Research Article

# Incidence of Patulin-Producing Fungi in Some Foods: Isolation, Patulin Analysis and Risk Assessment

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**Abstract** | A variety of fungal species produces the mycotoxin patulin (PAT) and represents a health hazard contaminant in several food stuffs. The aim of this study was to isolate and identify PAT- producing fungi, determine the incidence of PAT in different juices, and assess PAT's associated risk. In this work, a number of fungi were isolated from different food stuffs collected from local markets in the Great Cairo Governorate, Egypt. The isolated fungi were examined for PAT production and the PAT-producing ones were phenotypically and genotypically identified. The fungal isolates were grouped into 14 morphotypes; out of these morphotypes, 10 were tentatively related to the genus *Penicillium* and the others belonged to the genera *Aspergillus* and *Talaromyces*. Phylogenetic analysis applying the Internal transcribed spacer (ITS) gene sequencing conclusively identified these PAT-producing fungi as 10 strains of *Penicillium* with different 10 accession numbers and two strains of *Aspergillus* and *Talaromyces* having two different accession numbers. The maximum PAT amount (5.1 µg/ g) was produced *in vitro* by *P. expansum* S7 while a minimum PAT content of 0.15 µg/ g was determined in the culture of *A. flavus*. The amount of PAT in apple juice ranged between 13.91 and 79.35 µg/ l, while the highest PAT range (27.36 to 234.30 µg/ l) was determined in orange juice samples. Grapes and cocktail juices contained as low as 1.68 and 0.56 µg/ l PAT, respectively. Using the highest PAT values found in each juice, a risk assessment was performed for the examined juices. The calculated Estimated daily intake (EDI) were below the Provisional Maximum Tolerable Daily Intake (PMTDI) (0.4 µg/kg BW/day) for all tested juice types except orange juice with values (1.029 µg/kg BW/ day) for children and 0.812 µg/kg BW/day for adolescents. While the Target Hazard Quotient (THQ) values were 2.573 for children and 2.029 for adolescents, indicating a potential non-carcinogenic risk for these two groups, whereas no such risk was observed for adults (THQ = 0.70). As a conclusion PAT can be detected in juice samples with levels exceeded the MPLs in some types, which can threaten the public health especially the children and adolescents.

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## Introduction

During pre-harvest or storage, mycotoxins, which are secondary metabolites of fungi, contaminate a variety of food products (Yin *et al*., 2021). Several fungal species from the *Aspergillus* and *Penicillium* genera produce patulin (PAT) (Shinde *et al*., 2021; Shekhar *et al*., 2025). The most popular pathogen that produces PAT is *P. expansum* (Zouaoui *et al*., 2015; Patriarca, 2019; Shinde *et al*., 2021; Gal *et al*., 2025). PAT concentration in raw material is higher than that of processed food products, as various processing steps help to reduce its levels such as filtration, clarification, and enzymatic treatment (Gomes *et al*., 2021). PAT can also be found in several food products, including apples and corn. It is now recognized as a global contaminant, leading many countries to carefully observe its levels in food (Gomes *et al*., 2021). PAT has also been detected in silage and feed intended for ruminants, and it has been responsible for cattle deaths reported in France (Bhat *et al*., 2010). Corn, cereals, oilseeds, malted barley, wheat, and coffee bean seeds are just a few of the foods and feeds that naturally contain PAT (Bokhari and Aly, 2009).

In general, maintaining control throughout all steps of apple processing including homogenization, crushing, pasteurization, and hygienic packaging can help reduce the final levels of PAT (Gomes *et al*., 2021). Apples offer consumers numerous health benefits and help lower the prevalence of chronic illnesses (Zhong *et al*., 2018). Apples and apple products (juices, concentrates, compotes, purees, and ciders) rated the 17$^{th}$ in the list of the most produced commodities globally, according to the Food and Agriculture Organization (FAO) (FAOSTAT, 2012). Children under the age of 12 are a group at risk, because the amount of PAT in apple-based foods for children has been determined to be five times lower than the safe limit for adults. In addition to being more exposed per kilogram of body weight, children are more susceptible due to their unique physiology (Raiola *et al*., 2015; Gonya *et al*., 2024).

Patulin has neurotoxic, teratogenic, and mutagenic effects. Additionally, it has been connected to a number of health risks, such as digestive problems, genotoxicity, and immunotoxicity (Shinde *et al*., 2021). PAT is a fast-absorbing enteropathogenic mycotoxin that damages the intestinal mucosa by causing inflammation and ulceration. In people and animals, it results in a number of health issues, such as edema, ulceration, inflammation, vomiting, bleeding, and even death (Bayraç and Camizci, 2019; Xiao *et al*., 2019). The provisional maximum tolerated daily intake (PMTDI) for PAT has been established at 0.4 µg/kg BW/ day by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (Zhong *et al*., 2018; Saleh and Goktepe, 2019; Shinde *et al*., 2021; Gomes *et al*., 2021). The maximum level of PAT for apple juice and apple juice constituents is set at 0.05 mg/kg by the CODEX Alimentarius Commission (Zhong *et al*., 2018; Saleh and Goktepe, 2019). In animals, PAT has an oral lethal dose 50% ($LD_{50}$) of 29 - 55 mg/kg body weight (Noman, 2022). In order to identify fungal taxonomy at the species and intra-species levels, the ITS region of nuclear DNA is normally sequenced (Chinnasamy *et al*., 2023). In regions where food manufacturing lacks hygiene regulations, the hazards associated with mycotoxins impact the entire community. All racial, gender, and age groups are impacted by elevated patulin levels. Consuming patulin causes potential health hazards, such as genotoxicity, carcinogenesis, and neurotoxicity (Saleh and Goktepe, 2019).

The current work aimed to isolate and identify PAT producing fungi obtained from some food stuff using morphological and molecular techniques, determine the incidence of PAT in different juices, calculate the PAT dietary intake, and assess the PAT associated risk.

## Materials and Methods

*Sampling*

Different samples of fruits, cereals, and juices were randomly collected from local markets in Cairo Governorate, Egypt, to isolate the PAT -producing fungi. The collected 31 samples included 10 samples of apple juice, six samples of ripping apple, four samples of white flour samples, three samples of corn, two samples of each of pears, rice, and bananas, and one sample of each of grape and wheat. A total of 78 juice-manufactured samples (28 apple, 26 orange, 12 grape, and 12 cocktail juices) were collected from local markets in the Great Cairo Governorates during season 2024 for determination of patulin.

*Isolation of PAT -producing fungi*

The pour-plate technique was used for fungal isolation according to Ottow (1972). 5 g from each sample were serially diluted in 45 ml peptone buffer.

