin Mycotoxin from strawberry fruits

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## Abstract

*pencillium expansum* is the most common fungi isolated from strawberry furits. The frequency and visibility of *pencillium expansum* is (40 and 86) respectively,on Sabouraud dextrose agar.

Patulin is extracted from the *penicillin expansum* that grows in the strawberry fruit. Concentration of patulin in the Strawberry fruits is (14.158-24.843) µg/ml

HPLC results show the retention time of major peak of *patulin* standard toxin located at 5.098 minutes, while a filter extract of *p.expansum* from (5.095) which is located in the same area .

The UV-VIS spectroscopy results of *p.expansum* and the standard *patulin* toxin, the major peak of standard *patulin* toxin is located with wavelength 276 nm and 2 absorbance; while the extract of *p. expansum* which is producing *patulin* toxin it located at a wavelength of 277 nm and 2.3 A.

## Keywords

Strawberry fruits **,** *pencillium expansum* **,** Mycotoxins **,** patulin toxin

## 1-Introduction

The strawberry (*Fragaria ananassa*) could be a part of the *Rosaceae* family, health impacts of strawberries depend on their momentous substance of phenolics, flavonoids, and micronutrients such as folate, vitamin C, and minerals. (Stewart and Folta, 2010).

Young strawberry leaves contain much more polyphenolic compounds than the fruits and mature leaves(Yang *et al*., 2011) and are a wealthy source of flavonoids and procyanidins (Bensa *et al.*, 2021). Flavonoids appear to play an important role in human health and to possess beneficial effects in the prevention of human diseases**.**(Chen and Chen, 2013) **.**

*Penicillium expansum* is one of the foremost hurtful post-harvest pathogens of pomaceous

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fruits and the causal operator of blue spoil infection. amid contamination, *P. expansum* produces the poisonous auxiliary metabolites *patulin* and *citrinin* (Tannous *et al.*, 2020). *Penicillium expansum* is the major capable of fruit pome rotting in cold capacity. Strawberry damage by *P. expansum* are anticipated to contain *patulin*, a *mycotoxin* which is demonstrated to affect human health (el Alami *et al.*, 2020)**.** *P. expansum* usually happens in soil and is related with few moldy fruits and vegetables, particularly spoiled apples and figs. It is the main source of *citrinin* and *patulin* in natural products (Awuchi and Ogueke, 2019).

*Penicillium expansum* could be major patulin producing pathogen of putaway, especially pome fruits and derived products. *Patulin* defilement postures a major risk for children, who devour large quantities of fruit juices (Zheng *et al*., 2017).

Mycotoxins are secondary metabolites basically created by fungi belonging to the genera of *Aspergillus, Penicillium, or Fusarium(*Kifer *et al*., 2020).

Patulin (PAT) a mycotoxin found basically in developed apples, is produced by different species of fungi, mainly *Penicillium expansum*, and is found in different fruits and vegetables used to produce juice(Silwana *et al*., 2020). Essentially harms crucial organs such as kidneys, liver, gastrointestinal tract, organs of the immune system, and endocrine glands(Ramalingam *et al*., 2019). Few studies also reveal PAT actuated DNA variation, chromosome aberration, and micronuclei formation in mammalian(Drpic *et al.*, 2018).

A method was developed and validated in-house for the detection and quantification of patulin in strawberry juice concentrate using a charge coupled device (CCD) on thin-layer chromatography (TLC) plates (Welke *et al*., 2009)

HPLC(High Performance Liquid Chromatography) methods are complex processes; since, several variables mobile phase pH, buffer concentration, flow rate, column temperature, detector wave length, etc. are to be concurrently controlled in attaining the desired separation HPLC separation of each chemical entity from the sample mixture is based on its distinct affinity towards the adsorbent material in the column or the mobile phase, causing various constituents to travel at different velocities and separate(Sahu *et al.*, 2018). Separation by HPLC predominantly depends on some intrinsic tunable parameters of mobile phase like polarity, flow rate, pH, composition and some inherent properties of sample matrix, type and nature of stationary phase, environmental factors like temperature and detector type and settings.(Sahu *et al*., 2018).

