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Investigating the Ochratoxin Production Potential of *Aspergillus spp* in Indian Rice: A Comprehensive Study

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Rice (*Oryza sativa*), world's second most important cereal crop and most commonly consumed grains in the world. Though more than 100,000 species of fungi are known to exist, majority of mycotoxigenic fungi belong to the species of *Aspergillus, Penicillium,* and *Fusarium.* Among *Aspergillus spp*, *Aspergillus niger* and *Aspergillus ochraceus* are known to produce Ochratoxin A (OTA), a notable mycotoxin having adverse effects due to ubiquitous presence, renal toxicity and lengthy persistence. Totally eighty-one samples including unpolished (n = 36) and polished rice samples (n = 45) were collected in various districts of Tamil Nadu to identify ochratoxigenic fungi particularly *Aspergillus spp*. Out of 81 samples, 62 % (50/81) of samples including unpolished (n = 24) and polished rice (n = 26) documented the occurrence of ochratoxigenic fungi. Extraction of

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OTA was carried out by agar plug method and the ability of fungus to produce OTA was quantified by RP HPLC-FLD analysis. Among the fungal genera, *Aspergillus spp* (80 %) recorded the predominant fungus in rice. Molecular confirmation of ochratoxigenic fungi *A. niger* and *A. ochraceus* isolates was performed by 18s rDNA analysis. OTA producing ability by RP HPLC-FLD analysis revealed *A. ochraceus* of section *Circumdati* recorded the highest OTA production than *A. niger* isolates of section *Nigri.* The concentration range of OTA by *A. ochraceus* vary between 12.33 - 196.84 ng/g and 0.18 - 2.82 ng/g respectively in *A. niger* isolates. *Among the A. ochraceus* isolates, potent isolate AO 9 documented the highest OTA production (196.84 ng/g) followed by AO 6 (104.74 ng/g). Similarly, in *A. niger* AN 1 showed highest production of 2.82 ng/g followed by AN 5 isolate (1.25 ng/g) respectively. The objective of the study is to determine and quantify the ochratoxin production potential of *Aspergillus spp* in Indian rice by RP HPLC-FLD analysis. The occurrence of *Aspergillus spp* in rice and the subsequent ochratoxin production raise concern about possible health risk to animal and human environment upon consumption. Hence there is need for improved storage practices and regular monitoring to prevent the prevalence of *Aspergillus* fungus and OTA contamination in rice supply chain.

Keywords: Ochratoxigenic fungi; Aspergillus spp; RP HPLC-FLD; rice.

1. INTRODUCTION

India and China are considered as the main producers of rice worldwide, while India's production was 196.25 million metric tons during 2022. As of 2023/2024, India and Thailand were the main exporters of rice, however India had the highest export volume of 16.5 million metric tons. Among the total global consumption of milled rice in 2023/24, India ranked second with 118 million metric tons of rice consumption. In India, leading rice-producing states are West Bengal, Uttar Pradesh, Andhra Pradesh, Punjab, Tamil Nadu, Bihar, Chhattisgarh, and Odisha. West Bengal with the rice production of 15.75 million tons, stands as the largest rice producing Indian state, followed by Uttar Pradesh, Punjab and Tamil Nadu. The area under cultivation in Tamil Nadu was 2.04 million hectares with production of 7.98 million tons. (Statista, 2024).

Every year, nearly 12 million tonnes of rice are contaminated with mycotoxin, including ochratoxin, which results in a 25% global economic loss of agricultural commodities of which rice is major concern. According to FAO, 15 % of the rice harvest is lost annually, as a result of improper storage conditions that encourage the growth of fungi and mycotoxin contamination [1]. Fungal infection and toxin production have a significant impact on rice's nutritional value and quality, causing discolouration of the grains and viability loss, which completely reduce the rice's market worth [2].