1 ml of the appropriate dilution was cultured using Chloramphenicol Rose Bengal Agar (RBA) (Lab M, Neogen Company, UK) to determine the total fungal count, incubated at 25˚C for 7 d. After incubation, the hyphal tip technique was used for purifying the developed well separated colonies, subcultured on Rose bengal agar (RBA) plate, and incubated for 7 d. RBA was used to study the isolates morphological features. Every fungal isolate was identified to the species level using morphological and molecular methods according to Frisvad and Samson (2004). The isolates were maintained at -20˚C using glucose yeast extract broth supplemented with 20% glycerol. The slide culture was prepared for determination of the isolate's microscopic features (Okayo *et al*., 2020).

*Molecular identification of the isolated fungi*

Fungal isolates were identified molecularly using internal transcribed spacer (ITS) gene sequencing *via* polymerase chain reaction (PCR) analysis (Table 1), in reference to Luo and Mitchell (2002). The fungal isolates were cultured for 7 d at 25ºC in glucose yeast extract broth. Centrifugation was performed for 5 min at 12000 g to extract the fungal cells. Genomic DNA was extracted using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Lithuania). Following centrifugation, the DNA yield and purity were evaluated using a UV-Vis NanoDrop spectrophotometer (NanoDrop, 2000, Thermo Fisher Scientific, Germany). The PCR process involved an initial denaturation at 95ºC for 12 min., followed by 30 cycles of 95ºC for 30 sec, 55ºC for 30 sec, and 72ºC for 1 min., with a final extension step at 72ºC for 10 min. After PCR, the obtained products were checked using agarose gel electrophoresis (Bio-Rad, USA), purified with a gel extraction kit, and sequenced using automatic ABI 370×1 DNA Sequencer (Applied Biosystem, USA). The sequences were analyzed applying BLAST V2.0 software (http://www.ncbi.nlm.nih.gov/BLAST/).

**Table 1:** *Primers used for molecular identification of the isolated fungi (Luo and Mitchell, 2002).*

| Primer name | Primer sequence |
|---|---|
| ITS1 | 5′-TCCGTAGGTGAACCTGCG G-3′ |
| ITS4 | 5′-TCCTCCGCTTATTGATATGC-3′ |

*Phylogenetic analysis of the fungal isolates*

The phylogenetic analysis of the identified fungal strains was conducted to determine the genetic relationships between them and closely related strains. The phylogenetic analysis was performed using the Neighbor-Joining method. The Kimura 2-parameter approach was used to calculate the evolutionary distances, which were expressed in terms of the number of base substitutions per site. The phylogeny was tested through 1000 bootstrap replicates. Version 11 of Molecular Evolutionary Genetics Analysis was used to create the phylogenetic tree (MEGA11).

*Patulin mycotoxin Standard and solutions*

Patulin standard (5 mg) was purchased from Sigma-Aldrich Company (Germany). PAT stock solution (2 mg/ml) was prepared in acetonitrile which was used also in preparation of the working standard patulin (25-500 mg/ml) and stored at -20 ºC until use.

*Patulin extraction and determination*

Using the procedure described by Neri *et al*. (2010), PAT was extracted from the isolated fungal species. Yeast Extract Sucrose agar medium (YES) (Scharlab, Barcelona, Spain) was used to cultivate each isolate. Five agar plugs of mycelia (6 mm) were cut using a sterile cotk borer from the growing margins of freshly-grown fungal cultures after 7 days of incubation at 25 ˚C. For 60 min., 2 ml of ethyl acetate/formic acid (200:1, v/v) solution was used for the PAT extraction. The extract was centrifuged at 4500 rpm for 10 min (SIGMA Laborzentrifugen Gmbh, Germany) and then dried in a rotating vacuum evaporator (LABOROTA 4000, Heidolph Germany). A 0.45 µm membrane filter was used to filter the dried residue after it had been dissolved in 1 ml of methanol for 30 min. An analysis method of PAT in apple juice was conducted according to Cho *et al*. (2010). Five ml of juice sample were placed into centrifuge tube (50 ml) and extracted twice with 10 ml ethyl acetate by shaking for 1 min, using a vortex. The aqueous and organic phases were separated through centrifugation (1600 xg for 3 min), followed by separation of upper layer using a separating funnel. The total organic layers were transferred into a separating funnel and 2 ml of 1.5% sodium carbonate solution were added. The ethyl acetate extract was filtered through a Whatman filter paper, and the filtrate was evaporated at 40 ºC using a rotary evaporator (LABOROTA 4000, Heidolph Germany). The dry film was dissolved in 2 ml ethyl acetate, transferred into a vial, and evaporated to dryness using nitrogen stream. The residue was dissolved in 1 ml of acetic acid solution (pH 4.0) and filtered using 0.45 µm membrane filter. Determination

of PAT was executed using High Performance Liquid Chromatography (HPLC) without delay. HPLC analysis was carried out according to Cho *et al.* (2010) using Agilent Technologies 1260 Infinity II liquid chromatograph equipped with an auto sampler and a diode-array detector. The analytical column was Eclipse XDB-C18 (150 X 4.6 µm; 5 µm) with a C18 guard column (Phenomenex, Torrance, CA). The isocratic mobile phase consisted of acetonitrile: water (1:9, v/v). The flow rate was kept at 1.0 ml/min. The injection volume was 20 µl and analysis was performed at a wavelength of 276 nm. Acrodisc syringe filter 0.22 µm (Gelman Laboratory, MI) was used for filtration of samples before injection.

*Verification of PAT determination method*

The applicability of PAT method was evaluated in the aspect of linearity, limit of detection (LOD), limit of quantification (LOQ), and recovery. The linearity was checked using prepared PAT standard solutions at 8 concentrations, mainly 0.25, 0.50, 0.75, 1.0, 1.5, 2.0, 3.0, and 4.0 µg/ml that were used to construct a calibration curve. Linearity was determined as the coefficient of determination ($R^2$). LOD was calculated as a 3:1 signal-to-noise ratio, and limit of quantification (LOQ) was calculated as a 10:1 signal-to-noise ratio. The recovery was analyzed by spiking 3 concentrations in apple juice samples (25, 50 and 100 µg/l) in three replicate. The % recovery value was determined using the following equation (Iqbal *et al.*, 2018):

$$\%\ \text{Recovery} = \frac{\text{Measured concentration of spiked sample}}{\text{Spiked (added) concentration}} \times 100$$

*Risk Assessment of patulin detected in juice samples*

*Estimated daily intake (EDI)*

The estimated daily intake (EDI) of PAT in the four juice types was calculated based on the maximum detected concentrations (C µg/l) in each juice type. The EDI (µg/kg body weight/day) was determined using the following formula (Saleh and Goktepe, 2019).

$$\text{EDI} = \frac{C\left(^{\mu g}/_{L}\right) \times \text{AoF} \times \text{IGR}\left(^{g}/_{day}\right) \times \text{EF}\left(^{day}/_{yr}\right) \times \text{ED(yr)} \times \text{CF}(0.001)}{365(\text{day in year}) \times \text{AT(Yr)} \times \text{BW(kg)}}$$

Where: EDI= Intake of PAT (µg/kg BW/day), C= PAT contamination level in juice samples (µg/l). The maximum detected levels in this study were used, AoF = Bioavailability (unitless) was used in this work as reported by Torović *et al.* (2018), IGR = Ingestion rate of food (g/day or L/day) for each age group was provided by EPA (2011a), EF = Exposure frequency (days/year) by assuming daily exposure (365 d/year), ED = Exposure duration (year) provided for each age group (Children 7 year, Adolescents 13 year, and adults 50 year), CF= Conversion factor (used in this work was $10^{-3}$ to unify the unit between C and IGR) (Saleh and Goktepe, 2019), AT= Average time period of exposure (e.g. hour, day, month, year), BW= Body weight of the assessed age group (kg) as provided by EPA (2011b).

