Risk assessment of aflatoxin in red peppers from selected districts of Amhara region, Ethiopia

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Abstract: Red pepper is most widely consumed food in the world and easily damaged by mycotoxic fungi during growing, harvesting, transporting, storage and processing stages. Red pepper is susceptible to degradation by aflatoxins-producing fungi which have serious health effects on humans and livestock. Therefore, in this study, the levels of aflatoxin (B1, B2, G1 and G2) were determined and the potential health risk in red pepper samples collected from selected districts of Amhara Region, Ethiopia, were evaluated. The levels of AFB1, AFG1, AFB2 and AFG2 were ranged from 2.51–63.20, 0.96–5.29, 1.71–32.79 and 0.53–2.04 µg/kg, respectively. Among 18 investigated red pepper samples, 44.5% (8 samples) contaminated with aflatoxins. All the contaminated red peppers contained AFB1 and AFG1. Three-fourth (75%) of the total aflatoxins detected in red pepper were greater than maximum permissible levels set by European Union (10 µg/kg). Estimated Daily Intake (EDI) ranged from 0.00013 to 0.015800 µg/kg b.w/day. The Margins of Exposure (MOE) values for all aflatoxins were far from the safe margin (< 10,000), indicating potential health risk due to red pepper consumption. Therefore, public institutions, non-governmental and private organizations should give attention in order to raise awareness of aflatoxins’ effects on human health.

Subjects: Environment & Health; Food Additives & Ingredients; Food Chemistry

Keywords: Aflatoxins; estimated daily intake; margin of exposure; red pepper

1. Introduction

Red pepper (Capsicum annuum L.) is the second widely cultivated in the world next to tomatoes (Qiu et al., 2019). It is a main source of essential vitamins, minerals, carotenoids, flavonoids, capsaicinoids, ascorbic acid and tocopherols, which are known to prevent inflammatory diseases, diabetes, back pain and acute tonsillitis (Kim et al., 2019). In addition, it is used as seasoning and flavoring ingredients in cheese (Schick et al., 2010).

Red pepper is widely grown in various regions of Ethiopia under various environmental and climatic conditions, and it is the main source of income for smallholder farmers (Gobie., 2019). It can also be available on daily dish of every Ethiopian home in the form of paste or sauce to modify the flavor, color and aroma of almost every cuisine (Hunduma et al., 2020). Since larger quantity of red pepper is produced, it accounts to a substantial share of Ethiopian income from the international markets (Getahun & Habetie, 2017). For instance, around 68% of production in Amhara region are for commercial purpose (Aiko & Mehta, 2015).

The major challenges of red pepper production are lack of improved varieties, pure seed supply and susceptibility of local varieties to diseases. Numerous diseases exist at each growth stage of
pepper by bacteria, fungi, insects and pests, which emerged as serious threat of this crop in the major producing areas (Alioto et al., 2020; Tesfaw et al., 2013).

Aflatoxins are toxic secondary metabolites mainly produced by Aspergillus family fungi, the common types of aflatoxins are aflatoxin B1 (AFB1), aflatoxin G1 (AFG1), aflatoxin B2 (AFB2) and aflatoxin G2 (AFG2). AFB1 is mostly available in food and it is considered as the most toxic to human and animals (Al-Ghouti et al., 2020; Marijani et al., 2019). Contamination of foods by aflatoxins can cause significant potential health risk like cancer in humans and livestock, as well as reducing individuals' immune resistance, causing outbreaks of type B viral hepatitis (Alamu et al., 2020). Owing to this, International Agency for Research group on Cancer classified aflatoxins as group 1 carcinogens even at very low levels (International Agency for Research on Cancer (IARC), 2012). The productions of aflatoxin by moulds are strongly enhanced in various growing conditions such as temperature, humid, water activity, light intensity and pH (Kumar et al., 2021). Additional factors are growing, harvesting, transporting, processing stages and storage condition and time influence of fungal growth and aflatoxin production (Zhao et al., 2013).

