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Dietary Risk Assessment of Patulin in Cow Milk from Urmia of Iran

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HIGHLIGHTS

- The mean concentration of patulin in milk samples was measured at 0.34±0.49 µg/L.
- Patulin wasn't detected in 17 out of 30 analyzed milk samples.
- The highest recorded concentration of patulin in milk samples was 1.57 µg/L.

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Abbreviations
HI=Hazard Index
HPLC=High-Performance Liquid Chromatography
LOD=Limit of Detection
LOQ=Limit of Quantification
PMTDI=Provisional Maximum Tolerable Daily Intake
TDI=Tolerable Daily Intake

ABSTRACT

Background: Food contamination with mycotoxins is a global concern. Patulin, a mycotoxin secreted by molds, such as *Penicillium expansum* and *Aspergillus clavatus*, poses significant health risks. This study aimed to determine the presence of patulin in cow's milk in Urmia, Iran, and to assess dietary intake of patulin and the associated Hazard Index.

Methods: A total of 30 individual cow milk samples were collected during the summer of 2020 from a village near Urmia, Iran. Patulin levels were measured using High Performance Liquid Chromatography-Diode Array. Data was analyzed through SPSS.

Results: Incidence rate of patulin in milk was 43.3%, with the mean of 0.34 µg/L. The Provisional Maximum Tolerable Daily Intake exceeded the estimated dietary intake, which ranged from 0.000063 for adults to 0.00086 µg/L for children. The Hazard Index was below one, indicating no non-carcinogenic health hazards.

Conclusion: Patulin contamination was quantified in milk samples from Urmia, Iran. Given the potential health implications of patulin contamination, it is essential for government authorities and regulatory agencies involved in milk production to monitor mycotoxin residues and implement hazard control measures throughout the food supply chain.

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Introduction

Mycotoxin contamination in food is a widespread issue, particularly in developing countries. Notably, climate change has amplified this risk in developed countries (Schrenk et al., 2020). Patulin ($C_7H_6O_4$) is a mycotoxin produced by some fungi, such as *Penicillium expansum*, and *Aspergillus clavatus*, under suitable conditions of humidity and temperature (Wei et al., 2020). While patulin is mainly measured in apple and its derivatives, including juices, nectar, and beverages, it has also been detected in other rotten fruits and vegetables, including cherries, pears, blueberries, peaches, strawberries, mulberries, and plums (Wright, 2015). The highest average level of patulin contamination was measured in apples in Iran (620 µg/kg) (Montaseri et al., 2014), followed by grapes in the Czech Republic (328.5 µg/kg) (Ostry et al., 2018). A High-Performance Liquid Chromatography (HPLC) investigation of juices in Argentina revealed patulin contamination levels ranging from 3 to 19,622 µg/L (Oteiza et al., 2017). In addition, an investigation of orange and grape juices in South Korea indicated that 12.5% were contaminated with patulin (Pattono et al., 2013). A study employing HPLC-Ultra Violet (UV) showed that 50% of 214 sampled fruit juice products in Tunisia were contaminated, with 22% exceeding the maximum levels (50 µg/Kg) (Zouaoui et al., 2015). Another study in Northwest Iran, investigated more than one hundred juices from four different fruits by HPLC and detected patulin in the samples (Fathi Achachlouei et al., 2009). Although patulin is generally associated with apples and their products, studies have suggested that it also pollutes fruits like figs and kiwis; grains like rice, wheat, and corn and dairy products like cheeses (Assunção et al., 2016).