Rice plays a crucial role in the context of nutrition, human health and remains a primary focus of interest among the Indian population as it contributes nearly 70% of daily calories requirement, 56% of protein intake. The proximate composition of brown rice and white rice composed of 77.8 % carbohydrates, 6.7% protein, total dietary fibre (6.7 % in brown rice 2.2 % in white rice) and 3.3 % fat in brown rice [3]. Fungal invasion may occur during plant growth in a contaminated environment (pre-harvest contamination) as well as during transport and storage (post-harvest contamination) [4]. Although rice crop may become infected with ochratoxin producing fungus prior to harvest, OTA problem occurs during storage and do not arise prior to harvest [5]. Rice is typically grown under flood irrigation, which creates high moisture conditions that encourage the growth of fungi. Improper storage condition combined with meteorological events like flooding and intense rain during harvest increase the fungal attack. Sun-drying of rice, commonly practiced by majority of the farmers but this method is insufficient to lower the moisture level, leaving rice more vulnerable to fungal invasion [6].

Van der Merwe et al. [7] first identified ochratoxins (OTs) in *Aspergillus ochraceus* cultures [8]. The ochratoxigenic fungi in rice attributed to the genus *Aspergillus*, particularly section *Circumdati* (*A. ochraceus*), *Aspergillus* section *Nigri*, and a few *Penicillium* species which are responsible for the majority of OTA production [9]. The most critical factors which are essential for the growth of fungus and OTA production are water activity, temperature, moisture content and nutrients. For rice grain or seed stored for two to three weeks, the recommended moisture level is 14 – 18 %; for

eight to twelve months, it is 12 – 13 %; and for more than a year, it is less than 9 %. *Aspergillus* and other storage fungus can often grow at a temperature of 25 – 35 °C and a relative humidity of 70 – 90 % [10]. The optimal temperature required for the *A. ochraceus* growth and OTA production was 25 – 30 °C, and optimum water activity (a_w) for growth was 0.96 - 0.98 a_w and OTA production at 0.98 a_w. For *A. niger*, the optimum temperature and water activity was 35 °C and 0.99 a_w for growth, OTA production respectively [11].

The presence of ochratoxin A in rice poses a risk to public health, necessitating further research and detection techniques in rice. The preferred chromatographic method of ochratoxin A detection involves High Performance Liquid Chromatography with fluorescence detector (HPLC-FLD) because of its sensitivity and accuracy over other methods [12]. The detection of OTA in rice was analysed by ultra-high-performance LC (UHPLC) fluorescence detection (FLD) based method as reported by Troestch et al., [13]. Other methods are high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) as documented by Arce-lopez et al., [14], high performance liquid chromatography-electrospray tandem mass spectrometry (HPLC - ESI - MS/ MS), ultra-high performance liquid chromatography/time-of-flight mass spectrometry (UHPLC/TOFMS) are widely used in detection of ochratoxin A [1]. Dhanshetty and Banerjee, [15] develop an ultra-high-performance LC (UHPLC) fluorescence detection (FLD) based method to detect OTA contamination in rice.

The fungal infection and consequent OTA production reported to have serious ill effects on human beings. Nephrotoxicity, neurotoxicity, immunotoxicity, and cause of the deadly disease known as Balkan Endemic Nephropathy (BEN) are among the toxicological consequences of OTA on humans [16]. The International Agency for Research on Cancer has categorised OTA as a human carcinogen in the class 2B family [17]. Despite the significance of rice as a staple food for Indians and the documented report of ochratoxins, there is little data on toxigenic fungal occurrence in rice and its toxin production ability. Hence present study was carried out to identify the ochratoxigenic fungi particularly *Aspergillus spp* and the possible production potential of OTA by these fungi in Indian rice.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Sterile disposable plates (Himedia, Mumbai) were utilised for culture maintenance and were autoclaved twice at 121 °C for 15 minutes each time before being disposed of. Ochratoxin A standard purchased from Sigma, USA (1 mg/ml) were stored in screw-cap amber vials (Agilent Technologies, USA). Glass wares, such as funnels, beakers, and amber vials, used for ochratoxin A extraction, were washed by soaking in 1% NaOCl for two hours and then soaking in 5% acetone for half an hour.

2.2 Sample Collection

Totally 81 samples including unpolished (n = 36) and polished rice (n = 45) which are intended for human consumption were collected randomly from wholesale, local retail market and petty shops of different districts (Coimbatore, Erode, Salem, Trichy, Thanjavur, Kanyakumari, Namakkal, Madurai, Dindigul, Karur, Nilgiris) of Tamil Nadu, India during 2022 – 2023. These establishments are often patronized by local customers and represent significant source of rice in the community. The purpose of sampling is to isolate and identify the ochratoxigenic fungi (*Aspergillus spp*) and to assess the ochratoxin A (OTA) production potential by *Aspergillus spp* using RP HPLC-FLD analysis. The samples were collected in sterilized polythene bags, labelled and stored at 4 °C.

