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Variation of patulin levels in citrus fruits from central cities of Punjab and Northern cities of Pakistan, and estimation of dietary intake

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Abstract: The research aims to discover the natural occurrence of patulin (PAT) in selected citrus fruits from the central cities of Punjab and Pakistan's Northern cities. Total 2970 samples of twelve citrus fruits; kinnow, orange, grapefruits, bitter orange, mausami, red blood, pineapple, sweet orange, rough lime, sweet lime, kagzi lime and lemon were examined using liquid chromatography fitted with UV detector. The limit of detection (LOD) and quantification limit was 0.04 and 0.12 µg/kg, respectively. The results have shown that 56% of samples of citrus fruits from Punjab's central cities, Pakistan, were discovered to be infected from PAT. The elevated amounts of PAT ranging from 0.12 to 1150 µg/kg were found in citrus fruit samples from Multan cities. Furthermore, 31.7% of samples of citrus fruits from Northern cities of Pakistan were discovered to be infected with PAT, and the elevated amounts were found ranging from 0.12-320 µg/kg from Swat city. About 22.1% of samples of citrus fruits have levels of PAT greater than the suggested limits established by the European Union (EU). PAT's dietary intake levels ranged from 0.10-1.11 µg/kg bw/day from the central cities of Punjab, Pakistan, and 0.13-1.93 µg/kg bw/day were documented from Northern cities of Pakistan.

Keywords: Citrus fruits; patulin; dietary intake; variation in patulin; liquid chromatography

Highlights for Review

- A total of 2970 samples of twelve citrus fruit types were analyzed for patulin (PAT) contamination.
- About 56% & 31.7% samples of citrus fruits from central cities of Punjab & Northern cities of Pakistan were found positive.
- About 22.1% of samples of citrus fruits were greater than the proposed limits of EU.
- The daily intake levels of PAT were ranged from 0.10-1.11 µg/kg bw/day from central cities of Punjab,
- Furthermore, the levels of dietary intake of 0.13-1.93 µg/kg bw/day were documented from Pakistan's Northern cities.

1. Introduction

Citrus fruits belonging to the *Rutaceae* family covering 130 genera are well-known worldwide due to their diversified potential in fruits, juices, confectionaries, and fresh fruits consumption. Citrus fruits can be cultivated in tropical, subtropical, and temperate environmental conditions, and these are produced almost in 137 countries. The essential citrus fruits are oranges, tangerines, limes, mandarins, grapefruits, lemons, citrons, and many hybrid varieties, with pleasant flavors, aroma, and attractive colors. Citrus fruits are enriched with many health-promoting bioactive compounds such as ascorbic acid (vitamin C), phenolic acids, flavonoids, carotenoids, and pectin. Furthermore, calcium, phosphorous, iron, potassium, zinc, copper, and sodium are also present in citrus fruits [1, 2]

Pakistan is ranked the 10th biggest country for the production of citrus fruits in the world. The high water content and the respective nutrient composition of the citrus fruits make them susceptible to infections by microbial pathogens during the periods between harvest, transportation, storage, and subsequent consumption. The presence of mold in fruits and juices is a burning issue for the health consequences and the economic point of view. Pakistan is known to produce good quality fruits with good flavor and taste and famous worldwide. The fruits are grown in tropical and subtropical climatic conditions and available throughout the year. The fruits are produced in an area of 800.000 hectares with a production of about 7.05 million tons. Almost 10% of fruits were exported during crop seasons (2017-2018) [45, 3]. The post-harvest diseases in citrus fruits are responsible for the massive economic losses throughout the world. It is estimated that 40% of whole citrus fruits produced in Pakistan were wasted during the storage or processing stage. The fungi reduce the shelf life and affect the acceptability of the fresh citrus fruits that leads to the rejection of fruits [4, 5].

Mold growth in the citrus fruit leads to producing hazardous chemical compounds known as mycotoxins [6]. Mycotoxins are the secondary metabolites produced during pre-and post-harvest conditions. These metabolites possessed a very high bioaccumulation level and have high thermal stability [7, 8]. The fungal species such as *Aspergillus*, *Penicillium,* and *Fusarium* are documented to produce different mycotoxins. Mycotoxins are highly diversified, with about 450 different structural categories are already identified and classified [9]. The most important mycotoxins concerning their food and feed presence include aflatoxins, ochratoxin A, fumonisins, zearalenone, trichothecenes, and patulin [10]. Studies have shown that mycotoxins are resistant to different processing stages like fluctuations in pH, water content levels, ions, temperature variations, heating rate, and heating times. FAO (Food and Agriculture Organization) has documented that almost 25% of food is contaminated with mycotoxins [11].