Continuous exposure to aflatoxins even at low doses over a lifetime can lead to chronic diseases, with the most frequent and severe of which is cancer (Benkerroum, 2020). East African Community (EAC) countries have recognized aflatoxin regulatory standard values of 10 μg/kg as maximum tolerable limit (MTL) for total aflatoxins and 5 μg/kg MTL for AFB1 set by European Union (Commission Regulation, 2010).

In 2017, red pepper that costs 10 million USD returned back to Ethiopia from European countries once it had been contaminated with aflatoxins and ochratoxins (Hunduma et al., 2020). Similarly, Germany blocked a large amount of pepper from entering its markets (Mamo et al., 2020). Although there are high consumption and demand of red pepper in Ethiopia, only very limited studies are found in the literature about the occurrence of aflatoxins. In one of the few such studies, Fuffaand Urga (2001) reported that the Ethiopian red pepper contained AFB1 from 26 to 75 μg/kg and AFG1 from 32 to 120 μg/kg, at levels exceeding the maximum permitted levels (MPL) set by the European Union. Similarly, a recent report by Hunduma et al. (2020) showed that only AFB1 and AFG1 were found in red pepper samples with level of 1.90 and 1.80 μg/kg, respectively. In another study, the total aflatoxin level in red pepper collected from market was found to be 11.7 μg/kg, which is a little bit higher than the MPL value set by European Union (Tsehaynesh et al., 2021). Therefore, this study aimed to determine the levels of aflatoxin and assess Margin of Exposure as a consequence of consuming red pepper collected from local markets of Central Gondar, South Gondar and West Gojjam Administrative zones of Amhara Regional States.

2. Materials and methods

2.1. Sample collection
A total of 18 red pepper samples were collected randomly during October to December 2020, from markets in six districts, namely, Ayikkel, West Dembia, East Dembia, Fogera, Dera, Bure and Jabi Tehnan (Figure 1). Form each sampling sites, 1.5 kg dried red pepper samples of various sized pods were collected directly from markets in each district. The collected pepper samples were placed in clean polyethylene bag and kept at room temperature in an open air to control moisture content in the laboratory until they were analyzed.

2.2. Chemicals and reagents
Analytical grades of aflatoxin standards (B1, G1, B2 and G2) used in the experiments were purchased from Sigma-Aldrich (German). High-performance liquid chromatography grade acetonitrile (Fisher Scientific), n-Hexane, methanol (>99.0%, Sigma Aldrich), sodium chloride (37%, Fisher Scientific), sodium phosphate dibasic and sodium dihydrogen phosphate (≥98%, Blulux laboratories) were also used in this study.
2.3. Preparation and extraction of sample

Sample preparation procedures and extraction were carried out according to AOAC (2005). To make homogeneity, samples were mixed and then finely ground. After and before grinding, the grinder was cleaned to prevent cross-contamination. A 50 g samples were taken on each sampling sites throughout the comminuted laboratory sample.

Aflatoxin extraction was carried out based on AOAC (2005). Briefly, 20 g of the grounded sample added to 2 g NaCl and extracted in 100 mL methanol/water (4:1, v/v) and blended with 50 mL n-Hexane for 5 min at high speed in a blender jar.

The mixture filtered with 42 mm Whatman filter paper and the filtrate was collected. A 7 mL of the filtrate diluted with 50 mL PBS solution (pH 7.2). A 10-mL filtrate was passed through column at a speed of 2 mL/min. After that, it washed with 10 mL of distilled water and air was drawn through the column until dry. An injection volume of 50 µL of the diluted extract passed through the HPLC equipped with a C18 column (5 µm, 250 mm x 4.6 mm, Reversed Phase). A mixture of acetonitrile: methanol: water (3: 5: 12, v/v/v) was utilized as mobile phase, and the flow rate was set at 1.2 mL/min with an oven temperature of 39°C. The calibration curve for AFB1 and AFG1 was constructed from 0.4, 2, 4, 8, 16 and 20 ppb, while for AFB2 and AFG2 0.2, 0.5, 1.0, 2.0, 4.0 and 5.0 ppb were considered. The level of each aflatoxins was calculated as the mean of triplicate analyses.