2.3 Identification of *Aspergillus spp* and Molecular Confirmation

Colonies of *Aspergillus spp* were identified and pure culture was maintained on Czapek Yeast Agar medium (CYA), incubated at 25 °C for 15 days under dark. Based on colony morphology, species level of *Aspergilli* was determined as described by Pitt and Hocking, [18]. The morphologically identified *Aspergillus* isolates were subjected to molecular confirmation by characterizing the ITS (Internal Transcribed Spacer) region.

DNA extraction from mycelial mat of *Aspergillus niger* and *Aspergillus ochraceus* was done based on CTAB method as described by Allen *et al*. [19]. Using phenol-chloroform mixture, DNA was purified and precipitated with ethanol. The amplification of ITS region was performed using

ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′TCCTCCGCTTATTGATATGC-3′) primers. The amplified PCR products were sequenced by using sanger dideoxy sequencing method at Biokart, Bangalore. The identification of isolates was determined by comparing the acquired DNA sequences with the National Centre for Biotechnology and Information's (NCBI) database.

2.4 Quantification of toxigenic potentiality of *Aspergillus niger* and *Aspergillus ochraceus* by HPLC-FLD

2.4.1 Inoculum preparation

Spore suspension of *A. niger* and *A. ochraceus* was prepared from ten days old cultures grown in CYA medium at 25 °C. Spores were collected by addition of 10 ml of sterile distilled water containing 0.05% Tween 80 by rubbing the surface with a glass rod. The concentration of the spore suspension was adjusted to 10^6 conidia/ml using a hemocytometer and the spore suspension was used for further experiment [20].

2.4.2 Extraction of OTA

To determine the OTA producing ability of *Aspergillus niger* and *Aspergillus ochraceus* isolates, agar plug method was adapted as reported by Bragulat et al. [21] and documented by using RP HPLC-FLD analysis. Purified Ochratoxin A standard at concentration of 1 mg/ml was purchased from Sigma, USA used as reference standard. Isolates of *A. ochraceus* and *A. niger* that produce OTA serve as positive controls, whereas isolates that do not produce OTA included as negative controls.10 µl of spore suspension was inoculated centrally onto the plates containing CYA medium and incubated for 15 days under dark at 25 °C. Five agar plugs were removed aseptically from the internal, middle and external areas of the well grown fungal colony along with mycelium using a cork borer (6 mm diameter). For OTA extraction, the agar plugs were transferred to sterile 2 ml eppendorf tube and one ml of HPLC grade methanol was added. The mixture was homogenized by vortexing for one min and followed by 60 mins incubation. The mixture was then centrifuged at 10,000 rpm for 5 mins. The supernatant was separated carefully and filtered using Millex® syringe filter (0.22 µm). The methanol extract was dried under nitrogen gas at 35 °C and re-dissolved in methanol: water (50:50). Fifty microlitre sample was injected into a reverse- phase High-performance liquid Chromatography (RP HPLC) instrument (Agilent 1200 HPLC system, Agilent Technologies, USA).

2.4.3 HPLC condition

Ochratoxin A was determined by reverse- phase HPLC (Agilent 1200 HPLC system, Agilent Technologies, USA) equipped with fluorescence detector and auto sampler. The silica packed C18 column (250mm × 4.6mm, 5µm particle size; Agilent Technologies, USA) used for chromatographic separation was maintained at 40 °C. The mobile phase was prepared with the mixture of acetonitrile, water and acetic acid (51:47:2). The flow rate of mobile phase was maintained at 1 ml/ min. The excitation and emission wavelength of FLD detection were 333 and 460 nm respectively. Fifty microlitre from each sample was used as an injection volume [22].

2.5 Statistical Analysis

All data presented are means of three replicates, and each experiment was repeated thrice. Differences between means were evaluated with analysis of variance using the Tukey means separation test at 5% significance [23]. The statistical analysis was performed using SPSS software version 20.0 for Windows (SPSS Inc.).

