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ACTIVITY OF PLANT ESSENTIAL OILS AGAINST OCHRATOXIN A PRODUCING *ASPERGILLUS OCHRACEUS*

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ABSTRACT

Plant essential oils have been used in traditional medicines since ancient times to combat disease and in agro-food science to preserve food stuff. Antifungal activity of plant essential oils was evaluated using different substrates of varied moisture levels against ochratoxin A (OTA) producing *Aspergillus ochraceus*. *A. ochraceus* (n = 3) isolates were processed for antifungal activity of various essential oils including *Zingiber officinale*, *Curcuma longa*, *Eucalyptus globulus*, *Syzygium aromaticum, Nigella sativa*, *Elettaria cardamomum*, *Cinnamomum verum* and *Cuminum cyminum* extracted by hydro-distillation. To check the antimicrobial activity of essential oils, the highest zone of inhibition recorded was of *C. verum* (33.67±0.57mm) followed by *S. aromaticum* (30.33±0.57mm) and the least minimum inhibitory concentration (MIC) was of *S. aromaticum* (0.52±0.22 µg/mL) followed by *C. verum* (0.65±0.22 µg/mL). Antifungal activity was evaluated in term of log reduction and at the exposure time of 60 and 90 min, 6±0.00 log reduction was observed by *S. aromaticum*, *C. verum* and *E. cardamomum* with non-significant differences. At 15th, 30th, 45th and 60th day of experiment, toxin production by *A. ochraceus* at moisture contents (10, 20, 30, 40, 50, 60 and 70%) in un-inoculated groups and inoculated treated groups with *C. verum* Essential Oils were found non-significantly different to each other but significantly different from the OTA production in inoculated groups of wheat, maize and rice (intact and broken). At 10% moisture level, OTA production was low and reached to maximum level at 40% moisture level and again decline with increasing moisture level. Cinnamon has showed antifungal activity against *A. ochraceus* at all moisture levels. The confirmation of inhibition potential of *C. verum* was evaluated using thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) techniques. This study illustrates that cinnamon oil is effective to inhibit the growth of OTA producing *A. ochraceus* in stored grains to overcome the economic losses.

Keywords: Antifungal activity, *Cinnamomum verum*, Dimethyl sulfoxide, Log reduction, Minimum inhibitory concentration

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INTRODUCTION

The toxic secondary metabolite ochratoxin A (OTA) produced by *Aspergillus ochraceus* contaminates different agro-products and considered as the most abundant food contaminating toxin (Su *et al*., 2022). *Aspergillus ochraceus* is a saprophytic filamentous fungus having yellow to pale yellow-brown conidiophores and known to produce ochratoxin A which is the most abundant food contaminating toxin. It grows rapidly at 25°C temperature, 0.95-0.99 water activity level and microscopically appears as smooth or rough phialides arranged in biseriate pattern on conidial heads. Ochratoxin A, alkaloids and steroids are the secondary metabolites produced by *A. ochraceus* fungi (Hu *et al.,* 2021). *A. ochraceus* can contaminate stored wheat having more than 16% moisture content (Khan Achakzai *et al.,* 2017).

Until now, 20 different analogs of Ochratoxin have been studied, however, OTA has been reported as the most common and potent analogue (Hua *et al.,* 2014). The World Health Organization classified OTA as a carcinogenic agent in humans because of it can badly effect various systemic organs of humans and animals (Meng *et al*., 2020). The potential health risks associated with OTA exposure are nephrotoxicity, immunotoxicity, neurotoxicity. teratogenicity, and hepatoxicity (Laaziz *et al*., 2022). Different approaches have been used to prevent and restrict the contamination of grains with OTA producing fungi. Commonly, chemical agents such as benzimidazoles, aromatic hydrocarbons, and sterol biosynthesis inhibitors are used for this purpose (Mondal *et al*., 2022). Nevertheless, the usage of these antifungal

agents increases the risk of toxic residues in food. Moreover, inappropriate use of these fungicides results in fungal resistance (Baibakova *et al.,* 2019). To combat this issue, alternative strategies have been applied in the recent years. Naturally occurring antimicrobials and antifungal agents are the promising replacements to the synthetic fungicides (Geddes-McAlister and Shapiro, 2019). Essential oils of different aromatic plants received inordinate consideration due to their non-toxicity and can be used to control mycotoxin producing fungi in food without any health hazardous effects (Singh *et al*., 2019).

Several studies have reported essential oils as the best antifungal and antibacterial agents due to their broad-spectrum activity (Ferdosi *et al.,* 2022; Ferdosi *et al.,* 2021). Often these oils are obtained from plants including *Zingiber officinale*, *Curcuma longa*, *Eucalyptus globulus*, *Syzygium aromaticum, Nigella sativa*, *Elettaria cardamomum*, *Cinnamomum verum* and *Cuminum cyminum. Z. officinale* and *C. longa* are the effective essential oils having the OTA inhibition potential and antifungal activity can be evaluated by antimicrobial assays such as well diffusion assay, MIC and log reduction (Kalagatur *et al.,* 2020a). *Cinnamon* essential oil has the potential to inhibit *A. ochraceus* growth and OTA production potential (Wang *et al.,* 2018).

The present study was designed to evaluate the antifungal and antitoxic potential of plant essential oils against characterized OTA producing *A. ochraceus* isolates to prevent the economic losses. Moreover, effective essential oil was optimized for OTA inhibition potential for various grains experimental groups at varying range of moisture levels followed by detection and quantification of OTA.

MATERIALS AND METHODS

***A. ochraceus* isolates:** Three *A. ochraceus* isolates were procured from the Institute of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan. The isolates were revived from soil stocks by sprinkle plate method on Sabouraud dextrose agar (SDA) plates under sterile conditions. The plates were incubated at 25°C for 3-5 days.