2.4. Method validation

Linearity, recovery, limit of detection (LOD), limit of quantification (LOQ) and precision were carried out for aflatoxin analysis (Kilic, M. & Kilic, S., 2019). A recovery percentage determines the efficiency of the method for detecting all of the studied analyte in a sample. A recovery experiment was performed by spiking red pepper with aflatoxins standards and the same analytical procedure that was applied to the unspiked red pepper also applied to the spiked red pepper. Recovery was calculated using the following equation
Recovery (%) = [(A-B)/C] x 100 (1)

where A is the concentration of spiked sample, B is the concentration of non-spiked sample and C is the concentration standard added.

The LOD was calculated as three times the standard deviation of blank solution signal (3S/N). The LOQ was calculated as ten times the standard deviation of blank solution signal (Adefa & Tefera, 2020). In this study, the precision was evaluated by the standard deviation of triplicate readings. All analytical measurements were made through triplicate measurements.

2.5. Exposure assessment
Estimated daily intake (EDI) is the ratio of the product of aflatoxin level (µg/kg) by daily ingested red pepper (g/person/d) in Ethiopia, 15 g (Gobie, 2019) to the average body weight (60 kg; Adefa & Tefera, 2020; Hathout et al., 2020).

2.6. Carcinogenic risk assessment
Margin of exposure (MOE) is used to estimate the carcinogenic risk, which was computed by dividing Benchmark dose lower limit (BMDL10) of aflatoxins with EDI. The BMDL10 values for AFB1 (0.170 µg kg⁻¹bw d⁻¹), AFB2 = AFG1 = AFG2 = 0.250 µg kg⁻¹bw d⁻¹ (Yu-jiao et al., 2018). It is reported that when MOE value is less than 10,000, aflatoxins cause a potential risk to public health (Sandova et al., 2019; Zhang et al., 2020).

3. Results and discussion

3.1. Analytical validation
After the calibration curve constructed, aflatoxins determination was validated by evaluating its linearity, regression coefficient ($R^2$), LOD, LOQ and recovery (Table 1). The values of regression coefficient ($R^2$) were > 0.970 for all analytes, indicating that the data were fitted to the regression line. In this study, the LOD for AFB1, AFG1, AFB2 and AFG2 were 0.00713, 0.01947, 0.00081 and 0.00071, respectively. The recoveries of AFB1, AFG1, AFB2, and AFG2 were found to be 99.0 ± 2.50%, 88.30 ± 1.72%, 96.40 ± 5.31%, and 95.70 ± 3.56%, respectively.

3.2. Levels of aflatoxin in red pepper
As can be seen in Table 2, it was observed that 10 red peppers out of 18 contained aflatoxins (55.5%), while the remaining 8 were below LOD (44.5%). All the contaminated samples contained AFB1 and AFG1, while AFB2 and AFG2 were found only in three samples. Total aflatoxin in red pepper samples varied from 4.74 to 98.99 µg/kg.

Red peppers collected from An3, Mnk2 and Wor1 contained AFB1, AFB2, AFG1 and AFG2, while Ay1, Ay2, An2, Jig and Wor2 contained only AFB1 and AFG1. This contamination difference may arise due to humidity and temperature differences and poor transportation and storage. However, there were no aflatoxins detected in Kol1, Kol2, Kol3, Ch1, Ch2, Mnk1, An1, Bur1, Bur2 and Gor1. In general, the levels of AFB1 in red pepper were found in the order of: An2 < Ay2 < Jig < Mnk2 < Ay1 < Wor2 < An3 < Wor1, within the range of 2.51–63.20 µg/kg. The AFB2 contaminations were found in the increasing order of: Wor1 < An3 < Mnk2 ranged from 0.96 to 5.29 µg/kg. The levels of AFG1 in red pepper samples (1.71 to 32.79 µg/kg) were found in the order of: Mnk2 < An2 < Ay2 < Ay1 < Jig < Wor2 < An3 < Wor1. The levels of AFG2 were found in the range of 0.53–2.04 µg/kg within the order of: Mnk2 < An3 < Wor1. Among the studied samples, the red pepper collected from Wor1 contained highest amount of AFB1, AFG1 and AFG2; while Mnk2 contained highest amount of AFB2. However, the least amount of AFB1, AFG1, AFB2 and AFG2 were investigated in An1, Mnk2, Wor1 and Mnk2, respectively.