3. RESULTS AND DISCUSSION

The main focus of study was to enumerate the ochratoxigenic fungi (*Aspergillus spp*) in rice and its production potential of OTA. In the present findings, the species level of ochratoxigenic *Aspergillus* was identified based on molecular characterization. The molecular confirmation was done by 18S rDNA gene sequence analysis and the acquired sequence were submitted in NCBI database. Accordingly, ten isolates of *Aspergillus niger* and *Aspergillus ochraceus* were identified, subcultured on Czapek Yeast Agar medium (CYA) and incubated at 25 °C for 15 days under dark in order to analyse their OTA production.

The results of obtained accession number for ten isolates of *A. niger* (Table 1) and *A. ochraceus* (Table 2) were elucidated. As per the report of Gil Serna et al., [24] 18srDNA sequence analysis are widely used tool to differentiate the fungal species. The present investigation for the prevalence of ochratoxigenic fungi *A. niger* and *A. ochraceus* in rice samples are in accordance with the report of Pitt and Hocking, [25] who reported the Aspergillus section Nigri and Circumdati being the major OTA producer in cereals such as rice.

Table 1. Molecular confirmation of *Aspergillus niger* isolates

S.No.	Isolate	Organism	Per cent identity	Accession number
1	AN 1	*Aspergillus niger*	99.99	PP972327
2	AN 2	*Aspergillus niger*	98.50	PQ041726
3	AN 3	*Aspergillus niger*	98.50	PQ041957
4	AN 4	*Aspergillus niger*	99.50	PQ041979
5	AN 5	*Aspergillus niger*	99.99	PQ043199
6	AN 6	*Aspergillus niger*	99.50	PQ044824
7	AN 7	*Aspergillus niger*	99.00	PQ044826
8	AN 8	*Aspergillus niger*	99.00	PQ046209
9	AN 9	*Aspergillus niger*	99.00	PQ047131
10	AN 10	*Aspergillus niger*	99.00	PQ047139

Table 2. Molecular confirmation of *Aspergillus ochraceus* isolates

S.No.	Isolate	Organism	Per cent identity	Accession number
1	AO 1	*Aspergillus ochraceus*	99.50	PQ097751
2	AO 2	*Aspergillus ochraceus*	98.50	PQ097749
3	AO 3	*Aspergillus ochraceus*	99.99	PQ097748
4	AO 4	*Aspergillus ochraceus*	98.00	PP973738
5	AO 5	*Aspergillus ochraceus*	99.99	PQ097715
6	AO 6	*Aspergillus ochraceus*	99.99	PQ097712
7	AO 7	*Aspergillus ochraceus*	99.00	PQ097294
8	AO 8	*Aspergillus ochraceus*	99.00	PQ041718
9	AO 9	*Aspergillus ochraceus*	99.99	PQ097668
10	AO 10	*Aspergillus ochraceus*	98.00	PP967837