Macroscopic and microscopic identification: The purified fungal isolates were subjected to macroscopic and microscopic identification. Growth pattern, colony texture and color on obverse side whereas ridges and color on reverse side of plates was observed. Agar-drop method was used to prepare fungal culture on the slide. Sterile molten SDA agar drop was poured in center of sterilized glass slide, fungal spores placed on agar and cover slip was placed over it. Glass slide was placed in sterilized petri plate (90 mm) containing moistened cotton plug and incubated at 25±3°C for three to five days. Slides were observed on daily basis under bright field microscope at 4X, 10X and 40X to record microscopic characteristics (Heredero-Bermejo *et al*., 2020).

Extraction of Essential oils: The roots of *Z. officinale* and *C. Longa*; seeds of *N. sativa*; leaves of *E. globulus*; bark of *C. verum* and dried fruit parts *S. aromaticum, E. cardamomum*, and *C. cyminum* were used for oil extraction. Plant parts were dried and crushed to form course powder. The crushed powder (250 g) was hydro-distilled for 5 to 6 hours using Clevenger apparatus. The aqueous phase was extracted using dichloromethane and organic phase was dried using anhydrous sodium sulphate Essential oils were stored in screw capped dark glass vials at 4°C (Kalagatur *et al*., 2018).

Antifungal assays of plant essential oils: Essential oils were solubilized in dimethyl sulfoxide (DMSO) to final concentration of 5mg/mL and screened for their antimycotic activity. Antifungal activity of essential oils against *A. ochraceus* isolates was evaluated by agar well-diffusion method, MIC determination by broth microdilution method and log reduction assay.

Fungal Spore Inoculum Preparation: Spore inoculum of *A. ochraceus* isolates was prepared in normal saline. Fungal spores from pure cultures were transferred in normal saline test tubes, mixed properly by inverting test tubes and counted with the help of improved Neubauer chamber (sterilized by ethanol and air dried). Fungal spores were enumerated at 40X and standard spore inoculum containing approximately 10^6 spores/mL was prepared for individual pure culture. (Arunachalam and Sasidharan, 2021).

Agar Well Diffusion Assay: For agar well diffusion assay, fungal spore inoculum was swabbed onto SDA media plates, about 8mm diameter wells were cut out of swabbed agar using sterile well-borer and sealed from bottom using a drop of molten SDA. Plant EO's were mixed with DMSO and 20-50 µL was added in wells. After incubation at 25°C for 3-5 days zones of inhibition (mm) were measured (Iram and Edwin, 2022).

Minimum Inhibitory Concentration (MIC): To determine MIC of plant EO's against three *A. ochraceus* isolates broth microdilution method using 96 wells flat bottom micro-titration plate was employed. SD broth (100 µL) was pipetted in 1st to 12th wells then 100 µL of oil was poured in 1st well and two-fold serially diluted up to 10th well. A volume of 100 µL of fungal spore inoculum was dispensed in 1st to 11th wells each. Optical density was recorded at 530nm at zero time and after incubation at 25°C for 3-5 days (Yan *et al*., 2021).

Log Reduction Assay: Plants EO's showed antifungal activity in agar well diffusion assay were selected for log reduction. Spore inoculum of *A. ochraceous* and MIC of each EO's were mixed in 1:1 ratio. After 15, 30, 60 and

90-minutes contact time intervals 1mL volume was transferred in sterile phosphate buffer saline (PBS) and ten-fold serial dilutions were performed. A volume of 1mL was spread on SDA plate followed by incubation at the temperature of 25°C for 72 hours. Experimental results were noted as colony forming unit (CFU) and percentage log reduction was calculated for each time interval (Hossain *et al.,* 2014).

Antifungal activity of *C. verum:* Antifungal activity of *C. verum* against OTA producing *A. ochraceus* at different moisture levels such as 10, 20, 30, 40, 50, 60 and 70 percent on 15th, 30th, 45th and 60th day post inoculation was determined.

Experimental groups: In the experimental groups, intact and crushed grains of wheat, maize and rice were processed. A weight of 100 g of each grain sample was sterilized by using autoclave (Taye *et al.,* 2016). Each type of grain was divided into three experimental groups such as un-inoculated (sterilized grains), inoculated group (sterilised grains with *A. ochraceus* having OTA producing potential) and inoculated treated group (sterilized grains inoculated with toxigenic *A. ochraceus* as well treated with *C. verum* plant essential oil). Standard spore inoculum (1 mL) was added in inoculated and inoculated treated groups while MIC of *C. verum* EO was mixed in Inoculated treated group. Further, efficacy of cinnamon against *A. ochraceus* in relation to OTA (ng/g) production at different moisture levels such as 10, 20, 30, 40, 50, 60 and 70% maintained by using digital hygrometer apparatus was evaluated separately for each experimental group. All the experimental flasks of 3 groups were incubated at 25°C for 15, 30, 45 and 60 days in shaking incubator.

Detection and quantification of OTA: At 15th, 30th, 45th and 60th day of post inoculation, 25 g grain sample from each experimental group flask was separated and processed for quantification of OTA by thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) following a previously described method with minor modifications (Flajs *et al.,* 2009).

Statistical analysis: Results obtained from experimental results were statistically analysed by one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range test by the software student package for social sciences (SPSS) version 20.0.

RESULTS

Macroscopic and microscopic identification: Macroscopically *A. ochraceus* isolates were examined from obverse and reverse side of culture plate. From obverse side, yellowish and rough cottony structures were observed and colorless from reverse side (Fig. 1). Under the fluorescent microscope, septate, hyaline hyphae were observed as well presence of vesicle covered by conidiophores and circular conidia arranged in chain were recorded for *A. ochraceus* isolates at 4X, 10X and 40X objective lens.