Patulin (PAT, 4-hydroxy-4H-furo[3,2-c] pyran-2(6H)-one), is an unsaturated heterocyclic lactone, known as a toxic class of mycotoxins. It is a common contaminant of fruits such as apples, cherries, maybush, kiwi fruits, strawberries, grapes, mango, pears, apricots, tomatoes, etc. The fungi, *P. expansum*, *Penicillium*, *Byssochylamys, Aspergillus*, and *Paecilomyces* are major PAT producers. The highest stability of these toxins is at pH 4.0; therefore, the ripening stage of fruits is ideal for the attack and production of PAT producing fungi. The fungi attacked at rapture surfaces of fruits, and thus, it spread and contaminate the whole fruit. The accumulation of PAT is accelerated in fruits during storage periods [12-15]. The high solubility of this toxin makes its transfer easy from fruits to juices during processing steps. Also, in acidic medium, it possesses high stability, like in fruit juice, processing from fruits to their juices a considerable amount detected of this toxin [16]. Previous studies have documented that it induces various health hazards to human health, implicating many acute and chronic diseases. The acute symptoms include convulsions, dyspnea, agitation, edema, ulceration, pulmonary congestion, GI tract distension, intestinal bleeding, intestinal inflammation, hyperemia, epithelial cell degeneration, kidney damage, vomiting, and other gastrointestinal issues. Furthermore, it is immunotoxic, immunosuppressive, neurotoxic, teratogenic, and genotoxic [17]. The IARC (International Agency for Research on Cancer) has ranked PAT in Group 3 (not carcinogenic to human) [18]. Therefore, many countries have established maximum permissible for PAT in food and food products. The (JECFA) has executed a short-term maximum acceptable dietary intake (PMTDI) of 0.40 μg/kg body weight (bw)/day [19]. The Codex Alimentarius Commission (2003) [20] has set a maximum limit of 50 μg/kg in apple juice, as well as European Union (EU) has set the highest level of 50 μg/kg fruit juices, fruit nectars, concentrated fruit juices, along with cider, alcoholic drinks as well as the other fermented drinks produced from apples or apple juice. The limit of 25 μg/kg has been implemented in solid apple fruits and 10 μg/kg in the apple-derived products along with baby foods, and for the young children and infants [21]. However, no regulations are established for PAT in fruits or juices in Pakistan [22].

In our previous study [22], considerable PAT amounts were found in juices, smoothies, and fruits from Pakistan. However, no comprehensive PAT surveillance in citrus fruits was conducted, considering their importance and production in Pakistan. Therefore, the present survey is designed to analyze PAT's existence in selected citrus fruits from the central cities of Punjab, and Northern cities of Pakistan, to evaluate the concentrations of PAT with EU permissible limits and estimate the daily intake of PAT in regional inhabitants. The findings will be useful for smallholders, consumers, and exporters to understand the toxic effect of this toxin.

2. Materials and Methods

2.1. Samples

Total 2970 samples of 12 citrus fruits (kinnow, orange, grapefruits, bitter orange, mausami, red blood, pineapple, sweet orange, rough lime, sweet lime, kagzi lime, and lemon) were collected from the major citrus crops producing cities of Pakistan (Toba Tek Singh, Sargodha, Multan, Jhang, Sawat, Peshawar, Mirpur) during September 2019 till December 2019. There was a random collection of samples, and each sample's size was maintained at 1kg. The samples were stored in plastic bags with proper labeling and kept at -20 °C in the freezer until further analysis.

2.2. Reagents and chemicals

Patulin standard with the concentration of 100 mg/mL, in acetonitrile was obtained from Sigma Aldrich: Saint-Louis, USA. Ethyl acetate, sodium carbonate, and acetic acid were obtained (Sigma-Aldrich, France). The solvents, including methanol and acetonitrile, were of HPLC grade, obtained from Fisher Chemicals (France). PAT's standard curve was made with a dilution of the pure amount of methanol ranging from 0.4, 1, 5, 10, 50, 100, 150, 200, 250, and 300 μg/L. The standard solutions were kept and stored in the sealed vials at -20 ºC, till further analysis. The other reagents and solvents were all analytical grade.

2.3. The cleanup procedure

Patulin extraction from the citrus fruit samples was achieved following the [22] method, with some modifications. About 10 g of solid citrus fruit sample was added in 10 mL water and homogenized. Then, a mixture of 20 mL of ethyl acetate was added and extracted using a vortex mixer for 3 min. After that, the mixture was centrifuged (5 min; at 25 ºC) at 4500 rpm. The upper organic layer was shifted in a centrifuge tube along with the addition of 10 mL of 1.5% (w/v) sodium carbonate with vigorous mixing. Again the addition of 5 mL of ethyl acetate with vigorous shaking for 5 min was done. The solution pH was maintained at 4 with the addition of a few drops of glacial acetic acid followed by the evaporation using a nitrogen stream at 60 ºC. A 5% solution of acetonitrile (5 mL) was then added to the residue and purified with a 0.22 mm syringe filter (Millipore), followed by the complete dryness using nitrogen stream. Finally, the residue was dissolved in 500 μL of methanol, and 20 μL was injected for HPLC analysis.