Mycotoxins are also present in animal feed. A study showed the presence of these mycotoxins in the plasma of lactating cow (Winkler et al., 2014). This raises concerns about the potential carryover of these toxins into cow's milk (Flores-Flores et al., 2015). Milk contamination with aflatoxin M_1 due to the presence of aflatoxin B_1/G_1 in animal feed is a common issue (Rahimzadeh Barzoki et al., 2023). Patulin is a thermos-tolerant toxin and does not metabolize within animals (Saleh and Goktepe, 2019). Research indicates that while rumen flora can metabolize certain mycotoxins, such as ochratoxin A, deoxynivalenol, aflatoxin B_1, and zearalenone, into compounds with lower toxicity, others like patulin or fumonisins pass through the rumen unchanged (Fink-Gremmels, 2008). Furthermore, the rumen barrier's effectiveness can be changed by factors such as diseases, dietary changes, or high level of mycotoxin in animal feed (Pattono et al., 2011). Food contamination with patulin is a global issue; therefore, a maximum limit of 50 µg/L or kg for patulin has been established for apple, apple juice, and cider. For baby food, a lower maximum limit of 10 µg/L or kg is set (Wei et al., 2020). Regarding the highest level of patulin in milk and its products, only Slovakia has established 50 µg/L for patulin (Flores-Flores et al., 2015).

Most toxigenic fungi and mycotoxins are recognized as hazardous to health. Hazard analysis includes measuring both acute and chronic health effects (Majeed et al., 2018). Patulin has several adverse health effects, such as mutagenicity, genotoxicity, immunotoxicity, and digestive one. Moreover, patulin can damage various body systems and organs including the liver, kidneys, intestines, and immune system (Wei et al., 2020). Gastrointestinal symptoms associated with patulin exposure are ulceration, distension, and hemorrhage as observed *in vivo* studies (Piqué et al., 2013). Patulin is categorized as not carcinogenic to human (group 3 carcinogen) (IARC, 2018). A Tolerable Daily Intake (TDI) has been determined for non-carcinogenic mycotoxins. Measurement of exposure to mycotoxin relies on mycotoxin's content in foods and their absorption rates. Risk assessment of non-genotoxic mycotoxins is performed by comparing exposure assessments with TDI (Majeed et al., 2018). Patulin contamination affects individuals across all age groups, races and genders; however, certain groups may be more sensitive. Children are particularly vulnerable to contaminants due to their higher exposure rate and different physiology (Raiola et al., 2015). Pregnant women and their fetuses are also at risk, as mycotoxins can influence fetal development (Chan-Hon-Tang et al., 2013). Although all age groups are at risk of patulin, nursing infants face heightened risks. Consumption of patulin by breastfeeding mothers may lead to the transfer of toxins into breast milk at levels exceeding TDI for children, even if the mother's exposure is within TDI limits (Saleh and Goktepe, 2019). According to extensive literature reviews, most human exposure to patulin occurs through apples; and up to our knowledge; there is a possibility of patulin secretion in milk. The aim of the present study was to investigate the presence of patulin in cow's milk in Urmia, Northwest Iran. Additionally, the exposure to patulin was calculated, and a risk assessment was carried out for consumers.

Materials and methods

Chemicals and reagents

Patulin (5 mg, 98%) was obtained from Sigma-Aldrich (Missouri, United States). All chemicals used were HPLC-grade and sourced from Fisher Scientific (Pittsburgh, PA, USA). Other chemicals, including $NaHCO_3$, ethyl acetate, and Na_2SO_4, were of analytical grade and obtained from Merck (Merck KGaA, Darmstadt, Germany). A Milli-Q

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system (Millipore, Bedford, MA, USA) was used for purification of deionized water.

Quality control

A standard stock solution (100 mg/kg) was prepared by dissolving patulin in acetonitrile. Calibration series were established by diluting the stock solution in acetonitrile.

Method validation

Parameters such as linearity, accuracy, Limit of Detection (LOD), Limit of Quantification (LOQ), precision (repeatability and intermediate precision), and selectivity were assessed. Patulin-free milk samples served as experimental blanks to verify method selectivity through spiking. Peak identities were confirmed to calculate linearity, precision, and accuracy of the method. Accuracy was represented as the mean of the removed baselines, while precision was indicated by the standard deviation of the mean in manual baseline correction methods as functions of the Signal-to-Noise Ratio (SNR) (Jirasek et al., 2004).