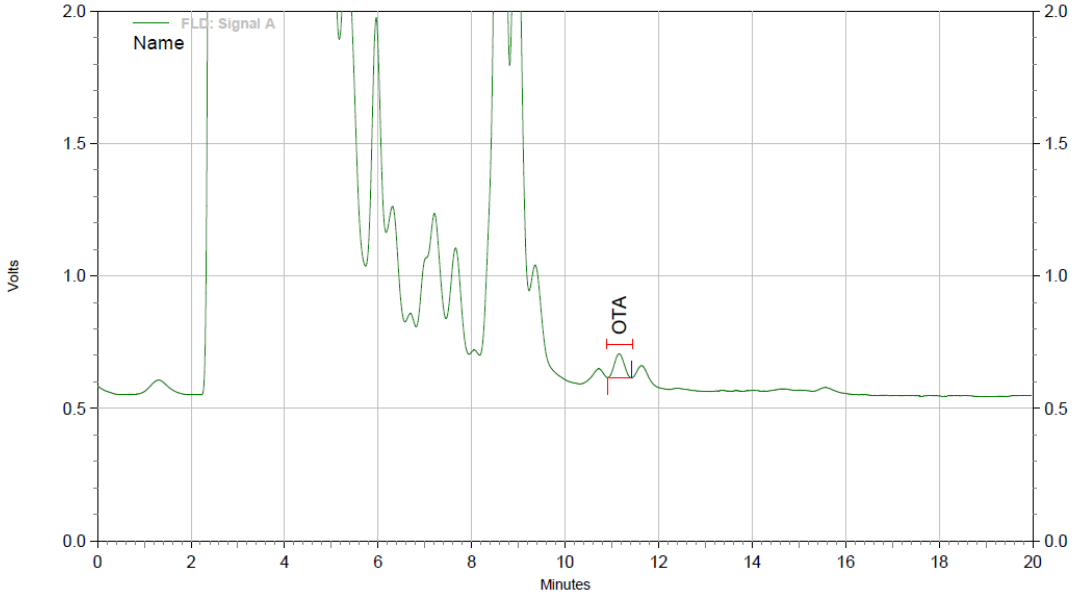

Fig. 1. HPLC chromatogram of OTA production by *A. niger* isolate AN 1

The current findings are in line with previous studies of several authors who reported the occurrence of ochratoxigenic fungi *A. ochraceus* in rice. Accordingly, among 457 strains of *A. ochraceus* isolated from Japanese moldy rice, two strains were recorded as highly ochratoxin A producers [26]. Similarly, Varga et al., [27] documented the presence of *A. ochraceus* in rice and van der Merwe et al.,[7] isolated *A. ochraceus* from African cereals. Frisvad et al., [28] documented *A. ochraceus* as the potent OTA producer in rice. The occurrence of *Aspergillus*

ochraceus and ochratoxin A production in rice was reported by Uchiyama et al. [29]. Rice's high nutritional content and water activity make it a great substrate for *Aspergillus* development. Upon infection, *Aspergillus* release enzymes and metabolites in the host. As a protective mechanism, this sets off the host's defences and the fungi start to produce mycotoxins as signal of defence [30].

The present study shows that the isolates of *A. niger* (AN 1) isolated from rice samples of Salem district showed highest OTA production of 2.82 ng/g and the least OTA production (0.18 ng/g) was recorded by AN 2 isolated from Erode district samples. Among *A. ochraceus* isolates, AO 9 isolated from Coimbatore district samples documented maximum production of 196.84 ng/g and AO 1 from samples of Nilgiris district recorded minimum OTA production (12.33 ng/g). Moreover, the OTA production was significantly varied within the isolates tested in the present investigation ($P< 0.05$). It is observed that *A. ochraceus* isolates were capable of producing higher OTA production potential than *A. niger*, as determined by RP HPLC-FLD analysis.