2.4. HPLC conditions

Patulin analysis of the citrus fruits was achieved on HPLC: Shimadzu LC-10A series, Kyoto, Japan, equipped with a UV detector (276 nm). A C18 column (4.6 X 250 mm, 5 μm) Discovery of Supelco, Bellefonte, USA, was used for evaluation. The mobile phase comprising the composition of 90% of acetonitrile, with a flow rate of 1 mL/min in the isocratic mode was employed.

2.5. Assessment of daily intake

The procedure for the estimation of dietary consumption in the citrus fruits was followed by our previously established method, i.e., Iqbal et al. [22]. The dietary consumption of patulin in the local consumers was calculated with a questionnaire based on food frequency, comprising 500 individuals. The recalling period of citrus fruits intake comprised of four weeks. Interviewees' memory was activated by collecting the information of citrus fruits intake by means of amount, size of fruits, or juices with glass or bowl measurements. Of 500 individuals, 450 individuals have returned the questionnaire with answers, 45 individuals have never responded, and five individual questionnaires were canceled due to the technical point of view. The body weight of the individual was 70 ±2. The daily intake was assessed utilizing the following procedure

$$\text{Dietary intake } \mu g/kg/day = \frac{\text{Daily intake of } \frac{\text{fruits}}{\text{juices}}\left(\frac{g}{day}\right) \times \text{Patulin in } \frac{\text{fruits}}{\text{juices}}\left(\frac{\mu g}{kg}\right)}{\text{Average individual weight (kg)}}$$

2.6. Method Validation

The method was validated through the parameters including precision, linearity, reproducibility, limit of quantifications (LOQ), limit of detections (LOD), and repeatability and reproducibility of PAT. The LOD was obtained from the 3:1 signal-to-noise ratio, and LOQ was quantified by the 10:1 signal-to-noise ratio [23]. To assess the recovery analysis, three fortified PAT levels were spiked to uncontaminated oranges samples.

2.7. Statistical analysis

PAT data in selected citrus fruits were presented as mean ± standard deviation, and all the samples were analyzed in triplicate. The straight-line equation and coefficient of determination R^2 was calculated using the simple linear correlation/regression analysis. To determine the significant levels of PAT in citrus fruits from central cities of Punjab and Northern cities of Pakistan, the student T-test was applied using SPSS (IBM, PAW-27, USA).

3. Results and Discussions

3.1. Validation of Method and quality control

Method validation was achieved through the quality control parameters. The recovery rate was obtained by spiking blank samples with three patulin concentrations: 50, 100, and 200 µg/L. Mean values of the recovery varied from 85.5 - 91.5%. The average relative standard deviation (RSD) variation ranged from 10.6 - 14.5 %, as presented in Table 1. The recoveries range was within the requirement (70-110%) recommended by EC regulation 401/2006 [24]. PAT's standard curve ranging from 0.4 - 300 µg/L has confirmed the linearity, with the coefficient of determination (R^2) value of 0.9947. The LOD and LOQ of PAT were 0.04 and 0.12 µg/kg, respectively. These values of LOD and LOQ are lower than the ones reported by [25], LOD and LOQ in sample matrix 0.5 µg/L and 2 µg/L, respectively. The accuracies of both these quantities mainly depend on the sensitivity of the instrument [26]. The chromatograms represented in Figure 1, showing the natural occurrence of PAT in orange (a), sweet orange (b), lime (c), and grapefruits (d).

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Table 1. Recoveries analysis of PAT in citrus fruits. 1

Patulin Fortified Level	**Recovery**	**RSD**	**Retention time**	**Coefficient of Determination**	**LOD**	**LOQ**	**Precision**	
(µg/kg)	(%)	(%)	(min)	R^2	(µg/kg)	(µg/kg)	Repeatability	Reproducibility
50	85.5	12.5	6.437 ± 0.050	0.9947	0.04	0.12	14	12
100	89.7	10.6					10	11
200	91.5	14.5					14	18

RSD = relative standard deviation, LOD = limit of detection, LOQ = limit of quantification. R^2 = coefficient of determination; LOQ = LOD x 3 Repeatability and reproducibility 2
are given as mean percent RSD (%). 3