Fig.1. Macroscopic visualization *A. ochraceus* isolate from obverse side

Antifungal Activity of Plant essential oils: EO's of eight indigenous medicinal plants were evaluated for antifungal activity against OTA producing *A. ochraceus* by agar well diffusion and the highest zone of inhibition recorded was of *C. verum* (33.67 ± 0.57mm) followed by *S. aromaticum* (30.33 ± 0.57mm) and *E. cardamomum* (12.67 ± 0 .57mm). Rest of the five tested oils did not exhibit antifungal activity. Statistically significant variations were recorded in zone of inhibition of oils having antifungal activity against *A. ochraceus.*

Essential oils showing antifungal potential against OTA producing *A. ochraceus* were selected for MIC determination (µg /mL). The least MIC was of *S. aromaticum* (0.52±0.22 µg/mL) followed by *C. verum* (0.65±0.22 µg/mL) and *E. cardamomum* (2.08±0.90 µg/mL). Statistically non-significant differences were observed in MIC values of *S. aromaticum* and *C. verum.*

Antifungal activity was also evaluated in term of log reduction. At 15 minutes exposure time, log reduction of 5.91±0.35, 5.66±0.05 and 4.30±0.00 was recorded for *S. aromaticum*, *C. verum* and *E. cardamomum*. After 30 minutes interaction of spores with *E. cardamomum,* a log reduction of 5.71±0.00 was recorded against *A. ochraceus* (*Aso-1*). At 15 minutes log reduction of 5.80±0.35 5.67±0.045 and 4.70±0.00 were shown *by S. aromaticum*, *C. verum* and *E. cardamomum* and after 30 minutes exposure a log reduction of 5.71±0.004 was observed for *E. cardamomum* against *A. ochraceus* (*Aso-2*). At 15 minutes log reduction of 5.80±0.35 5.67±0.045 and 4.70±0.00 were shown *by S. aromaticum*, *C. verum*

and *E. cardamomum*. After 30 minutes exposure a log reduction of 5.71±0.004 was observed for *E. cardamomum* against *A. ochraceus* (*Aso-3*). After 60 and 90-min exposure time of *A. ochraceus* spores of all three isolates with essential oil, completely inactivated fungal spores and 6±0.00 log reduction was observed by three oils with non-significant differences.

Antimycotoxigenic activity of *C. verum* against *A. ochraceous:* On 15th day of experiment, highest production of OTA was observed in maize broken (14.59±0.23 ng/g) as well as intact (14.04±0.08 ng/g) while least OTA production was detected in broken rice (2.30±0.12 ng/g) followed by intact rice (2.38±0.06 ng/g) in inoculated grains. High amount of OTA produced at 40% moisture level. At 10% moisture level, OTA production was low and increased with increasing moisture of grains up to 40%. After 40% moisture level, a decreasing pattern in OTA production was observed with increasing moisture. Cinnamon (*C. verum*) has showed antifungal activity against *A. ochraceus* at all moisture levels. The OTA quantities inoculated treated and un-inoculated groups of intact and broken wheat, maize and rice are found non-significantly different from each other at all moisture levels. On the other side, OTA production in inoculated group was significantly different from Inoculated treated and un-inoculated at all moisture levels from 10 to 70% (Table 1).

Table1: Efficacy of cinnamon against *A. ochraceus* in relation to OTA (ng/g) production at different moisture levels 15th day post inoculation.

S. No.	Substrates	Groups	Ochratoxin A (Mean±S.D.) at different Moisture Level (%)						
			10	20	30	40	50	60	70
01	Wheat Intact	Uninoculated	0.05±0.00^{a}	0.05±0.01^{a}	0.04±0.00^{a}	0.05±0.00^{a}	0.05±0.00^{a}	0.05±0.00^{a}	0.04±0.00^{a}
		Inoculated	3.34±0.11^{d}	6.33±0.19^{d}	10.23±0.11^{d}	12.54±0.11^{d}	9.77±0.10^{d}	7.58±0.08^{d}	4.15±0.15^{d}
		Inoculated Treated	0.04±0.00^{a}	0.05±0.00^{a}	0.04±0.00^{a}	0.05±0.00^{a}	0.05±0.00^{a}	0.05±0.00^{a}	0.04±0.00^{a}
02	Wheat Broken	Uninoculated	0.05±0.00^{a}	0.04±0.01^{a}	0.05±0.00^{a}	0.04±0.01^{a}	0.04±0.00^{a}	0.04±0.00^{a}	0.05±0.00^{a}
		Inoculated	3.05±0.07^{c}	5.95±0.19^{c}	9.94±0.05^{d}	13.27±0.17^{e}	10.10±0.11^{e}	7.68±0.40^{d}	4.64±0.12^{e}
		Inoculated Treated	0.04±0.00^{a}	0.04±0.00^{a}	0.04±0.00^{a}	0.0413±0.00^{a}	0.04±0.00^{a}	0.04±0.00^{a}	0.04±0.01^{a}
03	Maize Intact	Uninoculated	0.05±0.00^{a}	0.05±0.00^{a}	0.04±0.00^{a}	0.047±0.01^{a}	0.05±0.00^{a}	0.05±0.00^{a}	0.04±0.00^{a}
		Inoculated	3.56±0.09^{e}	6.49±0.18^{de}	10.66±0.12^{e}	14.04±0.08^{f}	11.01±0.34^{g}	8.22±0.17^{e}	4.99±0.12^{e}
		Inoculated Treated	0.05±0.00^{a}	0.04±0.00^{a}	0.05±0.00^{a}	0.05±0.00^{a}	0.05±0.00^{a}	0.05±0.00^{a}	0.04±0.00^{a}
04	Maize Broken	Uninoculated	0.05±0.00^{a}	0.05±0.00^{a}	0.04±0.00^{a}	0.04±0.01^{a}	0.04±0.00^{a}	0.04±0.00^{a}	0.04±0.00^{a}
		Inoculated	3.48±0.07^{e}	6.59±0.33^{e}	10.28±0.15^{de}	14.59±0.23^{g}	10.79±0.23^{f}	8.57±0.21^{f}	4.99±0.00^{e}
		Inoculated Treated	0.04±0.00^{a}	0.04±0.00^{a}	0.04±0.00^{a}	0.05±0.00^{a}	0.05±0.00^{a}	0.05±0.00^{a}	0.04±0.00^{a}
05	Rice intact	Uninoculated	0.05±0.01^{a}	0.04±0.00^{a}	0.04±0.00^{a}	0.04±0.00^{a}	0.04±0.00^{a}	0.04±0.00^{a}	0.04±0.00^{a}
		Inoculated	2.38±0.06^{b}	4.49±0.13^{b}	7.21±0.12^{b}	8.53±0.23^{b}	6.75±0.07^{b}	5.02±0.01^{b}	2.87±0.08^{b}
		Inoculated Treated	0.05±0.01^{a}	0.04±0.00^{a}	0.05±0.00^{a}	0.04±0.00^{a}	0.04±0.00^{a}	0.04±0.00^{a}	0.05±0.00^{a}
06	Rice Broken	Uninoculated	0.04±0.00^{a}	0.04±0.00^{a}	0.04±0.00^{a}	0.047±0.00^{a}	0.05±0.00^{a}	0.04±0.00^{a}	0.04±0.00^{a}
		Inoculated	2.30±0.12^{b}	4.33±0.11^{b}	7.88±0.95^{c}	8.75±0.38^{c}	6.94±0.14^{c}	5.42±0.26^{c}	2.99±0.01^{c}
		Inoculated Treated	0.04±0.00^{a}	0.04±.00^{a}	0.05±0.00^{a}	0.04±0.00^{a}	0.04±0.00^{a}	0.05±0.00^{a}	0.04±0.00^{a}