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## 2-1 Materials and Methods

This study has been conducted in the laboratories of the Department Biology / Faculty of Science / University of Kufa. Standard patulin was obtained from the Sigma - Aldrich Corporation.

## 2-2 Samples Collection

Samples have been collected from different locations of Najaf i.e. local markets(City center , AL-Munadhira district, Kufa district). Strawbbery fruits samples were put in Petri dishes containing culture S.D.A with a width of 9 cm in diameter, Then 5 pieces on each plate were put and incubated at temperature 25 ± 2 ° C for 7 days. Fungi were sub cultured, then were isolated by separation transporting the disk from each colony and cultured in a new petri dish. This process was repeated to obtain a pure culture.

## 2-3 Preparation of Culture Media

Media are used Sabouraud dextrose, Potato dextrose(agar and broth) were prepared according to the manufactures fixed on their containers and were sterilized by autoclave at 15 psi / inch2 in 121 ° C for 15 min, all types of media containing chloramphenicol antibiotic 100mg / Liter.according to (Al-Janabi *et al.*, 2019).

## 2-4 Diagnostic of the isolates fungi

Morphological features of growth colony which including color, texture, margin, colony reverse and pigments produced.

Microscopic Examination, observing fungi shapes and conidia and mycelium using light microscope and scanning electron microscope (SEM).

Evaluation of the Frequency and Visibility of the fungal isolation, visibility and frequency were calculated by using the formula below:

Visibility (%) = The number of samples that appeared genus or species X 100

The number of samples

Frequency (%) = Number of genus or species fungal isolates X 100

Totally of all fungal isolates

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### 2-5 Extraction of patulin

Pure isolated of the *p.expansum* grown on Sabouraud dextrose agar and incubated at 25± C for 14 days. By taking pure culture to detect produce toxic secondary metabolites of four repeated of each one, then the pure culture, cutting with sterile knife and mixing it with 20 ml ethyl estate. The mixture was shaken for 15 minutes by a shaker apparatus. The mixture was filtered through a filter paper whatman Nol. Filtrate puts it in separate funnel. Ethyl acetate were dried by reflex condenser. Filtrate then concentrated to reach about 1ml.

### 2-6 Separation of Secondary Metabolites

Put 1000 µl from each isolated *P. expansum* extracts the plate (TLC) as spots form. Leaving stains to dry and then place in a tank contains a mixture of toluene /ethyl acetate /formic acid (5:4:1, v/v) and observed until the arrival of solution at a distance of about 2 cm from the upper edge of the plate. Leave the TLC plate for air drying for 5 minutes. Examining the plate under UV light with wavelength 276 nm and each compound determined by sterilize needle.

### 2-7 HPLC Analysis

HPLC analysis was performed by using Shimadzu instrument type (LC - 20AD, CBM 20A, SPD - 20A), (250 mm + 150 mm x 4.6 mm) (Eclipse C18,5micron particle size. Analytical column. 38 plus a 12.5 mm guard column set at 40 ° C was used for the analyzes. The flow rate was set at 1.0 ml / min with an injection volume of 10ul for purifying patulin and 50 ul for the filter extracts.

## 1- Results and Discussion

### 4-1 Isolation of Fungi from strawberry fruits

The following fungi appeared in strawberry fruits which are *Aspergillus* ,*fusarium* ,*penicillium* ,*Rhizopus*. Most common fungi are isolated from strawberry fruits, which are *pencillium expansum*, The first species is found more visible and frequency than other fungi and visible have reached to 86% and a frequency 40% on(SDA) (Table 4-1). This result is in agreement with (Neri *et al*., 2010) .