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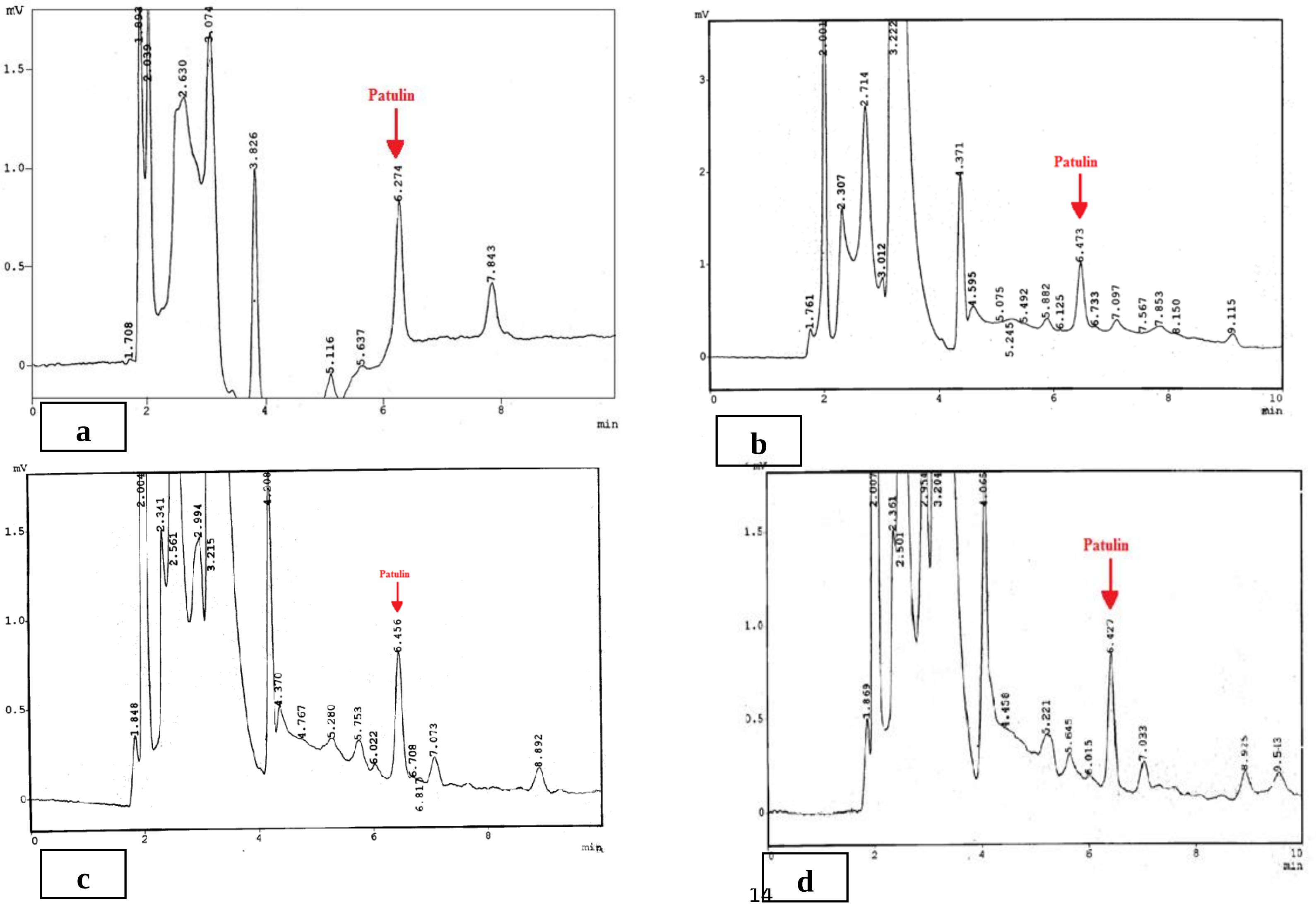

Figure 1. (a): The chromatogram showing the natural occurrence of patulin in orange (a), sweet orange (b), lime (c), and grapefruits (d).

3.2. Occurrence of PAT in the citrus fruit samples

The incidence levels of PAT in citrus fruit samples from central cities of Punjab, Pakistan, are shown in Table 2. The results have shown that 56%, i.e., 1101 out of 1967 samples of selected fruits samples, were positive with PAT. About 51.4%, 56%, 66%, and 50% samples of selected citrus fruits from Toba Tek Singh, Sargodha, Multan, and Jang cities were documented contaminated with PAT. The elevated amount of PAT 0.12-1150 µg/kg was found in sweet orange samples from Multan city.

The prevalence of PAT in selected citrus fruits from Northern cities of Pakistan is represented in Table 3. The results documented that 372 out of 1003, i.e., 37.1% of samples were found to be positive with PAT. The samples of 36.7%, 36.8%, and 37.8% were found positive from Mirpur, Peshawar, and Swat. The highest mean level of 163.3 ± 10.1 µg/kg was found in orange samples from the Swat City of Pakistan. About 657 out of 2970, i.e., 22.1% samples were found having a level of PAT greater than the EU permissible limit, and the highest 29.7% samples of citrus fruits were found levels higher than the recommended limit of EU. The PAT levels in citrus fruits from central cities of Punjab to Northern cities of Pakistan were found significant ($p < 0.05$).