Means within column having different superscripts differ significantly and with same differ non-significantly ($p \leq 0.05$)

Production of OTA by *A. ochraceus* in maize, wheat and rice grains in intact and broken form having different moisture contents (10, 20, 30, 40, 50, 60 and 70%) on 30th day of experiment. Highest OTA was quantified in maize broken (22.11±0.48 ng/g) with 40 % moisture contents followed by OTA in broken wheat (21.60±0.50 ng/g) at same moisture. An increase in OTA production was observed at moisture levels from 10 to 40% in wheat, maize and rice (both in intact and broken groups). From 50 to 70 % moisture inverse relation was observed in OTA production and moisture level. The least quantity of OTA (4.51±0.15 ng/g) was determined at 10% moisture level in intact rice followed by OTA production in broken rice (4.80±0.22 ng/g) at same moisture level. Statistical analysis revealed a significant difference in OTA production in inoculated group to inoculated treated and un-inoculated groups of wheat, maize and rice at all moisture levels from 10 to 70%. However, OTA production was non-significantly different in un-inoculated and inoculated treated groups (Table 2).

Table 2: Efficacy of cinnamon against *A. ochraceus* in relation to ochratoxin A (ng/g) production at different moisture levels 30th day post inoculation.

S. No.	Substrates	Groups	Ochratoxin A (Mean±S.D) at different Moisture Level (%)						
			10	20	30	40	50	60	70
01	Wheat Intact	Un-inoculated	0.04±0.01^{a}	0.04±0.00^{a}	0.05±0.00^{a}	0.05±0.00^{a}	0.05±0.00^{a}	0.05±0.00^{a}	0.05±0.00^{a}
		Inoculated	6.27±0.05^{d}	10.45±0.20^{c}	15.24±0.23^{cd}	21.24±0.36^{cd}	18.66±0.31^{e}	9.60±0.13^{e}	8.40±0.14^{d}
		Inoculated Treated	0.04±0.00^{a}	0.04±0.01^{a}	0.05±0.00^{a}	0.04±0.00^{a}	0.05±0.00^{a}	0.04±0.00^{a}	0.04±0.00^{a}
02	Wheat Broken	Uninoculated	0.04±0.00^{a}	0.04±0.00^{a}	0.05±0.00^{a}	0.05±0.00^{a}	0.04±0.00^{a}	0.05±0.00^{a}	0.05±0.00^{a}
		Inoculated	6.58±0.15^{e}	10.36±0.46^{c}	15.01±0.44^{c}	21.60±0.50^{d}	18.03±0.16	9.54±0.47^{e}	8.31±0.36^{d}
		Inoculated Treated	0.04±0.00^{a}	0.04±0.00^{a}	0.05±0.00^{a}	0.04±0.00^{a}	0.05±0.00^{a}	0.05±0.00^{a}	0.05±0.00^{a}
03	Maize Intact	Uninoculated	0.05±0.01^{a}	0.04±0.00^{a}	0.04±0.00^{a}	0.04±0.00^{a}	0.05±0.00^{a}	0.04±0.00^{a}	0.04±0.00^{a}
		Inoculated	6.81±0.18^{f}	11.08±0.14^{d}	15.40±0.46^{d}	21.05±0.80^{e}	17.85±0.16^{d}	9.11±0.33^{d}	7.95±0.07^{c}
		Inoculated Treated	0.05±0.00^{a}	0.05±0.00^{a}	0.04±0.00^{a}	0.04±0.00^{a}	0.04±0.00^{a}	0.04±0.00^{a}	0.04±0.00^{a}
04	Maize Broken	Uninoculated	0.05±0.00^{a}	0.04±0.00^{a}	0.05±0.00^{a}	0.05±0.00^{a}	0.04±0.00^{a}	0.05±0.00^{a}	0.05±0.00^{a}
		Inoculated	7.19±0.32^{g}	11.74±0.44^{e}	14.95±0.34^{c}	22.11±0.48^{e}	18.03±0.17^{d}	9.49±0.25^{e}	8.25±0.14^{d}
		Inoculated Treated	0.04±0.00^{a}	0.05±0.00^{a}	0.04±0.00^{a}	0.04±0.00^{a}	0.04±.00^{a}	0.04±0.00^{a}	0.05±0.00^{a}
05	Rice intact	Uninoculated	0.04±0.00^{a}	0.04±0.00^{a}	0.05±0.00^{a}	0.04±0.00^{a}	0.04±0.00^{a}	0.05±0.00^{a}	0.05±0.00^{a}
		Inoculated	4.51±0.15^{b}	8.78±0.02^{b}	14.23±0.11^{b}	16.48±0.28^{b}	13.41±0.18^{c}	8.16±0.14^{b}	4.92±0.14^{b}
		Inoculated Treated	0.04±0.00^{a}	0.05±0.00^{a}	0.05±0.00^{a}	0.04±0.00^{a}	0.04±0.00^{a}	0.04±0.00^{a}	0.05±0.00^{a}
06	Rice Broken	Uninoculated	0.04±0.00^{a}	0.05±0.00^{a}	0.05±0.01^{a}	0.04±0.00^{a}	0.04±0.00^{a}	0.05±0.00^{a}	0.05±0.00^{a}
		Inoculated	4.80±0.22^{c}	8.91±0.11^{b}	14.40±0.22^{b}	16.63±0.33^{b}	12.95±0.07^{b}	8.45±0.22^{c}	5.04±0.23^{b}
		Inoculated Treated	0.05±0.01^{a}	0.04±0.00^{a}	0.05±0.00^{a}	0.05±0.00^{a}	0-.04±0.00^{a}	0.05±0.00^{a}	0.05±0.00^{a}