*Target hazard quotient (THQ)*

The health risk associated with consuming juice samples was measured on the basis of the Target Hazard Quotient (THQ). The THQ is the parameter that is considered in assessing the non-carcinogenic effects as described by Saleh and Goktepe (2019); Salem *et al.* (2024). The THQ was calculated using the following equation:

$$THQ = \frac{EDI\ \left(^{\mu g/kg}/_{day}\right)}{PMTDI\ \left(^{\mu g/kg}/_{day}\right)}$$

Where: THQ= Target hazard quotient, EDI= Estimated daily intake (µg/kg BW/day), PMTDI= Provisional maximum tolerable daily intake of PAT set to 0.4 µg/kg BW/day (EC. 2006).

*Total target hazard quotient (TTHQ)*

Total target hazard quotient (TTHQ) was calculated by summing the THQ form different sources of PAT (all juice type tested in this work) according to Embaby *et al.* (2024).

$$TTHQ = THQ\ apple + THQ\ orange + THQ\ grape + THQ\ cocktail$$

*Statistical analysis*

The Web Agri Stat Package (WASP) at ICAR (Central Coastal Agricultural Research Institute) was used for the statistical analysis. The results were subjected to one-way analysis of variance (ANOVA) to analyze the difference between groups by applying the critical difference. All tests were treated in three replicate ($p < 0.05$) (Grech, 2018).

## Results and Discussion

*Isolation and morphological identification for the fungal species*

About 25 fungal isolates were obtained from the collected food stuff samples, showing the typical morphological and microscopic features of the genera

of *Penicillium* (18), *Aspergillus* (4), and *Talaromyces* (3) (Figure 1). Using a light microscope, the isolates were grouped into 8 morphotypes based on their phenotypic features such as colony color, growth patterns, surface, and spore dimensions. Morpghological identification gto the species level displayed that 2 isolates out of 14 were clearly identified as *P. expansum* and *P. citrinum.* The rest of of the isolates were identified as *Penicllium* spp., *Aspergillus* sp., and *Talaromyces* sp. Consequently, 12 isolates were subjected to further ITS sequencing.

*Molecular identification and phylogenetic analysis of the fungal isolates*

Phylogenetic analysis of the fungal isolates based on ITS region sequencing is presented in Figure 2. The ITS sequences of the identified strains showed high similarity to *Penicillium* spp.; except for the fungal strain S8 that showed high similarity to *Aspergillus flavus*. The accession numbers and the names of the identified strains are demonstrated in Table 2.

*Patulin determination in patulin-producing fungi*

The 14 identified fungal isolates were examined for their ability to produce PAT on YES agar. Using HPLC analysis, the 14 isolates displayed the ability to produce PAT. These species belonged to 3 genera; mainly *Penicillium* (12 species; 3 strains *P. melanoconidium,* 2 strains of each *P. aurantiogriseum* and *P. italicum*, 1 strain of each of *P. sizovae, P. neoechinulatum, P. expansum, P. citrinum,* and *P. polonicum*), *Aspergillus* (*A. flavus*), and *Talaromyces* (*T. islandicus*). Figure 3 illustrates the amounts of PAT (µg/ g) produced by the different fungal strains isolated from different food sources. The maximum PAT concentration 5.1 µg/ g was recorded by *P. expansum* S7, followed by *P. aurantiogriseum* S10 and S9, recording patulin concentration of 1.67 and 1.43 µg/ g, respectively. *P. melanoconidium* S15 produced PAT concentrations was 1.17 µg/ g While, the lowest PAT content was recorded by *A. flavus* S8, *P. italicum* S2, *P. italicum* S1, and *T. islandicus* S12, recording concentrations of 0.15, 0.16, 0.20, and 0.18 µg/ g, respectively. *P. neoechinulatum* S5, *P. citrinum* S13, and *P. sizovae* S6 produced PAT at concentrations of 0.88, 0.94, and 0.94 µg/ g, respectively.