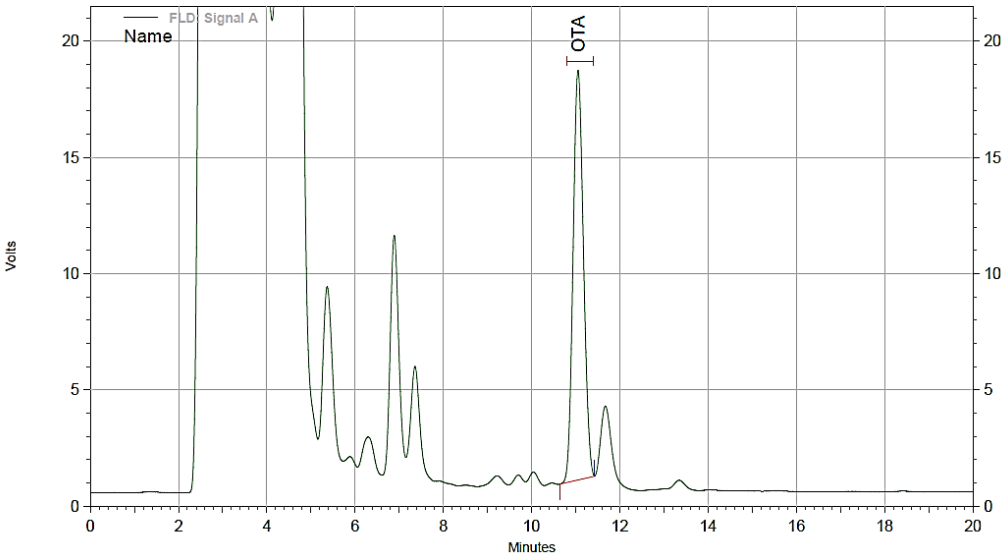

Fig. 2. HPLC chromatogram of OTA production by *A. ochraceus* isolate A0 9

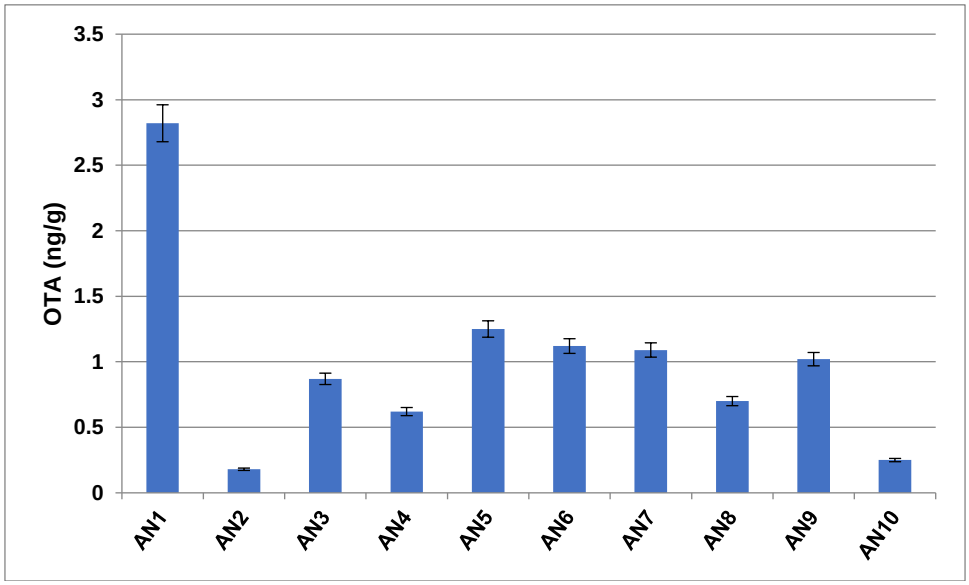

Fig. 3. Quantification of OTA production by *Aspergillus niger* isolates using RP HPLC-FLD analysis. Each value represents mean of three replicates

** OTA- Ochratoxin A RP HPLC-FLD - Reverse-Phase High- Performance Liquid Chromatography with Fluorescence Detector*

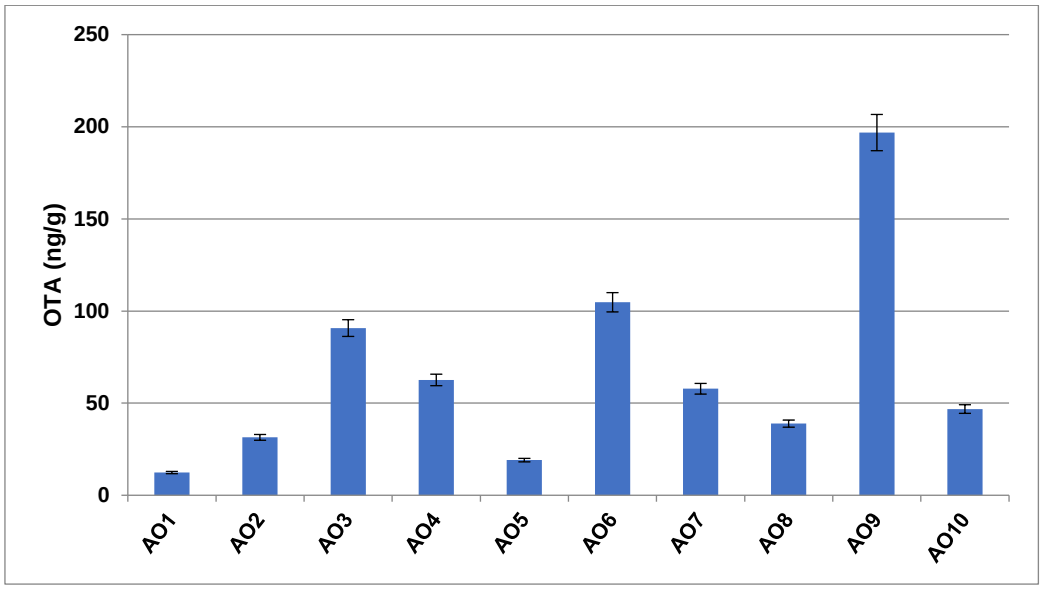

Fig. 4. Quantification of OTA production by *Aspergillus ochraceus* isolates using RP HPLC-FLD analysis. Each value represents mean of three replicates