Means within column having different superscripts differ significantly and with same differ non-significantly ($p \leq 0.05$)

On 45th day of experiment, the highest OTA was detected at 40% moisture level in intact maize (37.04±0.18 ng/g). While the least production of OTA (6.27±0.17 ng/g) was observed at 10% moisture in inoculated broken rice. OTA production increased with increase in moisture level up-to 40% in all experimental groups. OTA production by *A. ochraceus* was decreased with increase in moisture contents from 50 to 70%. Quantities obtained from HPLC on 45th day of experiment were processed for statistical analysis. It was revealed that the OTA in inoculated found to be significantly different not only to the OTA produced in un-inoculated and inoculated treated groups but also to OTA produced in each inoculated group (wheat, rice and maize). On other-side OTA produced by *A. ochraceus* in un-inoculated and inoculated treated groups are non-significantly different at all moisture levels in wheat maize and rice groups (Table 3).

Toxin production by *A. ochraceus* in un-inoculated groups and inoculated treated groups were close to each other even at 60th day of experiment. This shows that cinnamon oil is effective to inhibit the growth of *A. ochraceus* in stored grains. Statistically, the OTA values in these groups were found non-significantly different to each other but significantly different from the OTA production in inoculated groups of wheat, maize and rice (intact and broken). In inoculated groups the highest quantity of OTA (45.57±0.51 ng/g) was observed in intact wheat with 40% moisture contents. OTA was produced in the highest quantity at 40% moisture level. As the moisture level increased from 40%, a decrease in OTA production was found in all experimental groups. The least toxin production was recorded at 10% for all groups. However, the lowest quantity of toxin was 8.14±0.07 ng/g produced in broken rice by *A. ochraceus* at 10% moisture (Table 4).

Detection and quantification of OTA: Experimental group samples were processed for the detection and quantification of OTA. As n=02 samples detected positive for OTA production by TLC and lane=L1 labelled as OTA standard and L5 and L7 labelled positive for OTA production by grain samples (Fig. 2). Samples were also processed for OTA quantification and HPLC was performed for OTA standard (Fig. 3) as well for experimental grain sample (Fig. 4).

Table 3: Efficacy of cinnamon against *A. ochraceus* in relation to ochratoxin A (ng/g) production at different moisture levels at 45th day post inoculation.

S. No.	Substrates	Groups	Ochratoxin A (Mean±S.D) at different Moisture Level (%)						
			10	20	30	40	50	60	70
01	Wheat Intact	Un-inoculated	0.05±0.00^{a}	0.04±0.00^{a}	0.05±0.00^{a}	0.04±0.00^{a}	0.05±0.01^{a}	0.05±0.01^{a}	0.04±0.00^{a}
		Inoculated	9.19±0.23^{e}	15.31±0.14^{e}	30.12±0.11^{c}	36.62±0.17^{c}	27.40±0.26^{c}	21.03±0.78^{c}	12.33±0.31^{d}
		Inoculated Treated	0.05±0.00^{a}	0.04±0.00^{a}	0.04±0.00^{a}	0.04±0.01^{a}	0.04±0.00^{a}	0.04±0.00^{a}	0.05±0.00^{a}
02	Wheat Broken	Uninoculated	0.05±0.00^{a}	0.04±0.00^{a}	0.05±0.00^{a}	0.04±0.00^{a}	0.04±0.00^{a}	0.04±0.00^{a}	0.04±0.00^{a}
		Inoculated	9.33±0.08^{f}	15.61±0.18^{f}	30.03±0.39^{c}	36.53±0.65^{c}	27.68±0.15^{c}	20.82±0.39^{c}	12.23±0.21^{d}
		Inoculated Treated	0.04±0.00^{a}	0.05±0.00^{a}	0.04±0.00^{a}	0.05±0.00^{a}	0.04±0.00^{a}	0.05±0.00^{a}	0.04±0.00^{a}
03	Maize Intact	Uninoculated	0.05±0.00^{a}	0.04±0.00^{a}	0.05±0.00^{a}	0.05±0.00^{a}	0.05±0.01^{a}	0.04±0.00^{a}	0.05±0.00^{a}
		Inoculated	8.95±0.07^{d}	14.84±0.15^{d}	29.97±0.25	37.04±0.18^{d}	28.11±0.11^{d}	22.2±0.22^{d}	12.87±0.23^{d}
		Inoculated Treated	0.04±0.00^{a}	0.04±0.00^{a}	0.04±0.00^{a}	0.04±0.01^{a}	0.04±0.00^{a}	0.04±0.01^{a}	0.04±0.00^{a}
04	Maize Broken	Uninoculated	0.05±0.00^{a}	0.05±0.01^{a}	0.05±0.00^{a}	0.05±0.00^{a}	0.04±0.00^{a}	0.043±0.00^{a}	0.04±0.00^{a}
		Inoculated	9.04±0.07^{d}	14.72±0.06^{d}	29.89±0.11^{c}	36.77±0.29^{cd}	28.03±0.33^{d}	20.84±0.34^{c}	12.88±0.33^{d}
		Inoculated Treated	0.04±0.00^{a}	0.05±0.00^{a}	0.05±0.00^{a}	0.04±00.00^{a}	0.04±0.00^{a}	0.05±0.01^{a}	0.05±0.00^{a}
05	Rice intact	Uninoculated	0.04±0.00^{a}	0.04±0.00^{a}	0.05±0.00^{a}	0.04±0.00^{a}	0.05±0.00^{a}	0.04±0.00^{a}	0.04±0.00^{a}
		Inoculated	6.47±0.17^{c}	10.84±0.25^{c}	20.82±0.74^{b}	25.30±0.20^{b}	19.37±0.22^{b}	14.15±0.11^{b}	8.56±.01^{c}
		Inoculated Treated	0.04±0.00^{a}	0.04±0.00^{a}	0.04±0.00^{a}	0.04±0.00^{a}	0.04±0.00^{a}	0.04±0.01^{a}	0.04±0.00^{a}
06	Rice Broken	Uninoculated	0.04±0.00^{a}	0.05±0.01^{a}	0.05±0.01^{a}	0.04±0.00^{a}	0.04±0.01^{a}	0.05±0.00^{a}	0.04±0.00^{a}
		Inoculated	6.27±0.17^{b}	9.99±0.20^{b}	20.96±0.55^{b}	25.33±0.35^{b}	19.19±0.54^{b}	14.47±0.71^{b}	8.65±0.17^{c}
		Inoculated Treated	0.04±0.01^{a}	0.04±0.00^{a}	0.04±0.00^{a}	0.04±0.00^{a}	0.05±0.00^{a}	0.04±0.00^{a}	0.05±0.01^{a}