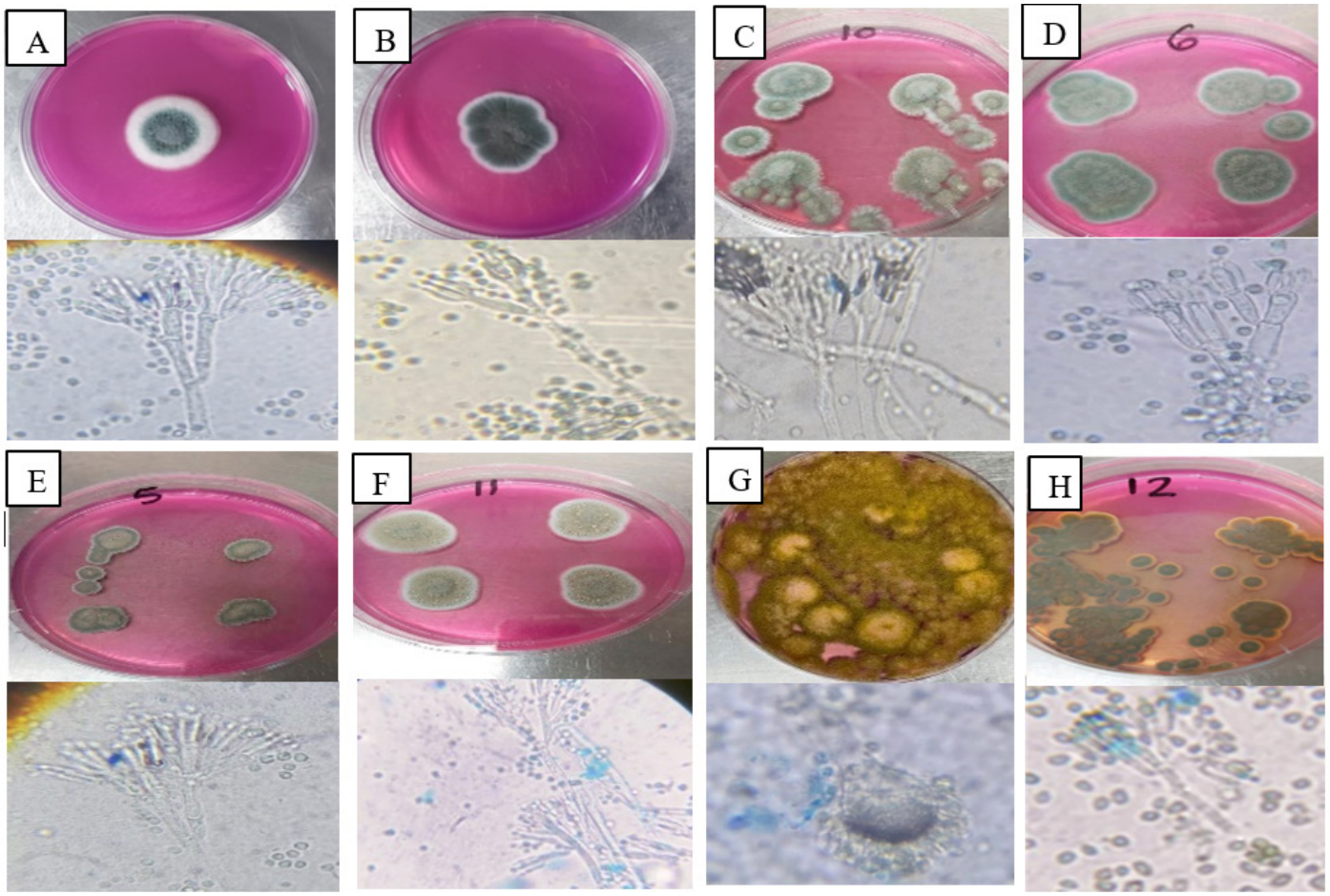


**Figure 1:** *Photomicrograph of the obtained fungal isolates showing the morphological features of the colony and the cells under light microscope (slide staining with mounting stain for moulds) with magnification power (1000x). (A) P. expansum, (B) P. citrinum, (C, D, E, and F) Pencillium spp., (G) Aspergillius sp., and (H) Talaromyces sp.*

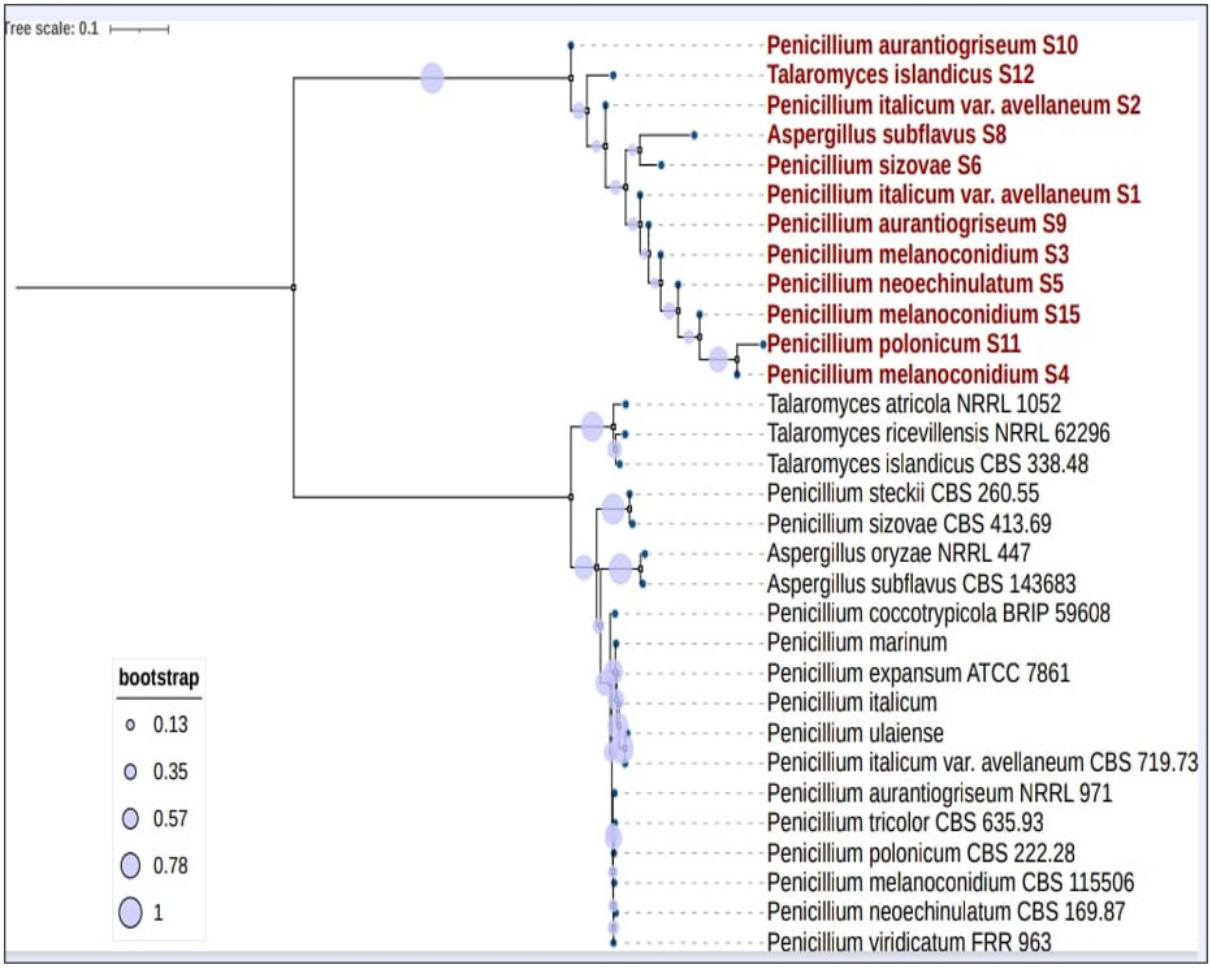


**Figure 2:** *A neighbor-joining phylogenetic tree based on ITS gene sequences of 12 fungal isolates (labelled with red color) with the closest hits obtained from the NCBI GenBank.*