Sampling and sample preparation

A total of 30 individual cow milk samples were randomly obtained from a village on the outskirts of Urmia in Northwest Iran during the summer of 2020, and stored at -18 ℃ before extraction. For extraction, 2.5 g of milk samples was mixed with 0.75 g $NaHCO_3$, five ml of ethyl acetate, and 2.5 g Na_2SO_4 in a falcon tube and left for 3 min. The mixture was then vortexed for one min and centrifuged at 3,800 rpm for three min. Ethyl acetate phase was obtained and dried under a Nitrogen (N_2) flow. The dried extract was subsequently dissolved in 200 µl acetonitrile and injected into the HPLC system according to standard ISO 8128-1:1993 (ISO, 1993).

Instrumentation

An agilent 1,100 series HPLC system equipped with a quaternary pump, network gasifier, and diode array detector (Agilent Technologies, Palo Alto, California, USA) was used. The separation was performed on a Supelco Octadecylsilane (C18-ODS) column (Bellefonte, Pennsylvania, USA; 250×4.6 mm inner diameter (ID), five µm particle size). Chromatographic data was analyzed by a computer with the Agilent ChemStation program for the liquid chromatography system. The chromatographic investigations were conducted under isocratic binary mobile phase conditions. Acetonitrile/water (10:90 v/v) served as the mobile phase with a flow rate of one ml/min after filtration through a 0.45 µm membrane (Whatman Limited, Maidstone, UK). The analysis was carried out at ambient temperature, and injection volume was 50 µl. LOD and LOQ were determined using a signal-to-noise ratio of 3:1 and 10:1, respectively. The recovery rate was determined using spiked samples and was found to be 100%.

Dietary exposure assessment

The dietary intake of patulin in milk was estimated by considering daily consumption and the average patulin content in milk using the following formula from the literature (Lien et al., 2020; Roila et al., 2021):

$$DI = \frac{C \times I}{BW}$$

Where, DI: is the total dietary exposure to patulin (µg/kg bw/day); C: is the patulin concentration in milk (µg/kg); I: is the intake of food item (milk) (0.038 kg/day) (INSO, 2021); and BW: is the average body weight of the consumer (kg), considered as 15 for children, 50 for adolescents, and 70 for adults.

Hazard Index (HI)

The HI of patulin in milk is calculated as follows (Sadighara et al., 2023):

$$HI = \frac{DI}{PMTDI}$$

In which, DI: is the Dietary Intake, and PMTDI: is the Provisional Maximum Tolerable Daily Intake of patulin, set at 0.4 µg/kg bw/d according to JECFA (1995). An HI<1 indicates no health risk, while an HI>1 signifies non-tolerable exposure risk (Lien et al., 2020).

Statistical analysis

Data was analyzed using SPSS version 22. Quantitative variable was expressed through mean ±Standard Deviation (SD).

Results and discussion

Table 1 shows the quantitative characteristics of the detection method for patulin and its precision in milk samples. The detection method demonstrated good LOD and LOQ. Table 2 outlines the accuracy of the method revealing that the mean concentration of patulin residues in milk samples was 0.34±0.49 µg/L. Figure 1 illustrates the chromatogram of a milk sample alongside patulin's standard. Notably, patulin wasn't detected in 17 out of the 30 samples analyzed, resulting in an incidence rate of 43.3%. The highest concentration of patulin recorded was 1.57 µg/L. The average concentration in positive samples was found to be 0.7 µg/L. Table 2 provides further accuracy data for patulin measurement, and Table 3 shows the mean and standard deviation of patulin content across different samples. Results from analysis of various fruit juices, including apple, pineapple, pear, peach, pomegranate, and both white and red grape juices, conducted in Iran showed that patulin contaminated 16.1%

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of the samples with concentrations ranging from five to 190.7 µg/kg (Rahimi and Rezapoor Jeiran, 2015). The mean concentration of patulin in contaminated samples was 34.5 µg/kg with approximately 2.5% of the samples exceeding the maximum limit (Rahimi and Rezapoor Jeiran, 2015).

Table 1: Quantitative characteristics of patulin detection method for cow milk samples.

Compound	Calibration curve	R^2	LOD (µg/L)	LOQ (µg/L)	Recovery	Intraday patulin measurement Mean±SE	Coefficient Variation %
Milk	Y=1.4399X+3.3229	0.9863	0.09	0.26	100%	0.77±0.04	5.38

LOD=Limit of Detection; LOQ=Limit of Quantification; SE=Standard Error.