**Table (4-1)** The frequency and visibility fungi of from strawberry furits in the media SDA on (25±2) $^{0}$C.

| NO | Fungi | Frequency (%) | Visibility (%) |
|---|---|---|---|

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| | | | |
|---|---|---|---|
| **1** | ***Aspergillus niger*** | **30.4** | **68** |
| **2** | ***A.flavus*** | **10.5** | **22** |
| **3** | ***Fusarium spp*** | **10.8** | **59** |
| **5** | ***Penicillium expansum*** | **40** | **86** |
| **6** | ***Rhizopus spp*** | **8.3** | **18** |
| **total** | | **100** | |

**4-2 Diagnosis of Isolated Fungi of *pencillium expansum***

**4-2-1 Diagnosis appearance of *pencillium expansum***

*pencillium expansum* appeares as blue mold colonies on Sabouraud dextrose agar medium at a temperature $(25\pm2)^0$C after 7 days of incubation. The colonies of *P. expansum* grow rapidly forming a velvety carpet of surface filamentous mycelium up to 2mm deep. The colonies are initially white becoming dull yellow green to blue green in 5–7 days. (Figure 4-1). This result is closer to that observed by *(Li et al*., 2020)**.**

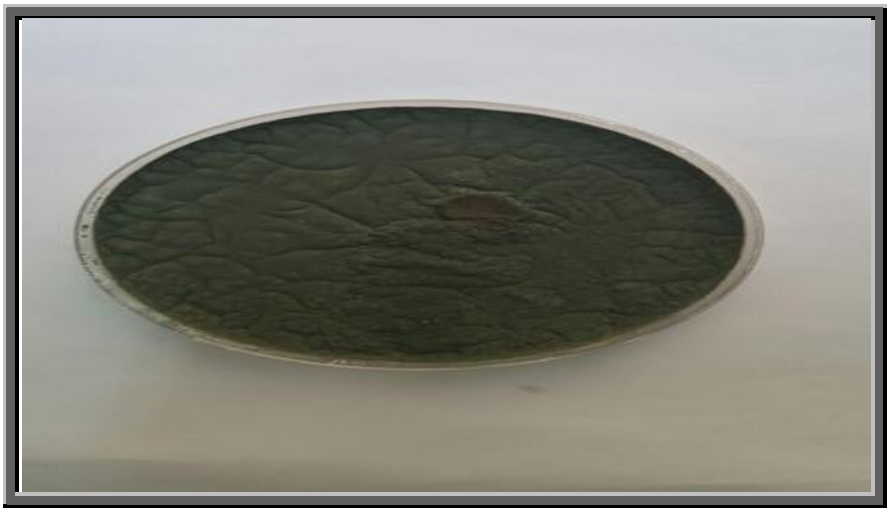

**Figure (4-1)** Colony of *pencillium expansum* fungus growing on the SDA medium $(25\pm2)^0$C after 7 Days of incubation.

**4-2-2 Microscopic diagnosis of *pencillium expansum***

Figure (4-2) shows the light microscopic examination of *p expansum*, conidiophores are determinats which are irregular branched, septate, microscopic study, observation of hyphae and conidia under a light microscope. The plate reverse is usually pale to yellowish. As to micromorphology, the conidial heads are asymmetric and once or twice branched (terverticillate) and conidiophores are smooth, 400–700µm long and with smooth elliptical conidia at 4–5 × 2.5–3.5µm.

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*P. expansum* conidia presented light green color with radially sulcate, velutinous texture, reverse yellow. Dull green conidia, surface texture from granular to floccose 7 days $(25\pm2)^0$C, it was observed differences in the diameter and coloration of colony reverses. *P. expansum* colonies produce green conidia, *P. expansum* conidia presented light green color with radially sulcate, velutinous texture, reverse yellow(Cardoso *et al*., 2007) . These results are closed to that obtained by (Li *et al*., 2020).