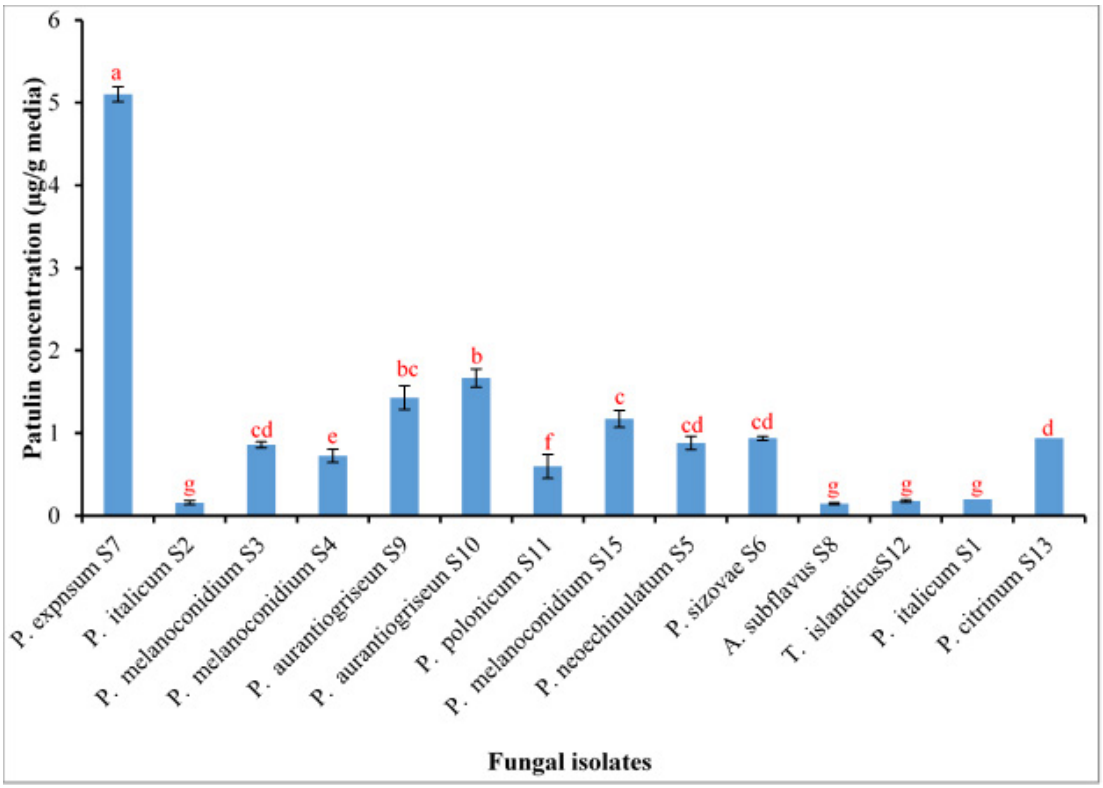


**Figure 3:** *Concentration of PAT (µg/ g) produced by the fungal strains obtained from different food sources on YES medium. Results are averages of three replicate (±SD). The columns with different letters are significantly different.*

**Table 2:** *Accession numbers and closest hits of the obtained fungal isolates from differenr food stuffs.*

| Isolate No. | Source of isolation | Strain name | Gene Bank accession number |
|---|---|---|---|
| S2 | Apple | *Penicillium italicum var. avellaneum* | PX021941 |
| S3 | Wheat | *Penicillium melanoconidium* | PX021942 |
| S4 | | *Penicillium melanoconidium* | PX021943 |
| S9 | Corn | *Penicillium aurantiogriseum* | PX021946 |
| S10 | | *Penicillium aurantiogriseum* | PX021947 |
| S11 | | *Penicillium polonicum* | PX021948 |
| S15 | | *Penicillium melanoconidium* | PX021949 |
| S1 | Wheat | *Penicillium italicum var. avellaneum* | PX061751 |
| S6 | Wheat | *Penicillium sizovae* | PX021945 |
| S5 | | *Penicillium neoechinulatum* | PX021944 |
| S12 | Corn | *Talaromyces islandicus* | PX061753 |
| S8 | Wheat | *Aspergillus flavus* | PX061752 |

Similarly, Samson *et al.* (2010) reported that the most fungal species associated with PAT production were *Byssochlamys nivea*, *A. clavatus*, *P. expansum*, *P. griseofulvum*, *P. funiculosum*, *P. roqueforti*, and *P. brevicompactum*. Rodriguez *et al.* (2011) indicated that *A. flavus*, *A. oryzae*, *A. tamarii*, *P. commune*, *P. expansum*, *P. polonicum*, and *P. verrucosum* were capable of PAT production. While, *P. brevicompactum* was considered the main source of produced PAT in tomato (Paterson *et al.*, 2003). Sabater-Vilar *et al.* (2004) found that *A. clavatus* isolated from malted barley grain had the ability to produce PAT at a concentration of 20 µg/ g of culture medium. Mansouri *et al.* (2014) stated that from 51 strains of *Penicillium* isolated from various cereals samples, only 6 strains of *P. expansum* produced PAT in a broth medium with range of concentration of 0.10 – 41.72 µg/ ml and one strain *P. purpurogenum* had a PAT content of 0.18 µg/ ml. The isolated fungi in this study were previously reported by several previous studies. In this regard, *A. flavus*, *P. aurantiogriseum* and *P. melanoconidium* were reported by Luque *et al.* (2011) as PAT producing fungi. Moreover, *P. aurantiogriseum* and *P. italicum* were reported by Okeke *et al.* (1993).

*Incidence of patulin in the juice samples*

The presence of PAT in juice samples is presented in Table 3. The obtained results showed that the highest percentage of positive samples with PAT were observed in apple juice (28.6%), followed by grape juice (25.0%) and orange juice (23.1%), while, cocktail juice recorded 16.7%. The concentration of PAT in apple juice ranged from 13.91 to 79.35 µg/ l, while the highest range was recorded in orange juice samples (27.36 to 234.30 µg/ g). On the other hand, PAT concentrations in both grape and cocktail juice samples were observed the at the lowest range of 1.68 to 1.88 ug/g and 0.56 to 1.08 ug/g, respectively. The percentages of juices samples that exceeded the maximum permissible levels (MPLs = 50 µg/l) were 7.14% and 15.38% in the apple and orange juice samples, respectively, while none of the grape and cocktail juice samples showed PAT concentration above the MPLs. In accordance with these findings, PAT was detected in apple at high levels in the range of 1120-6235 µg/ kg in South Africa by Brown *et al.* (1996). Moreover, PAT was detected in apple, grapes, orange, grains, pears, and other foods by Zouaoui *et al.* (2015) study, which revealed that the PAT levels in the fruit juice samples collected from markets in Tunisia ranged from 2 to 889 µg/kg, and about 22.0% of these contaminated samples exceeded the permissible limits (MPLs= 50 µg/l). In this regard, 50% of apple beverage samples collected by El Filali *et al.* (2024) from different markets in Morocco contained PAT levels that ranged from 0 to 16.36 µg/ l and none of the samples showed PAT concentration above the maximum permissible limit (50 µg/ l). Because of the heat-resistant nature of PAT molecule in acidic conditions, it can be detected in foods after various processes like heating, fermentation, and milling, and the sole way to prevent its incidence is to hinder growth of the PAT-producing fungi in foods (Lien *et al.*, 2020).