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Table 2. The incidence levels of patulin in selected citrus fruits samples from central cities of Punjab, Pakistan.

Citrus fruits type	**Toba Tek Singh**			**Sargodha**			**Multan**			**Jhang**		
	Total/ Positive (positive %)	**Mean µg/kg**	**Range µg/kg**	**Total/ Positive (positive %)**	**Mean µg/kg**	**Range µg/kg**	**Total/ Positive (positive %)**	Mean µg/kg	Range µg/kg	Total/ Positive (positive %)	Mean µg/kg	Range µg/kg
Kinnow	45/22 (49)	89.9 ±5.36	0.12-130	50/26 (52)	90.9 ± 6.32	0.12-140	40/22 (55)	104.5 ± 4.8	0.12-127	50/30 (60)	91.7 ± 5.6	0.12-115
Orange	35/18 (52)	140.4±6.70	0.12-220	40/19 (48)	150.6 ±7.21	0.12-225	44/25 (54)	142.6 ±4.36	0.12-245	55/32 (58)	150.3 ± 7.3	0.12-221
Grapefruits	55/ 22 (40)	150.9 ± 7.6	0.12-190	45/23 (51)	145.7 ± 8.1	0.12-198	50/26 (52)	125.8 ±7.5	0.12-178	35/10 (29)	115.9 ± 4.3	0.12-216
Bitter orange	40/ 16 (40)	190.6 ± 9.7	0.12-230	35/12 (34)	195.2 ±10.4	0.12-232	30/15 (50)	198.4 ±6.1	0.12-210	25/5 (20)	175.2 ± 4.7	0.12-198
Masambi	45/19 (42)	170.6 ±11.7	0.12-210	40/25 (63)	165.3 ±10.9	0.12-200	50/28 (56)	194.3 ±12.5	0.12-295	52/30 (58)	165.6 ±10.3	0.12-201
Red blood	30/10 (67)	89.9 ± 13.7	0.12-150	50/30 (60)	76.1 ± 12.8	0.12-153	45/22 (49)	75.4 ±10.6	0.12-135	46/25 (54)	77.4 ± 8.4	0.12-111
Pineapple	20/10 (50)	45.7 ± 15.7	0.12-120	30/15 (50)	50.2 ± 15.2	0.12-121	25/9 (36)	54.1 ± 14.3	0.12-110	20/9 (45)	65.7 ± 11.5	0.12-113
Sweet orange	30/17 (57)	55.7 ± 9.8	0.12-140	40/22 (55)	65.4 ± 10.1	0.12-155	45/26 (58)	45.2 ± 7.5	0.12-1150	25/6 (24)	55.2 ± 8.5	0.04-99
Rough lime	48/27 (56)	78.6 ± 10.7	0.12-150	45/27 (60)	65.3 ± 9.3	0.12-168	30/11 (37)	88.3 ± 9.6	0.12-146	30/12 (40)	97.4 ± 11.3	0.12-123
Sweet lime	50/28 (56)	99.7 ± 15.8	0.12-205	55/33 (60)	104.6 ±14.9	0.12-210	30/8 (27)	103.1 ±12.5	0.12-225	22/5 (23)	122.6 ± 8.9	0.12-197
Kagzi lime	43/22 (51)	110.6 ± 11.7	0.12-220	45/32 (71)	122.5 ±10.4	0.12-234	55/30 (55)	125.4 ±15.3	0.12-215	53/35 (66)	130.1 ±14.3	0.12-231
Lemon	40/26 (65)	85.5 ± 16.7	0.12-130	50/30 (60)	92.3 ± 15.9	0.12-145	54/36 (67)	106.7 ±15.3	0.12-155	50/32 (64)	125.5 ± 9.6	0.12-147
Total	**481/247 (51.4)**		**0.12-230**	**525/294 (56)**		**0.12-234**	**498/329 (66)**		**0.12-1150**	**463/231 (50)**		**0.12-231**
Total	**1967/1101 (56.0)**											

(positive %) = (positive samples percentage).

Table 3. The incidence levels of patulin in selected citrus fruits samples from Northern areas of Pakistan.