Table 2: Accuracy data for patulin measurement

	Variable 1	Variable 2
Mean	0.78	0.75
Variance	0.2	0.17
Observations	13	13
df	12	12
F	1.17	
P(F<=f) one-tail	0.39	
F Critical one-tail	2.69	

Table 3: Concentration of patulin in 30 milk samples (µg/L)

Mean+SD	Median	Interquartile range	Range (Lowest-Highest)	*PMTDI
0.34+0.49	0.00	0.61	1.57 (0-1.57)	0.4

*PMTDI=Provisional Maximum Tolerable Daily Intake

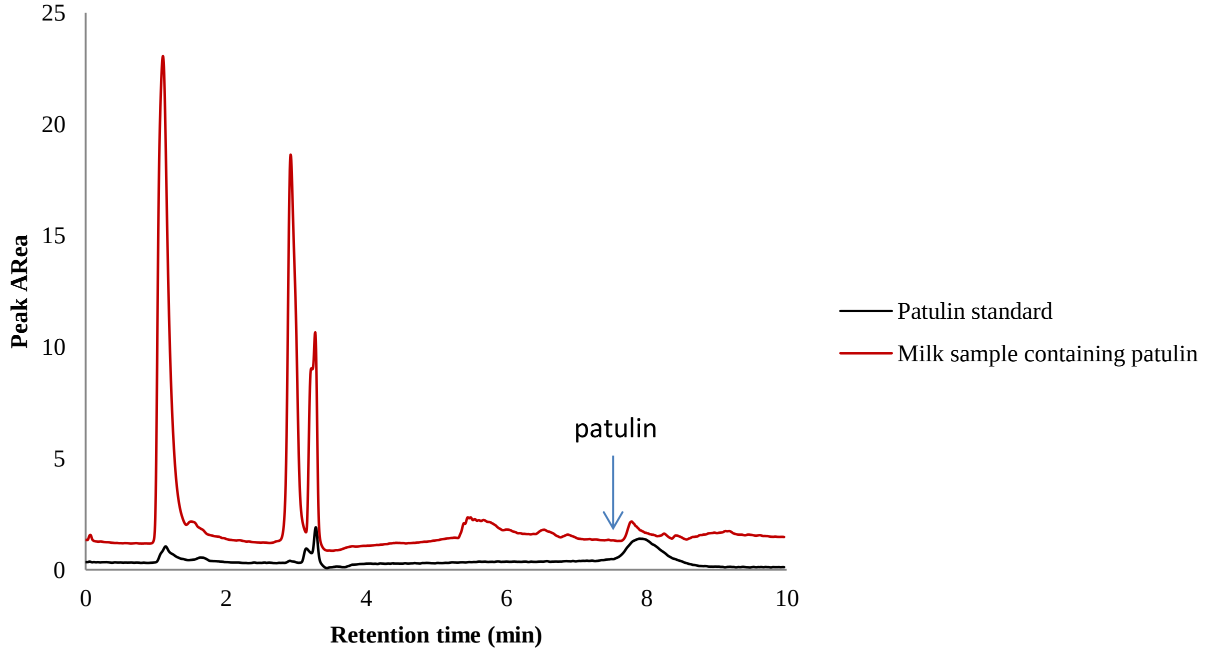

Figure 1: High-Performance Liquid Chromatography (HPLC) chromatograms obtained from cow milks in Urmia, Iran, and standard sample solution (17 µg/L)

Forouzan and Madadlou (2014) investigated the patulin content in apple juices from West Azerbayjan, Iran, and reported contamination in all analyzed samples at an average concentration of 48.64±4.4 µg/L, with values ranging from 29.58 to 151.2 µg/L. Notably, 29% of these samples had concentrations exceeding 50 µg/L (Forouzan and Madadlou, 2014). A study conducted in Northwest Iran, recorded the highest patulin content in a single

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sample of grape juice as 450.3 µg/L. Apple juice concentrates were also found to contain patulin levels above the maximum permitted levels, ranging from 89.45 to 110.25 µg/L. The authors concluded that the amount of patulin in natural fruit juice concentrates, especially apple juice concentrate, is about twice the permitted level (Fathi Achachlouei et al., 2009). Piqué et al. (2013) detected patulin in apple juice samples with an average concentration of 5.5 µg/kg; however, none of the apple juice samples exceeded the maximum permitted levels (Piqué et al., 2013).