**Table 3:** *Incidence of PAT in juice samples collected from local markets.*

| Sample type | No. of samples | No. of positive samples | Mean ± SE (µg/ l) | Min (µg/ l) | Max (µg/ l) | No. of samples exceeded MPLs* |
|---|---|---|---|---|---|---|
| Apple juice | 28 | 8 (28.6%) | 52.27 ± 10.54$^{b}$ | 13.91 | 79.35 | 2 (7.14%) |
| Orange juice | 26 | 6 (23.1%) | 102.70 ± 15.44$^{a}$ | 27.36 | 234.30 | 4 (15.38%) |
| Grape Juice | 12 | 3 (25.0%) | 1.78 ± 0.07$^{c}$ | 1.68 | 1.88 | 0 |
| Cocktail juice | 12 | 2 (16.7%) | 0.82 ± 0.12$^{d}$ | 0.56 | 1.08 | 0 |

*Where; SE: standard error, p<0.05, *MPLs: Maximum permissible levels (50 µg/ l). Cocktail juice: Juice that contains one or more of apple, orange or grape juice.*

In Turkey, the results of long-term survey of PAT in apple juice concentrates collected from different manufacturers for 4 consecutive years (1996–1999) indicated that the maximum recorded PAT concentrations were 376, 153, 103, and 109 µg/ l in 1996, 1997, 1998, and 1999, respectively. About 48% and 36% of the apple juice concentrations produced in 1996 and 1997, respectively, exceeded the MPLs (50 µg/ l). Meanwhile, 8% of the samples collected in 1998 and 1999 exceeded the MPLs (Gökmen and Acar, 2000). Also, Yurdun *et al.* (2001) reported that PAT was detected in apple juice samples sold in Turkey, with concentrations ranging from 19.1 to 732.8 µg/ l. Notably, 44% of these samples had PAT levels exceeding the maximum permitted limits (MPLs). Cho *et al.* (2010) study indicated that 12.5% of the apple, grape, and orange juice samples collected from different sites in South Korea were contaminated with PAT at concentrations that ranged from 2.8 to 30.9 µg/ l.

*Patulin validation methods*

Figure 4 represents HPLC chromatogram of the PAT standard and spiked apple juice sample (100 µg/ l) with retention time of 3.83 min. The coefficient of determination ($R^2$) was 0.9997, indicating an excellent fit of the standard curve and confirming the system's linearity (Figure 5). The LOD and LOQ were 0.21 and 0.55 µg/ l, respectively. The LOD and LOQ determined in the current study were comparatively high compared to the results obtained by Iqbal *et al.* (2018); Aslam *et al.* (2021); Iqbal *et al.* (2024). Pernica *et al.* (2021) reported that the LOD and LOQ levels of PAT were 4.9 and 6.6 µg/ l, respectively. While, the values of LOD from 2.6 to 7.5 µg/ l and LOQ of 8.0–15.0 µg/ lwere reported in dried fruits, jams, and juices samples (Ji *et al.*, 2017).