One-way ANOVA test indicates that the level of AFB1 in all samples are significantly different (p < 0.05) except Ay1 with Mnk2 and Jig and vice versa with p-value > 0.05. The mean level of AFG1
Table 1. Method validation for the determination of AFB1, AFG1, AFB2 and AFG2 in red pepper (n = 3)

| Aflatoxin | Concentration range (ppb) | R²   | LOQ  | LOQ  | Recovery (%) |
|-----------|---------------------------|------|------|------|--------------|
| AFB1      | 0.4–20                    | 0.9891 | 0.00713 | 0.02375 | 99.00 ± 2.50  |
| AFG1      | 0.4–20                    | 0.9870 | 0.01947 | 0.0649  | 88.30 ± 1.72  |
| AFB2      | 0.2–5.0                   | 0.9975 | 0.00081 | 0.00269 | 96.40 ± 5.31  |
| AFG2      | 0.2–5.0                   | 0.9700 | 0.00071 | 0.00236 | 95.70 ± 3.56  |
Table 2. The concentration of aflatoxin detected in red pepper samples (mean ± SD)(µg/Kg)*

| Zone            | Districts       | study areas | Aflatoxins | Aflatoxins | Aflatoxins | Aflatoxins | Total  |
|-----------------|-----------------|-------------|------------|------------|------------|------------|--------|
|                 |                 |             | Code       | AFB1       | AFG1       | AFB2       | AFG2   |
| Central Gondar  | Ayikel          | Chilga1     | Ch1        | <LOD       | <LOD       | <LOD       | <LOD   |
|                 |                 | Chilga2     | Ch2        | <LOD       | <LOD       | <LOD       | <LOD   |
|                 | Gorgora         | Gorgora     | Gor        | <LOD       | <LOD       | <LOD       | <LOD   |
| East Dembia     | Koladiba1       | Kol1        | <LOD       | <LOD       | <LOD       | <LOD       | <LOD   |
|                 | Koladiba2       | Kol2        | <LOD       | <LOD       | <LOD       | <LOD       | <LOD   |
|                 | Koladiba3       | Kol3        | <LOD       | <LOD       | <LOD       | <LOD       | <LOD   |
|                 | Ayimba1         | Ay1         | 7.52 ± 0.37<sup>a</sup> | 5.83 ± 0.24<sup>a</sup> | <LOD       | 13.35<sup>a</sup> |
|                 | Ayimba2         | Ay2         | 2.57 ± 0.25<sup>b</sup> | 3.04 ± 0.03<sup>b</sup> | <LOD       | 5.61<sup>b</sup> |
| South Gondar    | Dera            | Anbesame1   | An1        | <LOD       | <LOD       | <LOD       | <LOD   |
|                 |                 | Anbesame2   | An2        | 2.51 ± 0.05<sup>b</sup> | 2.23 ± 0.06<sup>f</sup> | <LOD       | 4.74<sup>f</sup> |
|                 |                 | Anbesame3   | An3        | 19.59 ± 0.06<sup>c</sup> | 17.75 ± 0.31<sup>g</sup> | 1.81 ± 0.01<sup>e</sup> | 1.65 ± 0.13<sup>b</sup> | 40.8<sup>g</sup> |
|                 |                 | Woreta1     | Wor1       | 63.20 ± 2.55<sup>f</sup> | 32.79 ± 1.59<sup>d</sup> | 0.96 ± 0.17<sup>d</sup> | 2.04 ± 0.08<sup>d</sup> | 98.99<sup>d</sup> |
|                 |                 | Woreta2     | Wor2       | 18.83 ± 0.13<sup>d</sup> | 14.82 ± 0.47<sup>d</sup> | <LOD       | <LOD   |
| West Gojjam     | Bure            | Bure1       | Bu1        | <LOD       | <LOD       | <LOD       | <LOD   |
|                 |                 | Bure2       | Bu2        | <LOD       | <LOD       | <LOD       | <LOD   |
|                 |                 | Jiga        | Jig        | 4.88 ± 0.21 | 10.14 ± 0.45 | <LOD       | 15.02<sup>c</sup> |
|                 |                 | Mankusa1    | Mnk1       | <LOD       | <LOD       | <LOD       | <LOD   |
|                 |                 | Mankusa2    | Mnk2       | 5.21 ± 0.19<sup>c</sup> | 1.71 ± 0.16<sup>c</sup> | 5.29 ± 0.16<sup>c</sup> | 0.53 ± 0.014<sup>c</sup> | 12.74<sup>c</sup> |