Means within column having different superscripts differ significantly and with same differ non-significantly (p≤0.05)

Table 4: Efficacy of cinnamon against *A. ochraceus* in relation to ochratoxin A (ng/g) production at different moisture levels 60th day post inoculation.

S. No.	Substrates	Groups	Ochratoxin A (Mean±S.D.) at different Moisture Level (%)						
			10	20	30	40	50	60	70
01	Wheat Intact	Un-inoculated	0.05±0.00^{a}	0.04±0.00^{a}	0.04±0.00^{a}	0.04±0.00^{a}	0.05±0.01^{a}	0.05±0.01^{a}	0.04±0.01^{a}
		Inoculated	12.47±0.18^{d}	23.43±0.31^{c}	38.05±0.45^{c}	45.57±0.51^{d}	32.20±0.23^{c}	26.99±0.13^{c}	14.85±0.24^{c}
		Inoculated Treated	0.04±0.01^{a}	0.05±0.00^{a}	0.04±0.00^{a}	0.05±0.01^{a}	0.04±0.01^{a}	0.05±0.00^{a}	0.04±0.01^{a}
02	Wheat Broken	Un-inoculated	0.04±0.01^{a}	0.04±0.00^{a}	0.04±0.00^{a}	0.05±0.01^{a}	0.05±0.01^{a}	0.04±0.01^{a}	0.05±0.01^{a}
		Inoculated	12.58±0.27^{d}	23.41±0.65^{c}	38.78±0.19^{d}	45.15±0.17^{d}	32.16±1.02^{c}	26.75±0.95^{c}	14.96±0.24^{c}
		Inoculated Treated	0.05±0.01^{a}	0.04±0.01^{a}	0.05±0.01^{a}	0.04±0.01^{a}	0.05±0.01^{a}	0.04±0.01^{a}	0.05±0.01^{a}
03	Maize Intact	Un-inoculated	0.04±0.01^{a}	0.05±0.01^{a}	0.04±0.01^{a}	0.05±0.01^{a}	0.04±0.01^{a}	0.05±0.01^{a}	0.05±0.01^{a}
		Inoculated	13.06±0.12^{f}	23.98±0.12^{c}	37.81±0.61^{c}	45.19±1.15^{d}	31.67±0.56^{c}	27.06±0.56^{c}	14.72±0.58^{c}
		Inoculated Treated	0.04±0.01^{a}	0.04±0.01^{a}	0.05±0.00^{a}	0.04±0.01^{a}	0.04±0.01^{a}	0.04±0.00^{a}	0.04±0.00^{a}
04	Maize Broken	Un-inoculated	0.05±0.01^{a}	0.05±0.01^{a}	0.04±0.01^{a}	0.05±0.01^{a}	0.05±0.01^{a}	0.05±0.00^{a}	0.05±0.00^{a}
		Inoculated	12.78±0.27^{e}	23.41±1.29^{c}	38.83±0.63^{d}	45.15±0.47^{d}	31.75±0.83^{c}	26.85±0.35^{c}	15.48±0.17^{d}
		Inoculated Treated	0.04±0.01^{a}	0.04±0.00^{a}	0.04±0.01^{a}	0.05±0.01^{a}	0.04±0.01^{a}	0.04±0.01^{a}	0.04±0.01^{a}
05	Rice intact	Un-inoculated	0.05±0.01^{a}	0.04±0.00^{a}	0.05±0.00^{a}	0.04±0.01^{a}	0.05±0.01^{a}	0.05±0.00^{a}	0.05±0.00^{a}
		Inoculated	8.43±00.09^{c}	15.18±0.18^{b}	26.04±0.46^{b}	31.07±0.73^{c}	21.81±0.45^{b}	18.92±0.18^{b}	10.16±0.14^{b}
		Inoculated Treated	0.05±0.01^{a}	0.05±0.01^{a}	0.04±0.01^{a}	0.05±0.01^{a}	0.04±0.01^{a}	0.05±0.01^{a}	0.05±0.01^{a}
06	Rice Broken	Un-inoculated	0.04±0.00^{a}	0.05±0.01^{a}	0.04±0.01^{a}	0.05±0.01^{a}	0.04±0.01^{a}	0.04±0.01^{a}	0.04±0.01^{a}
		Inoculated	8.14±0.07^{b}	15.49±0.18^{b}	25.59±0.77^{b}	30.39±0.59^{b}	22.14±0.41^{b}	19.16±0.36^{b}	10.08±0.66^{b}
		Inoculated Treated	0.04±0.00^{a}	0.05±0.01^{a}	0.05±0.01^{a}	0.04±0.01^{a}	0.04±0.01^{a}	0.04±0.00^{a}	0.04±0.00^{a}