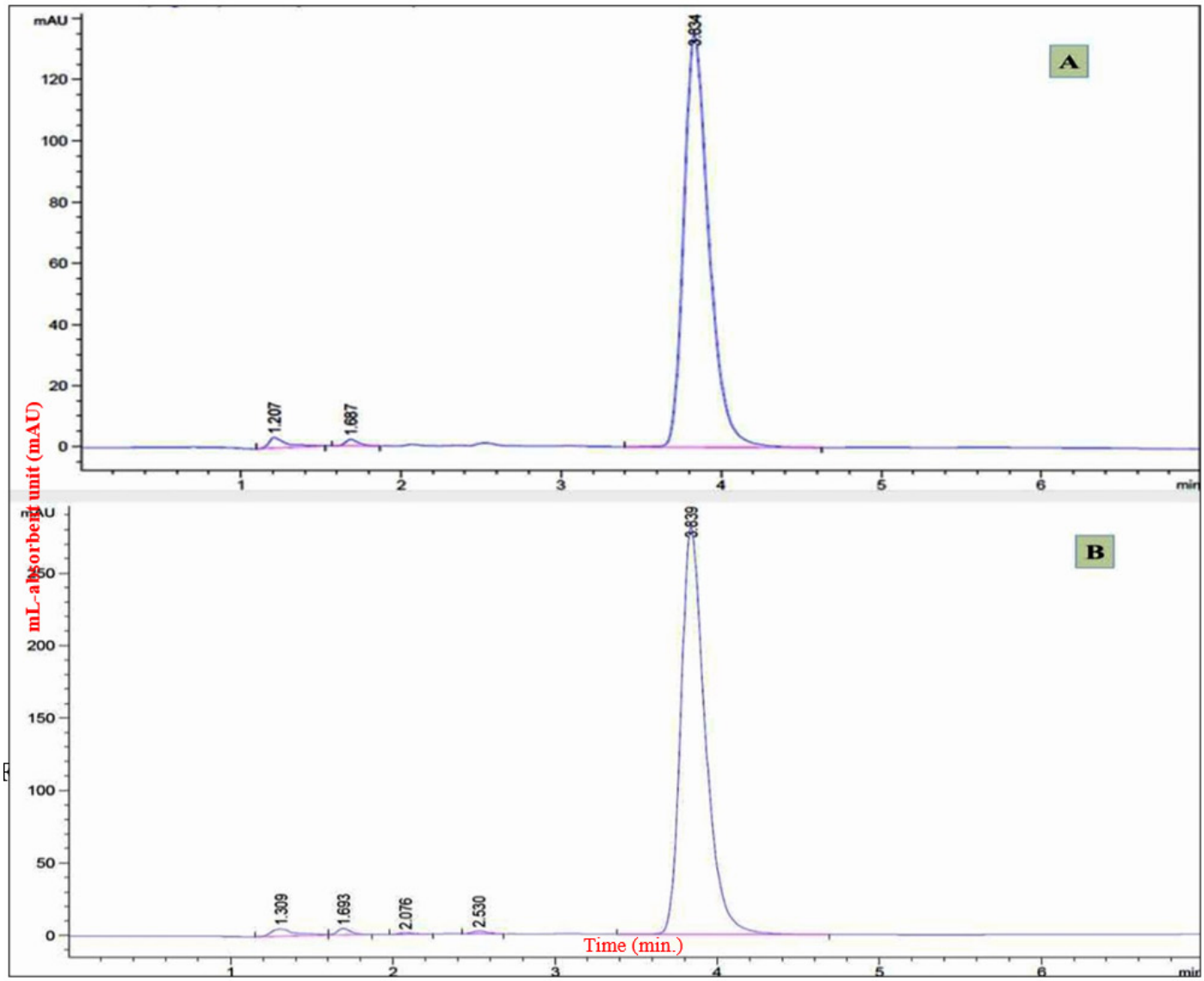


**Figure 4:** *HPLC chromatogram of PAT standard (A) and apple juice sample spiked with PAT 100 µg/ ml (B).*

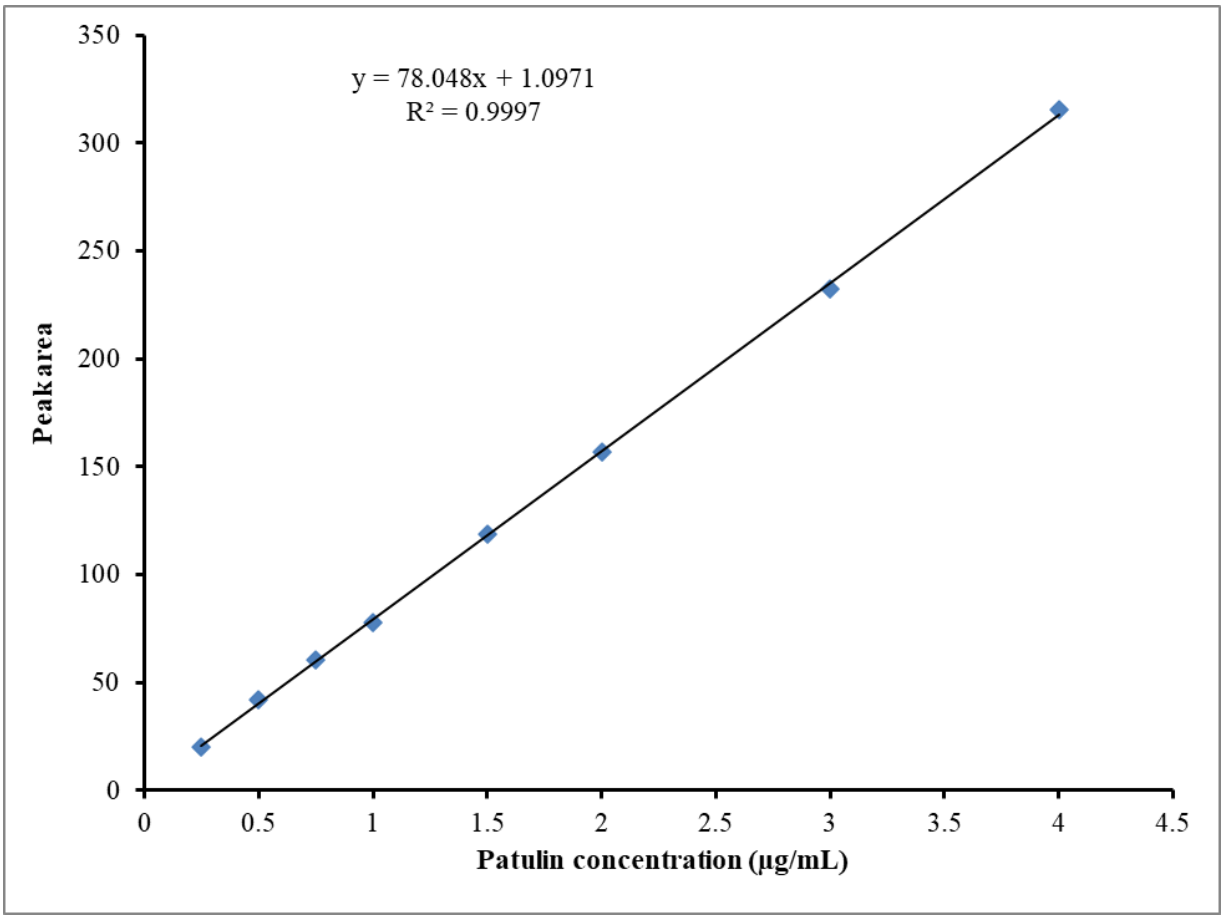


**Figure 5:** *Standard curve of PAT. The coefficient of determination (R2) was 0.9997, indicating an excellent fit of the standard curve and confirming the system's linearity.*

To confirm the accuracy of PAT determination method, the recovery rates were performed using spiked juice samples. Recovery was applied on blank apple, orange, grape, and cocktail juice samples spiked with PAT at three concentration levels: 25, 50 and 100 µg/ l (Table 4). The average of recoveries ranged from 98.84% to 102.79 % in apple juice, 98.81% to 101.74% in orange juice, 98.32% to 101.02% in grape juice, and from 96.87% to 99.23% in cocktail juice. While the relative standard deviation (RSDs) values varied from 4.65% to 11.37%. EC (2006) reported that the acceptable criteria for PAT recoveries were 50% to 120% for spiked samples with PAT less than 20 µg/ l, and 75% to 105% for spiked samples with PAT more than 50 µg/ l. These ratios are used to ensure the accuracy of the analytical methods applied to detect PAT in juices. Also, Cho *et al.* (2010) reported that the recovery rate of PAT in apple juice spiked sample ranged between 101.0% and 103.6% and in orange juice ranged from 102.4% to 115.7%, with RSDs values that ranged from 3.31% to 9.49%. Iqbal *et al.* (2024) revealed that the recovery rate of PAT using six concentrations of spiked apple samples varied from 79.8% to 104.5%.

**Table 4:** *Recovery and relative standard deviation (RSD) of patulin in different juices for three spiked concentrations.*

| Sample type | Spiked patulin (µg/ l) | Patulin concentration (µg/ l) | Recovery (%) | RSD (%) |
|---|---|---|---|---|
| Apple juice | 25 | 25.45 | 102.79 | 9.07 |
| | 50 | 49.42 | 98.84 | 6.71 |
| | 100 | 100.86 | 100.68 | 5.21 |
| Orange juice | 25 | 24.95 | 98.81 | 11.37 |
| | 50 | 49.81 | 99.62 | 6.33 |
| | 100 | 101.74 | 101.74 | 6.23 |
| Grape Juice | 25 | 24.58 | 98.32 | 4.68 |
| | 50 | 50.51 | 101.02 | 7.41 |
| | 100 | 99.11 | 99.11 | 10.62 |
| Cocktail juice | 25 | 24.22 | 96.87 | 4.65 |
| | 50 | 49.36 | 98.72 | 7.26 |
| | 100 | 99.23 | 99.23 | 5.93 |