Values described by different letters in the same column are significantly different at p < 0.05
<LOD = Less than Detection Limit
*Average of triplicate observations
in all detected samples was varied significantly. However, the level of AFG1 in red pepper collected from Jig was varied insignificantly with Ay1 and Mnk2. Similarly, there was an insignificant difference on the mean level of AFG1 between An3 and Wor2. Lastly, the level of AFB2 and AFG2 were varied significantly except An3 vs Mnk2 and An3 vs Wor1, respectively.

The finding of this research is also compared with other studies conducted in different countries. As can be seen in Table 3, the range of AFB1 values reported in literatures (Akhtar et al., 2020; Aydin et al., 2007; Jalili, 2016; Karaaslan & Arslangray, 2015; Ozbey & Kabak, 2020; Rosas-Contreras et al., 2016; Set & Erkm, 2014; Tosun & Arslan, 2013) was found within the range of

| Country  | AFB1       | AFB2       | AFG1       | AFG2       | References                                    |
|----------|------------|------------|------------|------------|----------------------------------------------|
| Mexico   | 0.23–10.62 | 0.05–1.04  | 0.02–9.21  | 0.05–3.35  | Rosas-Contreras et al., 2016.                |
| Turkey   | 0.025–40.9 | NR         | NR         | NR         | Aydin et al., 2007.                         |
| Pakistan | 0.1–93.00  | 0.1–2.5    | NR         | NR         | Paterson, 2007.                             |
| Turkey   | 23.4–46.6  | NR         | NR         | NR         | Tosun & Arslan, 2013.                       |
| Turkey   | 1–33.5     | NR         | NR         | NR         | Set & Erkm, 2014.                           |
| Iran     | 1.73–17.99 | 0.66–4.16  | 1.47–2.45  | ND-0.52    | Jalili, 2016.                               |
| Turkey   | 0.13–11.45 | 0.04–1.28  | LOD-2.05   |            | Ozbey & Kabak, 2020.                        |
| Turkey   | 0.24–165   | 0.15–11.3  | 0.15–3.88  | NR         | Golge et al., 2013.                         |
| Pakistan | 14.84      | NR         | NR         | NR         | Akhtar et al., 2020.                        |
| Turkey   | 6.9–77.13  | 0.505–6.69 | 0.145–4.23 | 0.122–1.542| Karaaslan & Arslangray, 2015                |
| Ethiopia | 2.1–63.2   | 0.96–5.29  | 1.71–32.79 | 0.53–2.04  | present study                               |

NG: not given  
NR: not reported

| EDI | Site  | AFB1 | AFG1 | AFB2 | AFG2 |
|-----|-------|------|------|------|------|
| Ay1 | 0.00188 | 0.00146 | - | - | |
| Ay2 | 0.00064 | 0.00076 | - | - | |
| An2 | 0.00063 | 0.00056 | - | - | |
| An3 | 0.00489 | 0.00444 | 0.00045 | 0.00041 | |
| Wor1 | 0.01580 | 0.00820 | 0.00024 | 0.00051 | |
| Wor2 | 0.00471 | 0.00370 | - | - | |
| Jig | 0.00122 | 0.00253 | - | - | |
| Mnk2 | 0.00130 | 0.00043 | 0.00132 | 0.00013 | |