Citrus fruits type	**Mirpur**			**Peshawar**			**Swat**		
	Total/ Positive (positive %)	**Mean µg/kg**	**Range µg/kg**	**Total/ Positive (positive %)**	**Mean µg/kg**	Range µg/kg	Total/ Positive (positive %)	Mean µg/kg	Range µg/kg
Kinnow	45 / 24 (53.3)	75.3 ± 4.1	0.12- 178	52/31 (59.7)	80.2 ± 9.6	0.12- 167	25/10 (40)	80.5 ± 7.3	0.12- 320
Orange	40 / 16 (40)	99.4 ± 8.5	0.12-156	44/ 22 (50)	144.2 ± 9.2	0.12-167	25/ 5 (20)	163.3 ± 10.1	0.12-204
Grapefruits	20 / 6 (30)	76.2 ± 11.4	0.12-187	25/ 3 (12)	88.4 ± 6.3	0.12-168	10/ 1 (10)	109.6 ± 5.8	0.12-121
Bitter orange	10 / 1 (10)	102.8 ± 9.4	0.12-199	16/ 1 (6.2)	133.4 ± 10.5	0.12-186	05/ 1 (20)	156.3 ± 6.3	0.12-209
Masambi	33/ 8 (24.2)	111.4 ± 11.7	0.12-150	35/ 7 (20)	125.8 ±15.2	0.12-221	40/23 (57.5)	145.2 ± 12.1	0.12-178
Red blood	30/ 6 (20)	72.1 ± 9.4	0.12-187	40/ 18 (45)	67.3 ± 3.8	0.12-196	20/ 6 (30)	56.7 ± 5.2	0.12-120
Pineapple	15/ 3 (20)	45.2 ± 11.8	0.12-165	15/ 2 (13.3)	36.4 ± 8.2	0.12-178	20/ 5 (25)	53.1 ± 13.7	0.12-101
Sweet orange	15/2 (13.3)	56.3± 8.8	0.12-133	14/ 1 (7.1)	65.2± 6.2	0.12-130	20/ 4 (20)	67.4± 12.4	0.12-102
Rough lime	30/ 10 (3.3)	88.9 ± 13.6	0.12-156	25/ 5 (20)	95.3 ± 12.7	0.12-168	35/13 (37.1)	86.9 ± 4.5	0.12-132
Sweet lime	20/ 4 (20)	56.8 ± 10.7	0.12-203	22/ 8 (36.3)	44.1 ± 5.3	0.12-199	27/ 10 (37)	35.8 ± 4.2	0.12-210
Kagzi lime	45/ 25 (55.5)	109.6 ± 8.7	0.12-225	40/ 21 (52.5)	125.4 ± 13.9	0.12-208	40/ 20 (50)	156.3 ± 11.4	0.12-234
Lemon	40/ 21 (52.5)	114.5 ± 15.8	0.12-221	33/ 14 (42.4)	125.2 ± 7.9	0.12-199	32/ 15 (47)	111.9 ± 13.4	0.12-178
Total	**343/ 126 (36.7)**		**0.12-225**	**361/ 133 (36.8)**		**0.12-221**	**299/113 (37.8)**		**0.12-320**
Total					**1003/372 (37.1)**				

(positive %) = (positive samples percentage).

The finding of current research on PAT levels is much higher than the previous study [27]. They documented that 74 out of 141 samples of orange fruits, juices, pulp, and orange jams were found contaminated with PAT and the elevated average amount was 8.7 ± 19.9 µg/kg in orange fruits. Only one sample was found to be contaminated with PAT with levels higher than EU limits. High levels of PAT in selected citrus fruits are observed because in Pakistan, Good Agriculture Practices are not followed, and during the growth of fruits, fungicides are not applied. During the ripening stage, wound on the skin of fruits would provide preharvest contamination of fruits, and in rotten or decayed areas, the levels of 113342 µg/kg were observed in apples [28]. In our previous study, Iqbal et al. [22] have observed 136 out of 237 (57.4%) samples of juices, smoothies, and fruits were observed contaminated with levels ranged up to 1100 µg/kg. The elevated mean level of 921.1 ± 22.4 µg/kg was found in red globe grapes, and 33.8% of samples were found to have levels higher than the EU recommended limit. Funes & Resnik [29] have studied that 21.6% of samples of solid, semisolid apple and pear products were found positive with PAT with average levels ranged from 17-221 µg/kg (mean levels 61.7 µg/kg). The elevated amount of PAT was observed in apple puree 123 µg/kg, and almost 50% of samples were observed to be positive with PAT.

Cho et al. [30], from South Korea, have analyzed 72 samples of fruits, including three apples, two orange, and four grapes samples, and reported the highest mean level of 30.9 µg/L in orange juice samples. Spadaro et al. [31] from Italy have shown that 47 out of 135 samples of different juices were contaminated with PAT with a mean level of 6.42 ± 4.48 µg/L, with the highest level of 55.4 µg/L. In another study from Greece, Moukas et al. [32] had reported the levels of PAT in orange juice samples ranging from 3.1-10.8 µg/kg.