*Where; *RSD = Relative standard deviasion.*

*Risk assessment of PAT detected in juice samples*

Data presented in Table 5 illustrate the calculated values of EDI, THQ, and TTHQ for PAT detected

in different juice types, evaluated individually for three age groups: children (7 year), adolescents (13 year), and adults (50 year). EDI and THQ are used for the assessment of non-carcinogenic risk of contaminants detected in the food. From Table 5, all obtained results for EDI were below the PMTDI (0.4 µg/kg BW/day) for all tested juice types except for the orange juice, where the calculated values were 1.029 µg/kg BW/day for children and 0.812 µg/kg BW/day for adolescents. The obtained results indicated potential non-carcinogenic risk resulting from the consumption of orange juice with such level of contamination at children and adolescents at a given dose and exposure data. If THQ values were below 1, this indicates there was no non-carcinogenic risk of consumption of such food at the detected contaminants levels. The THQ values for orange juice recorded 2.573 µg/kg BW/day and 2.029 µg/kg BW/day for children and adolescents, respectively, indicating a potential non-carcinogenic risk of the consumption of orange juice for children and adolescents at the given dose and exposure conditions. It is important to note that individual consumption of the other tested juices, including apple, grape, and cocktail was found to be safe. The TTHQ value above 1.0 suggested a potential health risk from non-carcinogenic effects associated with eating the contaminated food, recorded to be 3.473, 2.726, and 0.700 for children, adolescents, and adults, respectively, indicating an adverse health risk for children and adolescents but not for adults at the given exposure data.

It is worth mentioning that based on the minimum detected concentration, the obtained calculation values of EDI, THQ, and TTHQ revealed that all EDI values were below the PMTDI (0.4 µg/kg BW/day) and all THQ and TTHQ values were below 1, indicating no non-carcinogenic risk for all the tested age groups at the given exposure data (Supplementary Table S1).

The calculated EDI values for apple, grape, and cocktail juice were below the PMTDI (0.4 µg/kg/day) resulting in THQ values below 1, indicating the unlikelihood of adverse health effects occurrence *via* exposure to such juices at the given exposure data. These results were close to those obtained by a previous study performed by Saleh and Goktepe (2019).

**Table 5:** *Estimated daily intake (EDI), target hazard quotient (THQ), and total target hazard quotient (TTHQ) values of PAT detected in different juice types.*

| **Children ( Age 7 year, Body weight 25.1 kg, daily consumption 365 d/year)** | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Juice type** | **C (µg/l)** | **IGR (g/day)** | **Body weight (kg)** | **EDI (µg/kg/day)** | **PMTDI (µg/kg/day)** | **THQ** | **TTHQ** |
| Apple | 79.35 | 111.5 | 25.1 | 0.347 | 0.4 | 0.867 | 3.473 |
| Orange | 234.3 | 112 | 25.1 | 1.029 | 0.4 | 2.573 | |
| Grape | 1.88 | 111.5 | 25.1 | 0.008 | 0.4 | 0.021 | |
| Cocktail | 1.08 | 111.5 | 25.1 | 0.005 | 0.4 | 0.012 | |
| **Adolescent ( Age 13 year, Body weight 51.1 kg, daily consumption 365 d/year)** | | | | | | | |
| Apple | 79.35 | 173 | 51.1 | 0.269 | 0.4 | 0.672 | 2.726 |
| Orange | 234.3 | 177 | 51.1 | 0.812 | 0.4 | 2.029 | |
| Grape | 1.88 | 175 | 51.1 | 0.006 | 0.4 | 0.016 | |
| Cocktail | 1.08 | 175 | 51.1 | 0.004 | 0.4 | 0.009 | |
| **Adult (Age 50 year, Body weight 73.5 kg, daily consumption 365 d/year)** | | | | | | | |
| Apple | 79.35 | 72.5 | 73.5 | 0.078 | 0.4 | 0.196 | 0.700 |
| Orange | 234.3 | 62.5 | 73.5 | 0.199 | 0.4 | 0.498 | |
| Grape | 1.88 | 67.5 | 73.5 | 0.002 | 0.4 | 0.004 | |
| Cocktail | 1.08 | 67.5 | 73.5 | 0.001 | 0.4 | 0.002 | |
| Reference | Maximum levels detected in this study were used | EPA (2011b) | EPA (2011a) | Calculated from an equation reported by Saleh and Goktepe (2019) | EC (2006) | Calculated from an equation reported by Saleh and Goktepe (2019) | |

*Where; C: PAT concentration, IGR: Ingestion rate of food, EDI: Estimated daily intake, PMTDI: Provisional maximum tolerable daily intake THQ: Target hazard quotient, TTHQ: Total target hazard quotient.*

Torović *et al*. (2018) conducted a risk assessment for PAT in fruit juices by Serbian population for children (7-10 year), adolescents (11-14 year), and adults (15 + year), and recorded the estimated PAT daily intake means as 25.97, 8.32, and 2.82 ng/kg BW/day for children, adolescents, and adults, respectively. Also, a study carried out in China for PAT in apple juice and reported the PAT intakes to be 28.1, 67.5, and 110 ng/ kg BW/day for adults, children, and babies, respectively (Guo *et al*., 2013). The same line of results was detected in Qatari samples with PAT intake that ranged from 7.2 to 74.5 ng/kg BW/day (Saleh and Goktepe, 2019).

The obtained results in this study showed the same trend indicating that children were more susceptible with high daily intake than adolescent and adults, in agreements with a previous study (Torović *et al*., 2018). Although the calculated values for EDI and THQ are higher than that reported in a previous study (Saleh and Goktepe, 2019). The use of maximum detected PAT concentrations likely contributed to the obtained high EDI and THQ values. In contrast, calculations based on minimum detected concentrations yielded lower values, consistent with a previous study (Saleh and Goktepe, 2019) (Supplementary data Table S1). In this case, all estimated values for EDI, THQ, and TTHQ were below 1, indicating no potential risk from the consumption of such juices for each age group (children, adolescents, and adults) at the given exposure data.

## Conclusions and Recommendations

The present work points out to the possible incidence of PAT-producing fungi in different food and beverage stuffs. Phylogenetic analysis based on ITS sequences confirmed the predominance of *Penicillium* spp. among the recovered isolates, with two exceptions assigned to *Aspergillus flavus* and *Talaromysis islandicus*. Moreover, PAT was detected in 28.6%, 23.1%, 25.0%, and 16.7% of collected apple, orange, grape, and cocktail juice samples, respectively. This represents a human health threat which entails scientific concerns about the deterrence of production of such toxic fungal metabolite in food materials. Future studies are recommended to focus on the whole-genome characterization, demonstrating the genetic basis and regulatory mechanisms underlying the PAT biosynthesis, followed by using the main promising techniques for prevention, control. and detoxification of PAT.

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## Novelty Statement

The novelty of this study lies in providing a promising reference for the isolation, purificatuion, and identification of patulin-producing fungi. It also determined the patulin levels in different juices and assessed the potential risks of patulin consumption on humans.

## Author's Contribution

ASA, MNA, GME, SHS, DAM and OSB: Conceptualization, data curation, investigation, supervision, validation, roles, writing original draft, writing review and editing.

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The authors have declared no conflicts of interest.

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