EDI—Estimated Daily Intake (µg/Kg.bw/day).
TD₅₀ of total Aflatoxin = 1.3 µg/kg (Echodu et al., 2019))
Average body weight of an adult in Ethiopia = 60 kg (Adefa & Tefera)
Table 5. MOE for adults via consumption of red pepper

| Site  | AFB1    | AFG1    | AFB2    | AFG2    |
|-------|---------|---------|---------|---------|
| Ay1   | 212.7660| 171.2329| -       | -       |
| Ay2   | 622.5681| 328.9474| -       | -       |
| An2   | 637.4502| 446.4286| -       | -       |
| An3   | 81.6743 | 56.3063 | 555.5556| 609.7561|
| Wor1  | 25.3165 | 30.4878 | 1041.667| 490.1961|
| Wor2  | 84.9708 | 67.5675 | -       | -       |
| Jig   | 327.8688| 98.8142 | -       | -       |
| Mnk2  | 307.1017| 581.3953| 189.3939| 1923.077|

BDMLIC for AFB1 (0.170 µg kg⁻¹bw d⁻¹) and AFG1 = AFB2 = AFG2 (0.250 µg kg⁻¹bw d⁻¹; Yu-jiao et al., 2018)

this study (2.51–63.20 µg/Kg). However, the upper limit of AFB1 level reported by Golge et al. (2013) and Paterson (2007) was higher than the range of AFB1 reported in this study.

It was found that the level of AFB2 in this study were comparable with that reported from Iran (Jalili, 2016), Turkey (Karaaslan & Arslangray, 2015; Ozbey & Kabak, 2020) and Pakistan (Paterson, 2007). Reported from Turkey by Golge et al. (2013) was slightly higher than the values obtained in this study. However, study from Mexico was slightly lower than the one we obtained in this study (Rosas-Contreras et al., 2016). Except the values of AFG1 in red peppers collected from An3, Wor1, Wor2 and Jig, the AFG1 from other samples are comparable with values reported in literature (Golge et al., 2013; Jalili, 2016; Karaaslan & Arslangray, 2015; Rosas-Contreras et al., 2016).

In this study, the range of the levels of AFB2 in red peppers was comparable with the reported values (Jalili, 2016; Karaaslan & Arslangray, 2015; Ozbey & Kabak, 2020; Paterson, 2007; Rosas-Contreras et al., 2016), but lower than the upper limit reported by Golge et al. (2013). The level of AFG2 was comparable with the values reported from Mexico and Turkey (Karaaslan & Arslangray, 2015; Rosas-Contreras et al., 2016). However, the level of AFG2 was higher than that reported from Iran (Jalili, 2016).

The results of this study were also compared with international standard values. It was found that the levels of AFB1 in five red peppers (Ay1, An3, Mnk2, Wor1 and Wor2) out of seven samples exceeded the maximum legal limit values set by European Union for AFB1 (5 µg/kg) and total aflatoxin (10 µg/kg; Commission Regulation, 2010; Cagindi & Gurhayta, 2016; Massomo, 2020).

3.3. Risk assessment

The EDI values of aflatoxins as a result of red pepper consumption were determined during the experiment. As shown in Table 4, the EDI values for AFB1, AFG1, AFB2 and AFG2 ranged from 0.00064–0.015800, 0.00043–0.00820, 0.00024–0.00132 and 0.00013–0.00051, respectively. The results revealed that the highest values of EDI for AFB1, AFG1 and AFG2 were obtained in red peppers from Wor1, while the lowest EDI values of AFB1and AFG1 were from An2. However, the highest and the lowest EDI values of AFG2 were recorded in Wor1 and An3, respectively.

The MOE values of all aflatoxins were less than 10,000 (Table 5), which indicate that there is adverse health effects as a result of consuming red pepper collected from sampling areas for adult (Kortei et al., 2019).
4. Conclusion
The present study evaluated both non-carcinogenic and carcinogenic health risks of aflatoxins in red peppers collected from selected districts of Amhara Region, Ethiopia. Results in this study showed that most red pepper samples were not contaminated with aflatoxins. However, in the contaminated red pepper samples, the values of five AFB1 and AFG1, and one AFB2 were higher than the level set by European MPL, which poses a serious safety worry to the public as they consumed and marketed at the study areas. The MOE values through intake of red pepper indicated that the consumers are at higher carcinogenic risk of toxicity. These might arise from inadequate care during harvesting, production and storage conditions and relative unfavorable humidity. The findings of this study have presented valuable information for producers, sellers and the concerned organizations in preventing the growth of aflatoxin causing fungi.

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