Zouaoui et al. [33] from Tunisia have observed 214 samples (including concentrated juice, apple juice, pear juice, mixed juice, compote samples, apple jams, and pear jams samples). They documented PAT levels in 50% of analyzed samples with concentrations ranging from 2 to 889 µg/L and an average amount of 89 µg/L. However, 22% of samples have a PAT concentration that exceeded the limit proposed by the EU. Murillo-Arbizu et al. [34] from the Spanish market have found that 66% of samples of apple juice were observed affected from PAT (LOD 0.7 µg/L) with a mean level of 19.4 µg/L and levels were ranging from 0.7- 118.7 µg/L. They kept that 11% of the samples have PAT levels observed above the permissible limits of EU regulation. Similarly, very high levels of patulin are stated by Saxena et al. [35] from India, presenting branded juices including concentrate of apple, orange, guava, grape juice, etc. ranging 21–1839 µg/L, with an average level of 330 ± 141 µg/L. The maximum temperature of Northern cities of Pakistan remained 25 °C during summer and winter it drops below 0 °C, however, in central cities of Punjab, the weather during summers remains from 35 to 45 °C. During the winter season, it fluctuates from 7 to 15 °C. The climatic conditions would cause a high incidence of PAT in citrus fruits from the central cities of Punjab, Pakistan.

3.3. Daily intake assessment of patulin

The dietary intake of PAT from selected citrus fruits from central cities of Punjab, Pakistan, is presented in Table 5. The daily intake levels of PAT were ranged from 0.10-1.11 µg/ kg bw/day. PAT's highest daily intake was 1.11 µg/ kg bw/day in masambi fruits samples from Multan city. The levels of dietary intake of PAT from selected citrus fruits from Northern cities of Pakistan is presented in Table 6. The daily intake levels of PAT were ranged from 0.13-1.93 µg/kg bw/day. The elevated dietary intake level was 1.93 µg/kg bw/day in sweet orange from Sawt city. Rahimi & Jeiran [36] from Iran have analyzed different and estimated PAT's daily intake by fruit juice as 16.4, 45.9, and 74.6 ng/kg bw/day for Iranian adults, children, and babies, respectively, much lower compared the findings of current research. Saxena et al. [35], from India, have conducted a study to analyze apple juice's dietary intake among different consumer age groups and found the dietary intake ranging from 0.11-0.24 μg/kg bw/day, comparable to our research. In our previous study [22], low levels of daily intake of PAT were observed in fruits, juices, and smoothies 0.0049, and it was at least 0.0016, and at least 0.0014 μg/kg bw day, respectively. In China, Guo et al. [37] have estimated dietary intakes among adults, children, and babies of apple juice and found the levels of 28.1, 67.5, and 110 ng/kg bw/day, respectively. Piemontese et al. [38] from Italy have reported PAT's daily intake in different age groups and documented the levels ranging from 0.22 to3.41 ng/kg bw/day with a mean consumption of 21 g per day.

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Table 4. The percentage of sample of Patulin exceeds the recommended limits of European Union.

Ccitrus fruits type	**Toba Tek Singh**		**Sargodha**		**Multan**		**Jhang**		**Mirpur**		**Peshawar**		**Swat**	
	n ≥ 50 µg/kg	**N (%) ≥ 50 µg/kg**	**n ≥ 50 µg/kg**	**N (%) ≥ 50 µg/kg**	**n ≥ 50 µg/kg**	**N (%) ≥ 50 µg/kg**	n ≥ 50 µg/kg	N (%) ≥ 50 µg/kg	n ≥ 50 µg/kg	N (%) ≥ 50 µg/kg	n ≥ 50 µg/kg	N (%) ≥ 50 µg/kg	n ≥ 50 µg/kg	N (%) ≥ 50 µg/kg
Kinnow	12	26.7	14	28.0	10	25.0	18	36.0	13	28.9	14	26.9	5	20.0
Orange	11	31.4	10	25.0	12	27.3	16	29.1	9	22.5	10	22.7	0	0.0
Grapefruits	10	18.2	12	26.7	20	40.0	5	14.3	1	5.0	0	0.0	0	0.0
Bitter orange	9	22.5	8	22.9	10	33.3	0	0.0	0	0.0	0	0.0	0	0.0
Masambi	12	26.7	15	37.5	16	32.0	14	26.9	2	6.1	0	0.0	8	20.0
Red blood	7	23.3	18	36.0	11	24.4	12	26.1	0	0.0	7	17.5	0	0.0
Pineapple	7	35.0	7	23.3	4	16.0	4	20.0	0	0.0	0	0.0	0	0.0
Sweet orange	11	36.7	10	25.0	6	13.3	2	8.0	0	0.0	0	0.0	0	0.0
Rough lime	13	27.1	13	28.9	5	16.7	4	13.3	2	6.7	0	0.0	7	20.0
Sweet lime	14	28.0	16	29.1	4	13.3	0	0.0	0	0.0	0	0.0	5	18.5
Kagzi lime	12	27.9	18	40.0	13	23.6	14	26.4	6	13.3	10	25.0	13	32.5
Lemon	14	35.0	15	30.0	15	27.8	14	28.0	9	22.5	8	24.2	11	34.4
Total	132	27.4	156	29.7	126	25.3	103	22.2	42	12.2	49	13.6	49	16.4
Total	**657 (22.1)** [a]													

