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Research article

Mycofloral association and co-occurrence of ochratoxin A and citrinin in aromatic herbs and spices from Bihar state (India) detected by ELISA and LC-MS/MS

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Abstract

Mycofloral association and co- occurrence of ochratoxin A (OTA) & citrinin (CTN) contamination in 219 samples of 5 different aromatic herbs and spices (green cardamom, black cardamom, mace, cinnamon and caraway) were studied. *Aspegillus flavus* and *Aspergillus niger* were the most dominant species present in all types of spice samples. Mace has the highest incidence of fungal contamination followed by black cardamom, green cardamom and cinnamon. 33.3 % of *A. niger* was toxigenic and produce OTA whereas 51.4 % of *Penicillium citrinum* were produced CTN. Qualitative and quantitative detection of mycotoxins in spices were analyzed by Enzyme linked immunosorbent assay (ELISA) and further confirmed by LC-MS/MS. 66.6% of mace and 56.3% of black cardamom samples were contaminated with only OTA whereas co-occurrence of OTA and CTN was 20.0% and 10.9% respectively. CTN was not detected from cinnamon samples and very few samples of spices were CTN contaminated alone, they are generally detected along with OTA. The maximum amount of OTA was detected in mace (117 ppb), CTN in black cardamom (59 ppb). The results of this study suggest that citrinin producing fungi are generally produced with ochratoxin producing fungi and the detectable amount of mycotoxins were sufficiently high to induce carcinogenesis. This is the first report of natural occurrence of citrinin in black cardamom from India.

Keywords: Spices, Ochratoxin A, Citrinin, Toxigenic fungi, ELISA, LC-MS/MS.

1. Introduction

Spices are herbs and obtained from certain plant parts such as stem, root, bark, rhizomes, leaves, flowers, fruits, buds and bulbs, used as ingredient in food preparation for distinctive color, flavor and aromas. They are rich in carbohydrates and proteins whereas some other nutrients also present like vitamin A, vitamin K, vitamin C, magnesium, phosphorus and antioxidants compounds.

India is the largest cultivar of spices in the world and more than 78 types of spices are cultivated here in which green cardamom, black cardamom, mace, cinnamon and caraway are highly used as aromatic spices in food preparation and have also the medicinal properties in the ayurveda therapy [1]. Mixtures of these spices are commonly called Garam masala, which has distinct aroma and generally used to enhance the flavor of food.

Indian climate is divided into 6 major climatic subtypes these are alpine, humid subtropical, tropical wet-dry, tropical wet, semi-arid and arid in which the average temperature varies from 20°C to 27.5°C which is the optimum temperature for the growth of fungi and mycotoxin productions. Further handling, transportation, poor processing and storage system enhances contamination and quality deterioration in the spices. Mycofloral and mycotoxin contamination in spices generally occurs when they are not dried properly or stored in humid environment.

Mycotoxins are the secondary metabolites of fungi produced on wide range of foods and feeds. The most common fungal contaminants of spices are belonging to *Aspergillus* and *Penicillium* genera. Some species of these genera have potential to produce different mycotoxins such as OTA and CTN. OTA are generally produced by *Aspergillus ochraceus* and *Penicillium verrucosum* and also by some other species and CTN is produced by *Penicillium citrinum, Aspergillus ochraceus* and *Aspergillus terreus.* OTA is carcinogenic and causes hepatorenal carcinogenesis and CTN is nephrotoxic and co-occurrence of these two mycotoxins causes hepatorenal carcinogenesis [2, 3].

There are many reports available regarding mycofloral and mycotoxins contamination in other spices from different area of the world but these reports are mainly confined with AFT and OTA contamination in spices [4-6]. This is the first report regarding co-occurrence of OTA and CTN contamination in aromatic herbs and spices from India.

The present study was conducted to ascertain the predominant mycoflora associated with aromatic herbs and spices and there mycotoxin producing potentiality. The co-occurrence of ochratoxin A and citrinin was also examined in these spices. This is the first report of citrinin contamination in black cardamom from India.

2. Material and methods

2.1. Sampling

55 black cardamom, 42 green cardamom, 45 mace, 35 cinnamon and 42 caraway, total 219 samples belonging to 5 types of aromatic herbs and spices were collected from different markets of Bihar state, India. Each sample was put into the sterile cellophane bag and then put into the sterile brown envelop and stored at 4°C to arrest any mycotoxin formation before analysis.