Patulin contamination levels in Spain ranged from 4.2 to 8.1 µg/kg (Cano-Sancho et al., 2009). In Portugal, nearly 75% of cereal-based foods were found to be contaminated with patulin (mean: 2.33 µg/kg) (Assunção et al., 2016), all of which were below the maximum permissible level. In Qatar, all apple juice samples were contaminated with patulin, showing concentrations ranging from 5.27 to 82.21 µg/kg. About 11.11% of these samples exceeded permissible limits. Specifically, the patulin content in baby apple juice and baby apple compote was measured at 30.67 and 10.92 µg/kg, respectively (Hammami et al., 2017). In Pakistan, Hussain et al. (2020) reported that 61.7% of mango and 52.5% of orange samples were polluted with patulin. Their findings indicated that rotted mangoes were the most contaminated fruits with patulin. Moreover, processed products (mango juice, pulp, and jam) contained lower levels of patulin compared to the whole fruits themselves. About 21.8% of the mango samples and 0.7% of orange samples had patulin levels exceeding the maximum permissible limit (Hussain et al., 2020). Oteiza et al. (2017) reported high levels of patulin in fruit juices samples from Argentina. In Turkey, the content of patulin in soured apple reached as high as 1416 µg/kg (İçli, 2019). In addition, a total of 24% apple juices in India were found to be contaminated with patulin (845 µg/kg) (Saxena et al., 2008). Beretta et al. (2000) observed even higher levels of patulin in rotten apples (1,150 µg 1/kg) and noted that patulin was transmitted to unaffected parts of fruits by fungi. Yurdun et al. (2001) reported a patulin content of 733 µg/kg in apple juice.

All the studies mentioned above reported higher levels of patulin than those found in current study. Interestingly, mango juices from Malaysia were not contaminated with patulin (Abu-Bakar et al., 2014; Lee et al., 2014). The mean level of patulin was 1.7 and 1.4 µg/kg for apple juice and apple-containing beverages in Taiwan, respectively (Lien et al., 2020), which is lower than the findings of current study. Patulin was detected in 5.84% of the apple juice and 5.71% of apple-containing beverages (Lien et al., 2020). Influential factors contributing to patulin contamination in crops and products include agriculture practices (Piemontese et al., 2005), cloudiness of juices (Baert et al., 2007), and the addition of ascorbic acid (vitamin C) and other additives (Sant'Ana et al., 2008). The presence of ascorbic acid in juices has been shown to lead to the degradation of patulin (El Hajj Assaf et al., 2019; Sant'Ana et al., 2008).

Production of patulin mainly occurs during post-harvest and storage, but may also generate during the harvest of agricultural products. This may be due to damage from insects or mechanical damage to fruits, leading to fungal growth (Moake et al., 2005). Animal feed contamination with mycotoxin leads to detection of these toxins in plasma of dairy cows as well (Winkler et al., 2014), raising concerns about the potential transfer of these toxins into milk (Flores-Flores et al., 2015). The microflora in the rumen act as a defense against some mycotoxins; however, patulin passes through the rumen barrier unchanged (Fink-Gremmels, 2008). Additionally, changes in diet or high levels of mycotoxin contamination in animal feed can alter the rumen barrier's effectiveness (Pattono et al., 2011).

Golghasem Gharehbagh et al. (2021) investigated the influence of consuming apple pomace containing patulin in the diets of Mahabadi lactation goats. They found that the control group had the highest content of patulin (178 µg/kg), while groups treated with toxin binders exhibited lower levels (Golghasem Gharehbagh et al., 2021). Consequently, they concluded that due to the presence of patulin exceeding standard levels in apple pomace silage, using pesticide adsorbents alongside high levels of apple pomace in diets is essential to prevent negative effects on metabolism and ensure no transfer to milk (Golghasem Gharehbagh et al., 2021).