Means within column having different superscripts differ significantly and with same differ non-significantly (p≤0.05)

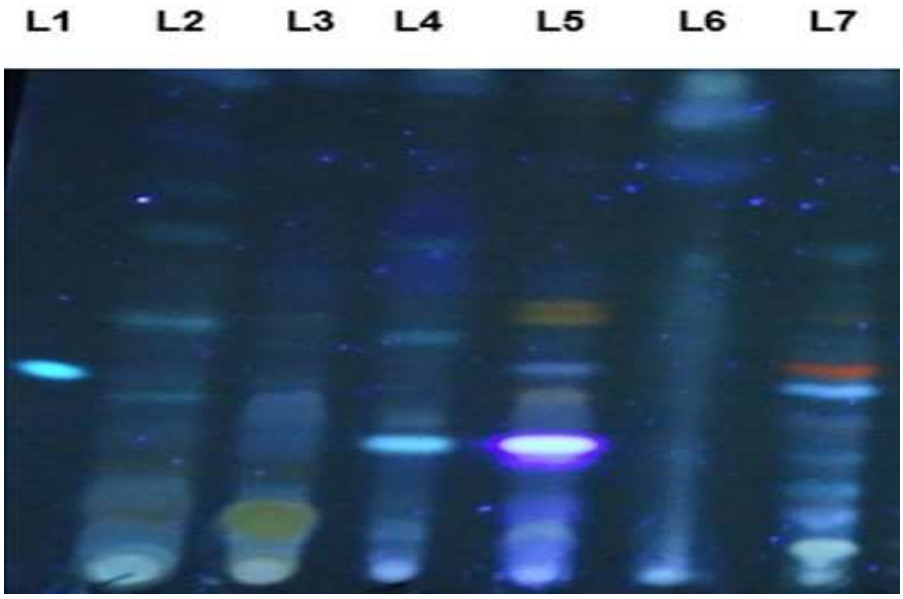

Fig.2. Chromatogram of Ochratoxin A detection by TLC

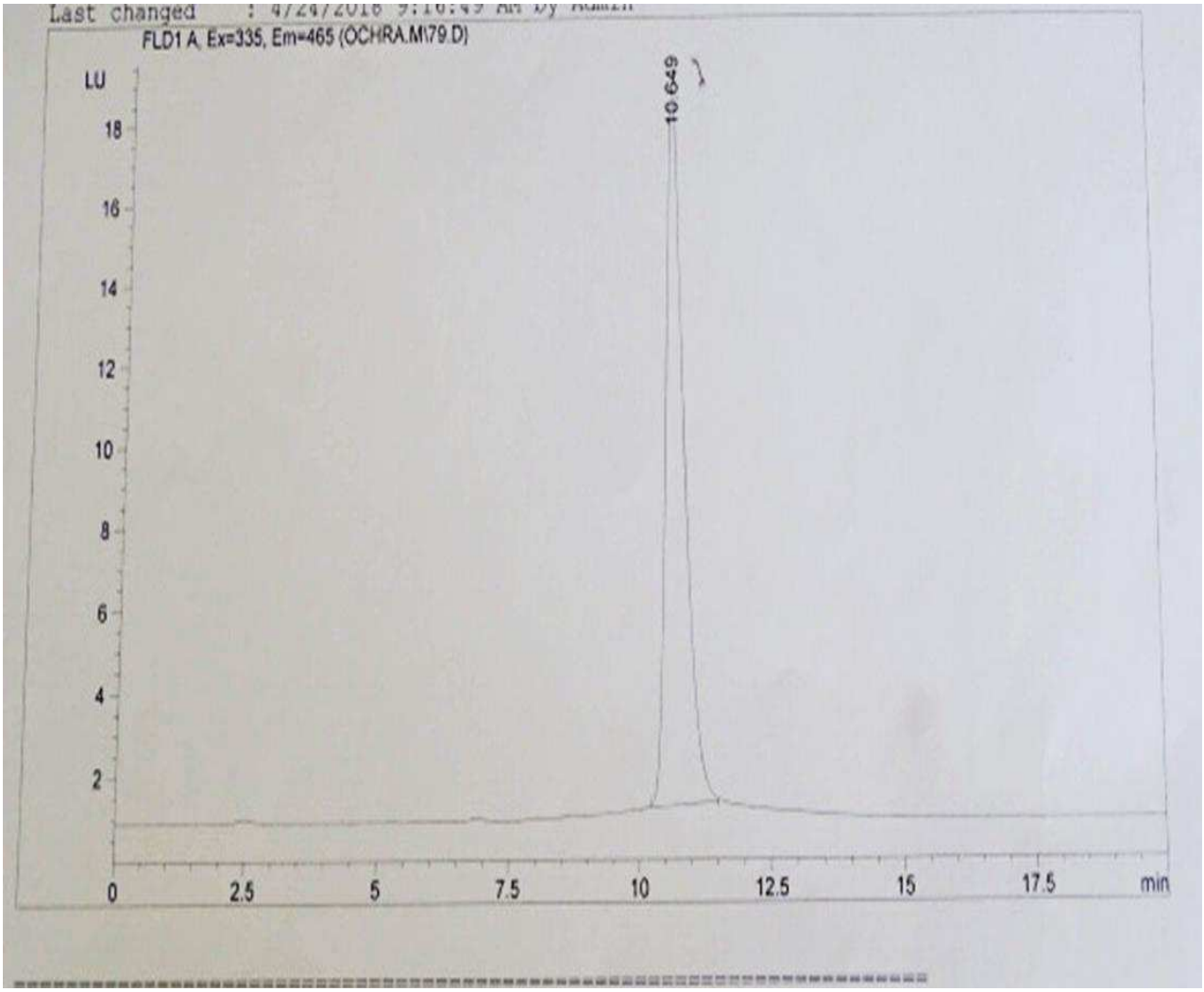

Fig.3. Chromatogram of OTA standard by HPLC analysis

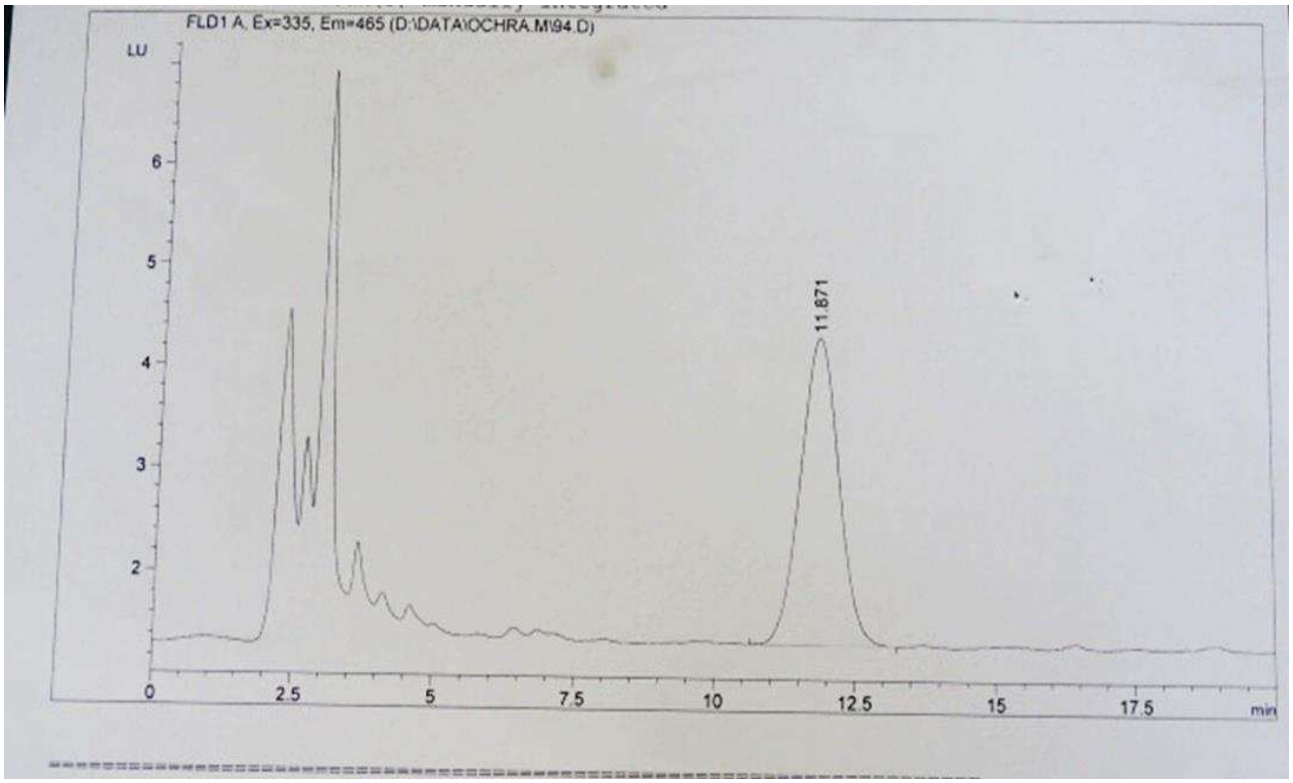

Fig.4. Chromatogram of positive sample for OTA by HPLC analysis.

DISCUSSION

In the current study, antifungal activity of eight plant EO's against OTA producing *A. ochraceus* isolates by agar well diffusion showed that highest zone of inhibition was of *C. verum* (33.67 ± 0.57mm) followed by *S. aromaticum* (30.33 ± 0.57 mm) and *E. cardamomum* (12.67 ± 0 .57 mm). The findings of study were consistent with previous studies (Achar *et al*., 2020; Hlebová *et al*. 2021). In the present study EO's showed antifungal potential against OTA producing *A. ochraceus* were selected for MIC determination (µg/mL). The least MIC was of *S. aromaticum* (0.52±0.22 µg/mL) followed by *C. verum* (0.65±0.22 µg/mL) and *E. cardamomum* (2.08±0.90 µg/mL). Statistically non-significant differences were observed in MIC values of *S. aromaticum* and *C. verum.* These results are in line with a recent study, showed that the inhibitory effects of Cinnamon, Clove, Thyme, Caraway and Cumin essential oils on the mycotoxin producing *A. ochraceus*. The essential oil of Cinnamon exhibited a more