2.3. Isolation and Identification of fungi

All the samples had randomly placed on the freshly prepared Potato dextrose agar (PDA), Rose Bengal agar media and on Blotter paper and incubated at 28 ± 2°C for 7 days and examined daily. The counts were recorded after 5 to 7 days. After incubation all plates were examine visually and by binocular stereomicroscope. Fungal colonies of different morphological type were sub-cultured by hyphal tip method culture tube containing PDA media. Identification was carried out by morphological characteristics and followed the taxonomic schemes of Maren [7] for genus *Aspergillus*, Pitt [8] for *Penicillium,* Nelson et. al [9] for *Fusarium* and Funder [10] for other genera.

2.4. Analysis for Potentiality of mycotoxin producing isolated fungi.

Mycotoxin producing potentiality of *A. niger*, *A. ochraceus, A. paraciticus, P. citrinum* and *P. verrucosum* were examined. The suspensions of isolated fungi were prepared by Macfarland in normal saline that each ml of saline contains 10^6 spores. In all cases 50µl of each suspension was inoculated in 25ml of freshly prepared broth media and incubated at 28 ± 2°C for 10 days. When vigorous growth of fungus occurred the medium was filtered with Watman No.1 paper and the cultured filtrate was extracted with 10 ml of chloroform. In case of CTN the culture filtered was acidified with 1N HCL to bring down the pH subsequently then it was extracted with chloroform. The chloroform extract was evaporated to dryness and residue was dissolved in 1 ml of chloroform and qualitative and quantitative estimation of mycotoxins producing potentiality of fungi were done by the method of Schwenk et. al. [11] and Davis et. al. [12] for testing OTA and CTN producing potentiality of *A. niger, A. ochraceus, P. citrinum* and *P. verrucosum*.

2.5. Qualitative and quantitative estimation of mycotoxins by ELISA and confirmed by LC-MS/MS

The Qualitative and quantitative detection for natural occurrence of OTA and CTN in spice samples were analyzed by enzyme linked immunosorbent assay (ELISA) and further confirmed by LC-MS/MS. Samples were analyzed by AgraQuant Ochratoxin (COKAQ2000) for OTA from ROMER LAB (ASTRIA) and RIDASCREEN FAST citrinin Assay (6302) for CTN . For the qualitative and quantitative estimation of OTA and CTN, 20 gm of sample were grinded and added 100 ml of 70% methanol blended for 3 minute. The solutions were filtered and the supernatant was collected. 4ml of extract was transferred through cleanup columns then the presence of OTA and CTN was detected with specific ELISA kits and the optical density was recorded by the ELISA reader (MERK mios mini) using a 450 nm filter with a differential filter of 630 nm. The minimum detectable amount of ELISA kit for OTA was 2ng/g and15 ng/g for CTN. Standard curve was prepared with standard solution provided with ELISA kits. The optical densities of the samples were compared to the optical density of standards and interpretative results were determined using dilution factor.

Positive samples were further confirmed by LC-MS/MS using Aligent poroshell 120 EC C18, 2.1 x 100mm column by the method of Ediage et. al [13].

3. Result and discussion

3.1. Mycofloral association with spices

In our present investigation, verities of mycoflora were associated with spices in which some were toxigenic. Total 219 samples of 5 types of spices were analyzed (green cardamom, black cardamom, mace, cinnamon and caraway) by agar plate method using PDA, Rose Bengal agar media and blotter paper method. The prevalence of mycoflora were observed in spice samples and isolated by hyphal tip method. A total of 5 different fungal genera belong to 16 species were isolated (Table 1). Fungi were identified on the basis of their culture and morphological characteristics, these were identified as *Aspergillus paraciticus, A. oryzae, A. niger, A. flavus, A. ochraceus, A. versicolour, A. sydowi, Penicillium citrinum, P. islandicum, P. verrucosum, P. cyclopium, Fusarium oxysporum, F. moliniforme, Rhizopus nigricans, R. oryzae* and *Mucor hiemalis*. Elshafie et. al. [14] also reported the some of the similar fungi from spices. Mace samples were the most contaminated samples followed by black cardamom and green cardamom. The present study also revealed the wide range of fungal contamination in spices in which *A. flavus* and *A. niger* was the most dominant and present in all 5 types of spice samples, the present finding supports the report of Bokari [15]. Moreover, the result from table 1 also shows that some of the fungi were only confined to specific spices. *A. paraciticus, A. niger, A. flavus, P. citrinum, P. verrucosum* and *F. oxysporum* were isolated from all 5 types of spices whereas A. oryzae was confined to mace and caraway samples, *A. sydowi* and *P. cyclopium* was only isolated from caraway smples. *A. ochraceus* was not present in mace samples. All these results indicate, it may be possible that some of the specific nutrients present in these spices will help in growth and mycotoxin production of some of the specific fungi or that may be that some of the nutrients present in spices will inhibit the growth of specific fungi. In mace samples, 11 species were isolated belong to 5 genera and *A. flavus* was the most dominated species (Fig. 1). In this context, Mandeel [16] also recorded some of the similar fungi from spice samples. Srivastava et. al.[17] recorded that *Aspergillus* followed by *Fusarium* genera were the most dominated mycoflora in spice whereas in our result *Aspergillus* was the most dominant genera followed by *Penicillium*. Our finding says that mace and black cardamom are susceptible substrate for growth of *Aspergillus niger, A. flavus, A. ochraceus, Penicillium citrinum, P. verrucosum, F. moliniforme* and *Rhizopus oryzae*.