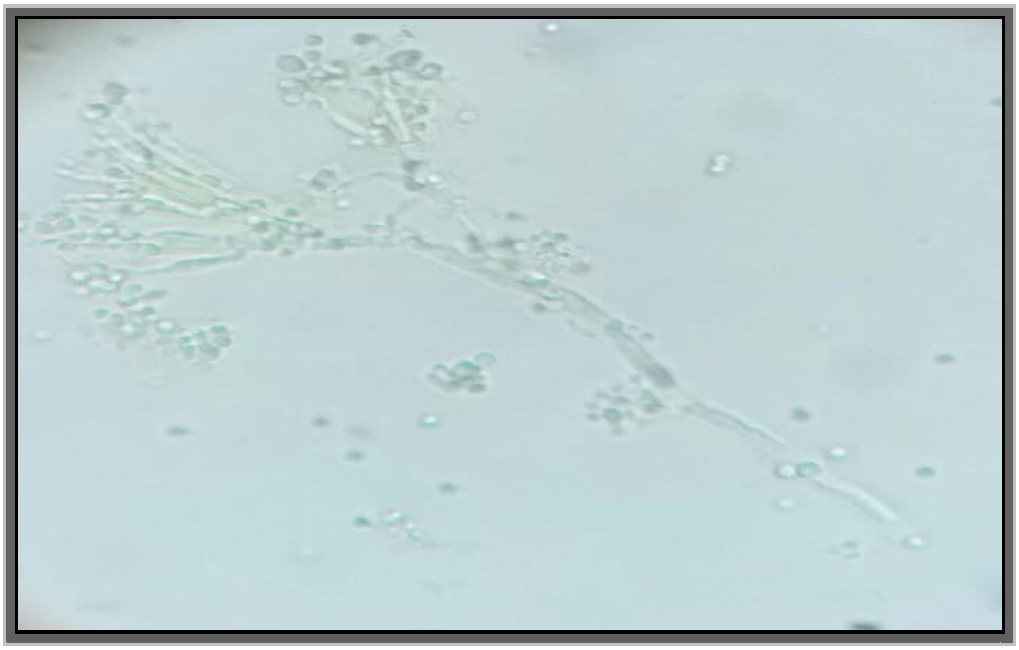

**Figure (4-2)** Light microscope examination of *pencillium expansum* (40x).

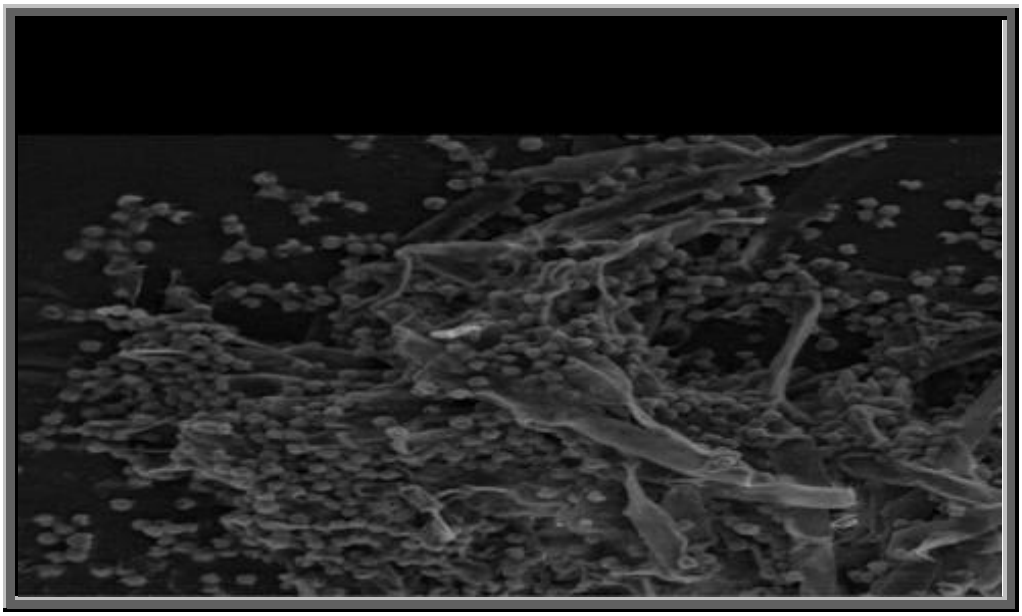

**Figure (4-3)** Electronic microscope examination of *pencillium expansum*(50 µm) .

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### 4-3 Thin layer Chromatography (TLC)