** OTA- Ochratoxin A RP HPLC-FLD - Reverse-Phase High- Performance Liquid Chromatography with Fluorescence Detector*

The chromatographic profiling of Ochratoxin A (OTA) production by isolates of *A. niger* (Fig. 1) and *A. ochraceus* (Fig. 2) were documented. The concentration of OTA produced by *A. niger* isolates was varied in the range of 0.18 - 2.82 ng/g (Fig. 3), while in *A. ochraceus*, the production lies in the range of 12.33 - 196.84 ng/g (Fig. 4) respectively. *A. ochraceus* isolate AO 9 recorded the highest OTA production of 196.84 ng/g followed by AO 6 isolate (104.74 ng/g). The potent OTA producer in *A. niger* was AN 1 which recorded 2.82 ng/g followed by AN 5 isolate (1.25 ng/g) respectively. Similarly, Bragulat et al., 2001 reported the production of OTA by *A. niger* isolates in CYA media. The current result was in accordance with the findings of Mateo, [31] who documented that *A. ochraceus* is the most potent ochratoxigenic species among *Aspergilli* and the second most OTA producing species belong to the section Nigri which includes *A. niger*.

The prevalence rate of ochratoxin A differs from one part of the world to another due to factors like humidity, temperature, and storage practices [32]. *Aspergillus* grows predominantly in tropical climate, with high temperature, high Relative Humidity and Water activity. Rice in tropical Asia is mostly contaminated with *Aspergillus* fungi such as *A. ochraceus* and OTA contamination is considerably prevalent in rice [1]. As India is bestowed with tropical climate, rice grown in various districts of Tamil Nadu, India is mostly contaminated with *Aspergillus* fungi such as *A. niger* and *A. ochraceus*. *A. ochraceus* has been reported to occur in cereals in tropics and subtropics. The presence or absence of *A. ochraceus* in cereals is probably more related to storage and climatic conditions [9].

The results and findings of several authors were supportive to our current investigation about the production of ochratoxin A by *A. ochraceus* and *A. niger* in rice samples. Accordingly, Zhihong et al., [9] reported *Aspergillus* section Nigri contamination of cereal grains including rice is a major issue in China. In connection with our study, Ochratoxigenic fungi *A. ochraceus* isolated from cereals reported to be potent OTA producer [11]. Laut et al., [33] stated that polished rice was contaminated with 80 % of *Aspergillus spp* and reported prevalence of *A. niger*, *A. ochraceus* in rice. As per the report of Raghu et al., [34], occurrence of *A. niger* in rice samples are documented in India. Han et al., [35] studied the ochratoxin producing ability of 27 strains of *A. niger* for four weeks and results shown nine strains found to be OTA producer. The findings of Sayed et al., [36] documented the occurrence of *Aspergillus* ochraceous (6.66 %) and *Aspergillus niger* (40 %), in rice samples.

As a consequence of *Aspergillus* infection and ochratoxin A production, the rice market value

deteriorates having significant impact on the rice quality, nutritional value and food safety aspects causing harmful effects to human health. OTA have high affinity to proteins particularly, serum albumins and possesses long half-life in humans (35 days). OTA can be absorbed from the stomach, gastrointestinal tract and small intestine, and the absorbed OTA is spread in human body through blood, and reaches the kidneys, liver [37]. Food safety which is an essential element of food security and hence more attention is needed during harvesting, storage, transportation and processing leading to safety of consumed rice.

4. CONCLUSION

The present investigation documented the rice samples in Tamil Nadu, India was contaminated with ochratoxigenic fungi such as *A. ochraceus* and *A. niger.* Quantification by RP HPLC-FLD analysis revealed *A. ochraceus* being the highest OTA producer (196.84 ng/g) than *A. niger* which recorded 2.82 ng/g respectively. The prevalence of ochratoxigenic fungi and its toxin production ability suggest the possible production of ochratoxin A in rice samples which affect rice quality and nutritional value. Further, fungal infestation and toxin production deteriorate rice market value and pose serious ill effects to human health. These findings could be used as a reference, since fungal infection has a major effect on rice quality and food safety. Hence there is need for focus on improved storage practices and strict statutory limits have to be implemented to control the wide spread of fungal infection and ochratoxin production in rice.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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