3.2. Mycotoxin producing potentiality of isolated fungi

Ochratoxin A and citrinin producing potentiality of *A.niger, A. ochraceus, Penicillium citrinum* and *P. verrucosum* were analyzed (Table 2). 33.3% of *A. niger*, 25.7% of *A. ochraceus* and 36.8% of *P. verrucosum* were found positive and produces OTA with the potential level ranges from 2.8-12.5 μg/l. Our fining supports the report of Essono et al. [18]. *Penicillium citrinum* shows 51.4% of toxicity and produces CTN with the level ranges from 0.8-3.7 μg/l. *Penicillium verrucosum* produces both mycotoxins i.e OTA and CTN. 13.1 % of *P. verrucosum* produces CTN and 36.8% were produces OTA with the potentiality ranges from 1.2-4.8 μg/l and 3.1-12.5 μg/l respectively. Our finding is well agreement with some other researchers [19, 20].

3.3. Co-occurrence of ochratoxin and citrinin in spice samples

All 5 types of spice samples were analyzed and found that same of the samples were only contaminated with OTA or CTN or in some samples co-occurrence of OTA and citrinin were detected. Numbers of positive samples with their mycotoxins is mentioned in Table 3. Out of 45 mace samples 30 samples were contaminated with OTA and only 4 with only CTN but 9 samples were positive to OTA along with CTN. Our result is supports the report of Ozbey et. al [21]. In all spice samples, citrinin was generally detected with OTA and very few samples were positive with only CTN (Fig. 2). The result says that CTN producing fungi are generally grows along with ochratoxin producing fungi and further mycotoxin produced or it may be that OTA can help in the growth of CTN producing fungi or CTN production. It is a matter of research and further research continued.

3.4. Natural occurrence of OTA and CTN in spices

The result of natural occurrence of OTA and CTN in different concentration in 5 types of spices has been shown in Table 3. During the analyses, 66.6 % of mace samples were highly contaminated with OTA followed by black cardamom (56.3%) and cinnamon (34.2%) has the lowest contamination. Highest amount of OTA was recorded in mace samples (117 ppb) (Fig. 3) where as in black cardamom and green cardamom samples, it was 98 ppb and 56 ppb respectively. It may be due to presence of high number OTA producing fungi in these spice samples (Fig. 4). The lowest concentration of OTA was recorded in cinnamon (42ppb). In our study, only 4 types of spices are contaminated with CTN and was maximum in caraway (76 ppb) (Fig. 5) followed by black cardamom (59 ppb). Our finding is well agreement with Jalili et. al [22]. In our result none of the cinnamon samples were positive to CTN. It may be possible that the essential oil of the cinnamon inhibited the growth of CTN producing fungi (*P. citrinum* and *P. verrucosum*) and CTN production. Zaied et al. [23] also report the OTA contamination in caraway, coriander, cumin, black pepper, red pepper and the average contamination level was 244, 206, 209,274 and 203µg/kg in Turkey.

4. Conclusion

On the basis of the present study, it may be concluded that the aromatic spices are rich substrate for fungal growth and OTA and CTN productions. All 5 types of spices were contaminated with OTA and CTN except cinnamon. During the investigation it has been observed that all the CTN contaminated spices was also contaminated with OTA but all the OTA contaminated samples were not contaminated to CTN (Fig. 6). It may be possible that the presence of OTA producing fungi help the CTN producing fungi to grow or in mycotoxin production. The present investigation will also help in the reduction of mycotoxin contamination in spices which will affect the Indian economy because India is the largest producer of the spices. In our result, OTA and citrinin were present in higher concentration and can cause carcinogenesis. This is the first report from India. It is very important to care in processing, handling, transportation and storage system to reduce the production of these hazardous mycotoxins in spices and aromatic herbs.