Chromatography is one of the most popular methods to analyze mycotoxins such as patulin. Figure (4-4) shows the result of the chemical analysis of the thin layer chromatography (TLC) which reveals that extract of *p.expansum* produce patulin which is compared with the standard of patulin, it can be seen in the same color and the same relative flow RF = 50%, which are appeared in yellow color spots and 276 nm under UV light this result is agree with those of(El Hajj Assaf, 2018)

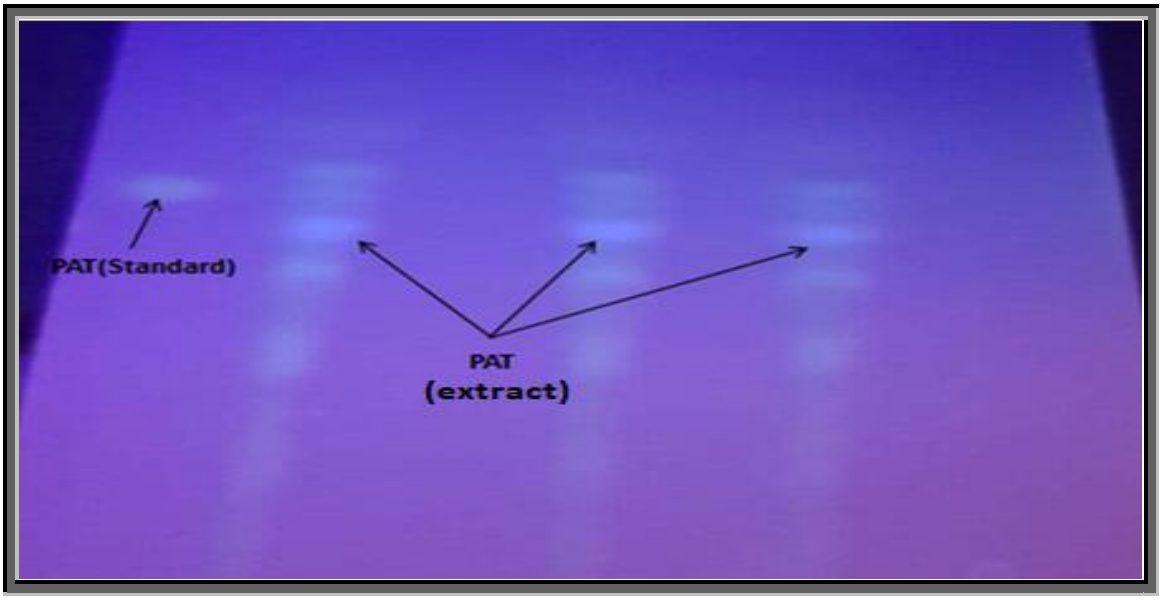


**Figure (4-4):** Thin layer chromatography (TLC) show the extract of *P.expansum* produce patulin.

### 4-4 Analytical Study Diagnosis of *P.expansum* and Separated Compounds.

#### 4-4-1 Liquid Chromatography( HPLC)

TLC and HPLC are the most used quantitative methods in research and routine analysis of patulin (Sohrabi *et al.*, 2021). *P.expansum* selected to produce patulin by (HPLC) analysis . HPLC results show that retention time of major peak of standard patulin located at ( 5.098 ) minutes (figure4-5) it is identical with patulin produced from a filter extract of *p.expansum* (5.095) in the same area. The obtained filter extract of *p.expansum* produced as shown in (figure4-6 ). These results confirmed the presence of patulin in the collected samples under study.