n= number of samples; N (percentage of individual samples to total number of samples)

a = total samples of all regions higher than EU regulations (percentage of total samples of all regions, higher than EU regulations)

Table 5. The dietary intake of patulin in citrus fruits samples from central cities of Punjab, Pakistan.

Citrus fruits type		**Toba Tek Singh**		**Sargodha**		**Multan**		**Jhang**	
	Consumption	**Mean**	**Patulin intake**	Mean	Patulin intake	Mean	Patulin intake	Mean	Patulin intake
	g/day	(µg/g)	µg/ kg bw/day	(µg/g)	µg/ kg bw/day	(µg/g)	µg/ kg bw/day	(µg/g)	µg/ kg bw/day
Kinnow	200	79.9	0.23	80.9	0.23	84.5	0.24	81.7	0.23
Orange	250	140.4	0.51	150.6	0.54	142.6	0.51	150.3	0.54
Grapefruits	100	150.9	0.22	145.7	0.21	125.8	0.18	115.9	0.17

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Bitter orange	300	190.6	0.82	195.2	0.84	198.4	0.85	175.2	0.75
Masambi	400	170.6	0.97	165.3	0.94	194.3	1.11	165.6	0.95
Red blood	250	89.9	0.32	76.1	0.27	75.4	0.27	77.4	0.28
Pineapple	150	45.7	0.10	50.2	0.11	54.1	0.12	65.7	0.14
Sweet orange	1000	55.7	0.80	65.4	0.93	45.2	0.65	55.2	0.79
Rough lime	400	78.6	0.45	65.3	0.37	88.3	0.50	97.4	0.56
Sweet lime	200	99.7	0.28	104.6	0.30	103.1	0.29	122.6	0.35
Kagzi lime	100	110.6	0.16	122.5	0.18	125.4	0.18	130.1	0.19
Lemon	100	85.5	0.12	92.3	0.13	106.7	0.15	125.5	0.18

The body average weight = 70 ± 2.

Table 6. The dietary intake of patulin in citrus fruits samples from Northern areas of Pakistan.

Citrus fruits type		**Mirpur**		**Peshawar**		**Swat**	
	Consumption	**Mean**	Patulin intake	Mean	Patulin intake	Mean	Patulin intake
	g/day	(µg/g)	µg/ kg bw/day	(µg/g)	µg/ kg bw/day	(µg/g)	µg/ kg bw/day
Kinnow	240	95.3	0.33	101.2	0.35	90.5	0.31
Orange	270	99.4	0.38	144.2	0.56	163.3	0.63
Grapefruits	200	76.2	0.22	88.4	0.25	109.6	0.31
Bitter orange	350	102.8	0.51	133.4	0.67	156.3	0.78
Masambi	300	111.4	0.48	125.8	0.54	145.2	0.62
Red blood	350	72.1	0.36	67.3	0.34	56.7	0.28
Pineapple	250	45.2	0.16	36.4	0.13	53.1	0.19
Sweet orange	2000	56.3	1.61	65.2	1.86	67.4	1.93
Rough lime	300	88.9	0.38	95.3	0.41	86.9	0.37
Sweet lime	400	56.8	0.32	44.1	0.25	35.8	0.20
Kagzi lime	200	109.6	0.31	125.4	0.36	156.3	0.45
Lemon	300	134.5	0.58	145.2	0.62	131.9	0.57

The body average weight = 70 ± 2.

4. Conclusions

In the current study, a considerable amount of PAT was observed in citrus fruit samples from central cities of Punjab and Pakistan's Northern cities. The results showed a significant difference in PAT levels from central cities of Punjab compared to Northern Cities of Pakistan. About 22.1% of citrus fruits from both locations have concentrations of PAT greater than the proposed limits established by the EU. The dietary intake of 1.11 and 1.93 µg/kg bw/day from masambi (Multan) and sweet orange (Swat) was determined. The high levels of PAT in citrus fruits could pose a significant health hazard for local consumers. Therefore, regular monitoring of fruits and their products would be desirable every sixth month, and recommendations should be shared with farmers, traders, exporters, and consumers.

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