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Table 1. Percent incidence of fungi content of different spices samples.

Fungal species	Name of the spices				
	Black cardamom	Green cardamom	Mace	Cinnamon	Caraway
Aspergillus parasiticus	2.5	1.8	2.9	1.9	1.4
A. oryzae	-	-	1.6	-	1.7
A. niger	12.5	3.7	20.3	7.5	4.2
A. flavus	10.7	9.5	16.6	7.9	7.3
A. ochraceus	1.1	2.4	-	3.1	3.2
A. versicolour	1.4	-	3.8	3.9	-
A. sydowi	-	-	-	-	4.0
Penicillium citrinum	3.8	3.6	2.3	1.4	2.7
P. islandicum	-	-	-	-	2.3
P. verrucosum	4.4	3.8	2.2	5.0	-
P. cyclopium	-	-	-	-	1.1
Fusarium oxysporum	4.2	1.7	2.7	2.6	1.6
F. moliniforme	2.4	3.2	3.1	4.0	-
Rhizopus nigricans	6.3	-	-	3.2	6.1
R. oryzae	1.8	1.7	2.8	3.1	-
Mucor hiemalis	3.3	3.6	2.8	4.7	-

Table 2. Detection of some isolated ochratoxin and citrinin producing toxigenic fungi and its potentiality.

Fungal Species	No. of isolate analyzed	No. of isolate Positive.	% toxicity	Mycotoxin detected	Potential Range(μg/l)
Aspergillus niger	24	08	33.3	Ochratoxin A	2.8 – 7.5
Aspergillus ochraceus	35	09	25.7	Ochratoxin A	3.2 – 9.8
Penicillium citrinum	35	18	51.4	Citrinin	0.8 – 3.7
	38	05	13.1	Citrinin	1.2 – 4.8
Penicillium verrucosum	38	14	36.8	Ochratoxin A	3.1 – 12.5

Table 3. Spice samples positive to ochratoxin A and citrinin in co-occurrence and invisibly.

Spices	N.S.A[a]	Ochratoxin A		Citrinin		OTA + CTN
		N.P.S[b] (% Con[c])	Mean Amount ± S.E. (ppb)	N.P.S[b] (% Con[c])	Mean Amount ± S.E. (ppb)	N.P.S[b] (% Con[c])
Black cardamom	55	31 (56.3%)	98 ± 7.5	3 (5.4%)	59 ± 9.8	6 (10.9%)
Green cardamom	42	20 (47.6%)	56 ± 12.8	2 (4.7%)	26 ± 5.4	3 (7.1%)
Mace	45	30 (66.6%)	117 ± 14.4	4 (8.8%)	45 ± 14.3	9 (20.0%)
Cinnamon	35	12 (34.2%)	42 ± 8.2	0	-	0
Caraway	42	18 (42.8%)	73 ± 6.3	2 (4.7%)	76 ± 11.2	4 (9.5%)

Figure 1: Mycotoxigenic fungal association in Mace, green cardamom and cinnamon samples.