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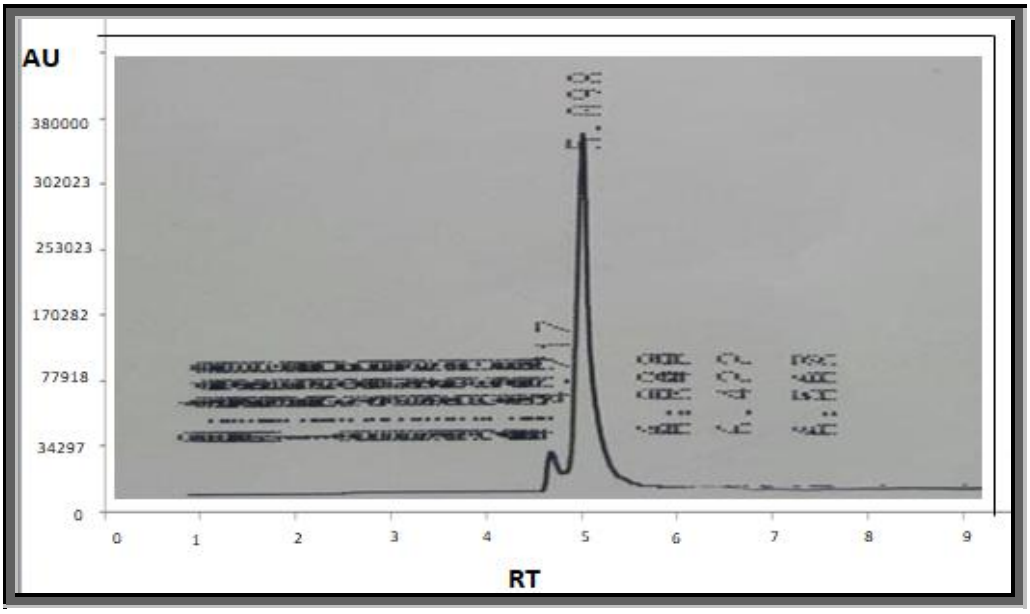
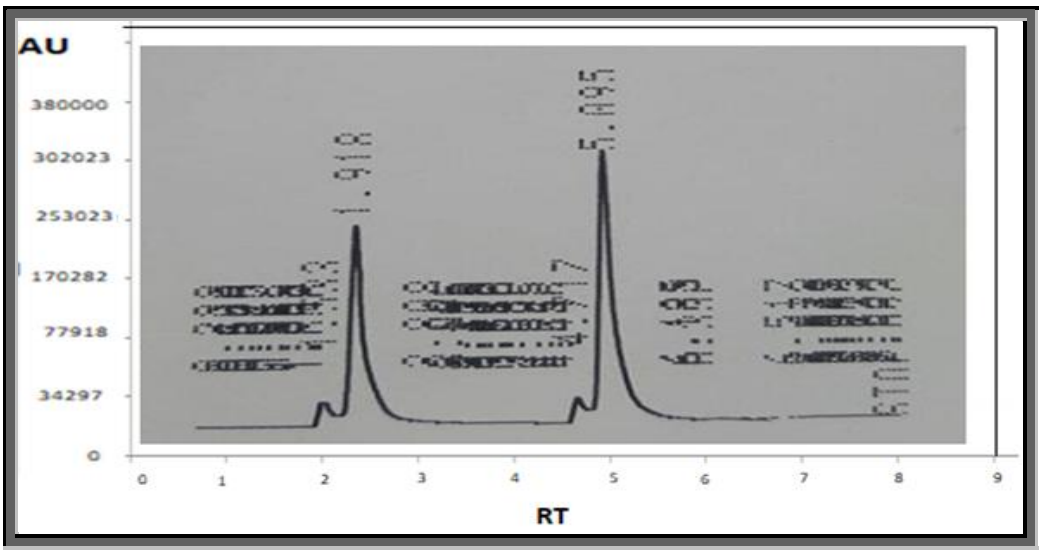



**Figure(4-5)** :HPLC Chromatography analysis shown major peak of standard patulin.




**Figure(4-6):** HPLC Chromatography analysis shown major peak of a filter extract of *p.expansum* which produced patulin.