The present research is the first study reporting the presence of patulin in cow milk in Iran. In this study, patulin contamination in milk can be attributed to livestock being fed with rotten apples, which are prevalent in the Uremia region. As noted earlier, rotten fruit contains higher levels of patulin that could be released into milk when cows are fed decayed fruit.

Health risk assessment of patulin

The daily intake of patulin was below the PMTDI (0.4 µg/kg). Table 4 shows the daily intake and HI of patulin through milk consumption. The calculated HI was lower than one, indicating no non-carcinogenic health hazard associated with the consumption of contaminated milk.

Table 4: Daily intake and Hazard Index (HI) of patulin through cow milk consumption

Age group	Daily intake (µg/kg bw/day)	HI
Children	0.00086	0.00215
Adolescents	0.00026	0.00065
Adults	0.000063	0.00016

Children are more vulnerable to milk contaminated with

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patulin. This finding was supported by other studies focusing on apple-based products (Baert et al., 2007; Cano-Sancho et al., 2009; Piemontese et al., 2005; Piqué et al., 2013). In one study, the daily intake of patulin for Iranian adults, children, and infants through fruit juice was reported as16.4, 45.9, and 74.6 ng/kg bw/day, respectively. Therefore, the primary concern regarding paulin arises from commercialized apple juice in Iran and rather than other fruit juices (Rahimi and Rezapoor Jeiran, 2015), which aligns with the findings of the present study. Piqué et al. (2013) reported that the daily intake of patulin was below the PMTDI across all population groups, consistent with our findings. Brandon et al. (2012) observed that children aged 12-20 months had the highest exposure to patulin through apple products in the Netherlands. Processed cereal-based food exhibited lower dietary exposure to patulin than the PMTDI in Portugal (Assunção et al., 2016). Lien et al. (2020) reported daily exposure to patulin ranging from 5.4 to 18.0 ng/kg bw/day for apple juice and 6.1 and 11.2 ng/kg bw/day for apple-containing beverages. The highest intake of patulin was observed in infants (0-3 years) and children (4-12 years old) due to their lower body weight (Lien et al., 2020). The HI for apple juice and beverages remained within safety level (Lien et al., 2020). Regarding aflatoxin M_1 in cheese and yogurt samples, although a high percentage of samples in Iran tested positive for contamination, no public health risk was identified due to a low HI (Heshmati et al., 2020; Mozaffari Nejad et al., 2020).

Children's increased vulnerability to milk contaminated with patulin. This finding is supported by other studies conducted on apple-based products (Baert et al., 2007; Cano-Sancho et al., 2009; Piqué et al., 2013). Susceptibility of children to toxins is attributed to their higher metabolism rates, reduced detoxification capacity, lower body weight, and underdeveloped tissues and organs (Drusch and Aumann, 2005). Ultimately, based on the findings of this study, patulin contamination at observed levels in milk does not pose health problems for consumers across any age group.

Conclusion

Recent studies have highlighted the contamination of fruit juices with patulin and identified associated health risks. The current study is the first attempt to document patulin contamination in milk. Given that livestock feeding on rotten apples contaminated with patulin have a significant role in this, it is crucial for government bodies and regulatory agencies to address this issue by implementing food safety management system aimed at reducing mycotoxin contamination in dairy cow feed and consequently decreasing mycotoxin residues in milk to ensure consumer safety. Moreover, further studies are advisable, particularly in areas related to preventive measures against hazards entering the food chain through toxin binders in dairy cow diets and toxicological evaluation of patulin contamination in milk. Future studies should also examine patulin levels in dairy products, such as flavored milk, flavored yogurt, or other dairy products, especially if derived from apples as a food component.

Authors contributions

R.T., M.G., and N.V.S. designed the study; R.T., M.G., A.A., and N.V.S. performed the experiment; T.Z. analyzed the data; R.T., M.G., N.V.S., P.S., A.A., and T.Z. drafted the manuscript; P.S., I.L., and T.Z. critically revised it. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare that there is no conflict of interest.

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Ethical consideration

Not applicable.

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