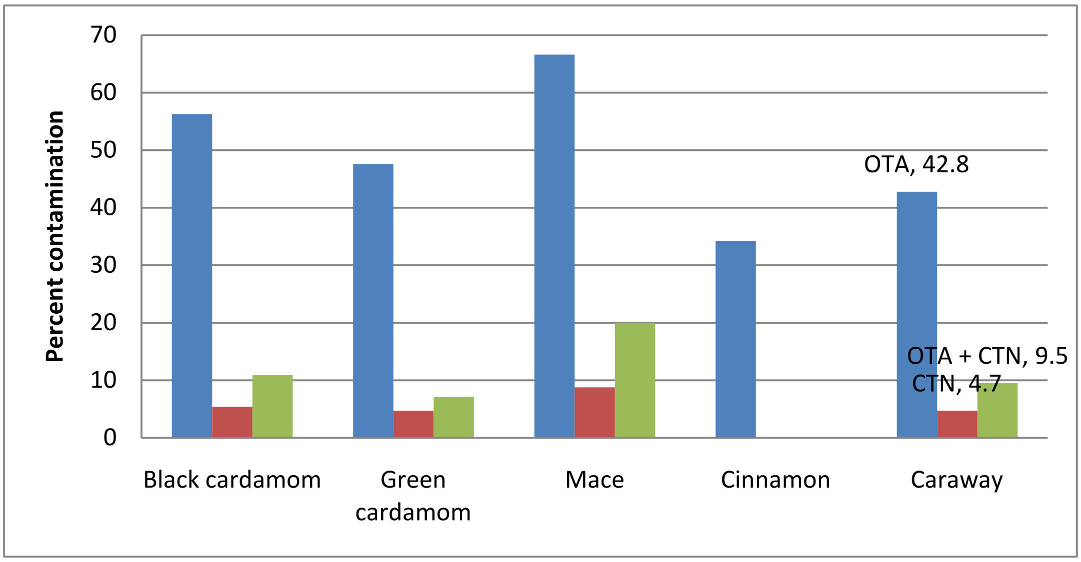

Figure 2: Percent contamination of OTA, CTN and co-occurrence in spice samples.

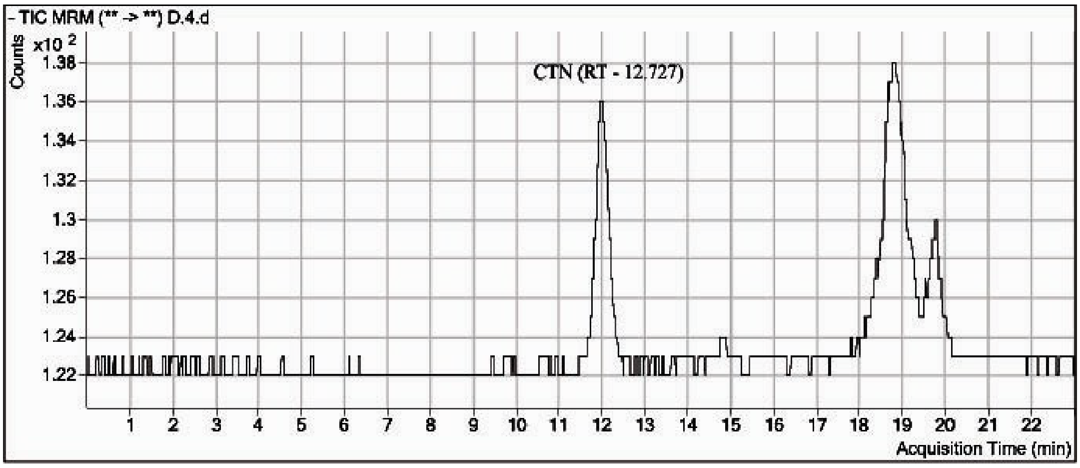

Figure 3: LC-MS/MS chromatogram of CTN for mace sample having maximum contamination.