**4-7-3 UV-VIS Spectroscopy Study**.

The UV-VIS spectroscopy results of *p.expansum* and the standard *patulin* toxin, the major peak of standard patulin toxin is located with wavelength 276 nm and 2 A absorbance; while for extract of *p. expansum* which is producing patulin toxin it located at a wavelength of 277 nm and 2.3 A (Figure 4-7). Most patulin standard are located at wavelength the range (200-320) nm. These results are compatible with (He *et al*., 2021).

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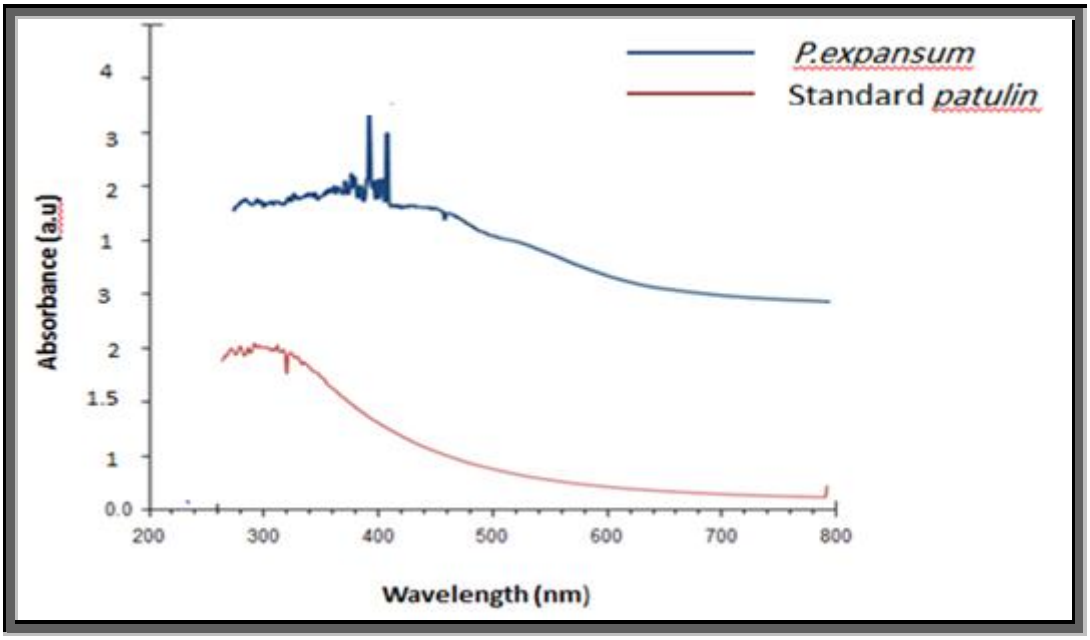


**Figure (4-7**) UV-VIS of Standard *patulin* toxin and extract of *p. expansum*..

### 4-4-2 Concentration of *patulin* in the strawberry fruits.

**Table (4-2)** Concentration of *patulin* in the Strawberry fruits**.**

| NO | Sample | Concentration µg/ml |
|---|---|---|
| 1 | Sample1 | 14.158 |
| 2 | Sample2 | 19.170 |
| 3 | Sample3 | 24.843 |
| 4 | Sample4 | 15,344 |
| 5 | Sample5 | 15.038 |
| 6 | Sample6 | 17.021 |
| 7 | Sample7 | 16.185 |
| 8 | Sample8 | 14.082 |
| 9 | Sample9 | 19.729 |

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| | | |
|---|---|---|
| **10** | Sample10 | 15.905 |
| **11** | Sample11 | 18.073 |
| **12** | Sample12 | 16.704 |

## Conclusions

1- *p.expansum* has show more frequency and visible than other fungi in Strawberry fruits.

2- Extracting patulin toxin from strawberry fruits

3- That UV-patulin extracted from strawberry fruits 277nm while UV-Standard Patulin was found 276nm

4- TLC(thin layer chromatography) test is a primary method for diagnosing toxin, because Rf for standerd patulin and patulin produced from a filter extract of *p.expansum* Rf=50%

5- HPLC test show that retention time of major peak of standard patulin located at ( 5.098 ) minutes while patulin produced from a filter extract of *p.expansum* (5.095)

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