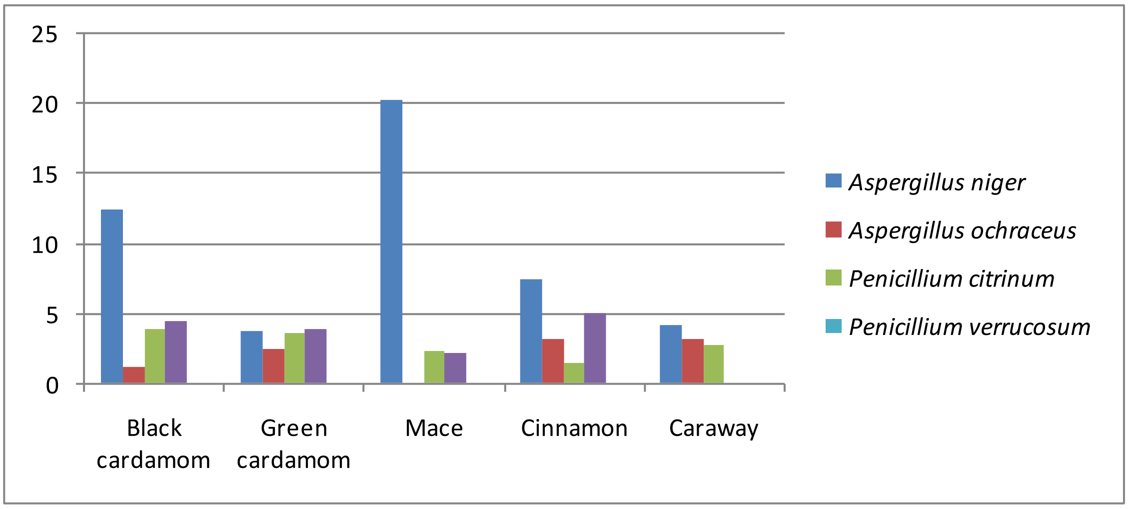

Figure 4: Percent incidence of OTA and CTN producing fungi in spices samples

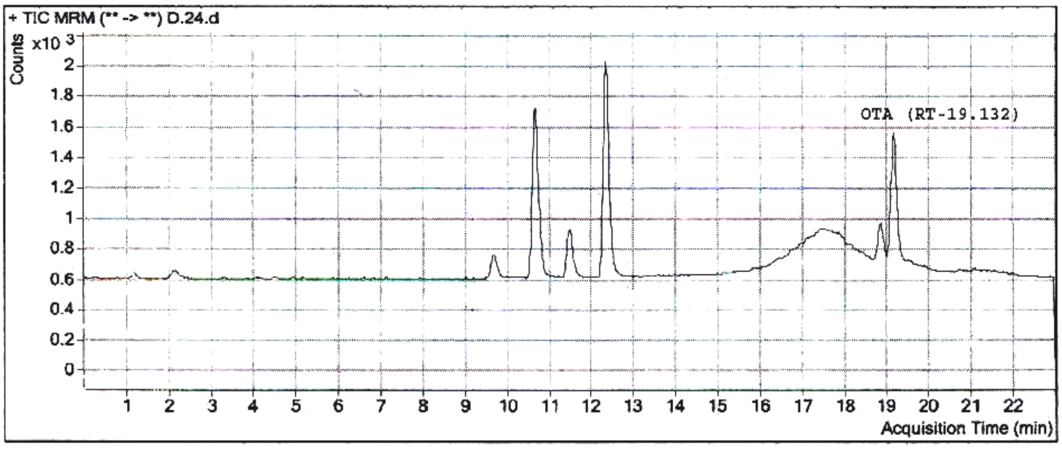

Figure 5: LC MS/MS chromatogram of OTA of mace sample having maximum contamination

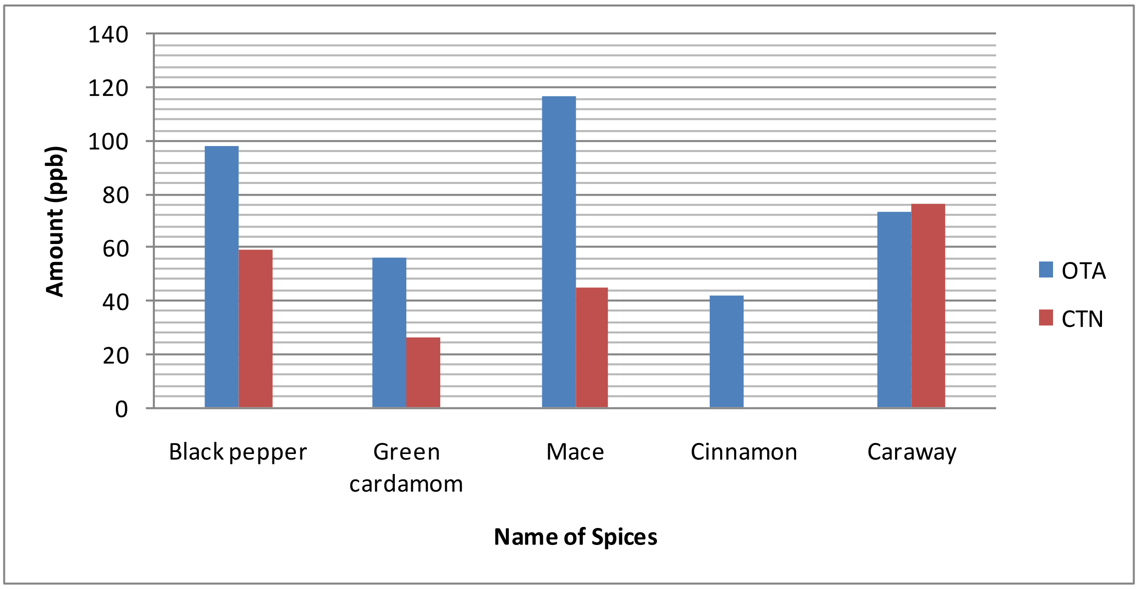

Figure 6: Natural occurrence of OTA and CTN in spice samples