inhibitory effect than other essential oils, especially caraway (Moghadam *et al.,* 2019). In another study antifungal activity of cinnamon, clove, anise, peppermint, citronella, camphor and pepper essential oils were investigated against three common fungi *Aspergillus niger*, *A. ochraceus*, and *A. oryzae*. Among all, the cinnamon EO showed highest antifungal activity for all three fungal species with the largest inhibition zone at concentration of 800 mg/mL and lowest MIC ranging from 0.0625 to 0.125 34 mg/mL, followed by clove EO in line with current study (Hu *et al*., 2019). Ju *et al*. (2018) reported that cinnamon and clove EOs showed strong antifungal activity against Penicillium spp. and Aspergillus spp. MIC of cinnamon EO against two mold genera were 0.21-0.83 µL/mL and clove's MIC ranges 0.21-1.67 µL/mL.

Antifungal activity was also evaluated in term of log reduction. At 15 min exposure time, log reduction of 5.91±0.35, 5.66±0.05 and 4.30±0.00 was recorded for *S. aromaticum*, *C. verum* and *E. cardamomum*. After 30 min interaction of spores with *E. cardamomum,* a log reduction of 5.71±0.00 was recorded against *A. ochraceus* (*Aso-1*). At 15 min log reduction of 5.80±0.35 5.67±0.045 and 4.70±0.00 were shown *by S. aromaticum*, *C. verum* and *E. cardamomum* and after 30 minutes exposure a log reduction of 5.71±0.004 was observed for *E. cardamomum* against *A. ochraceus* (*Aso-2*). At 15 min log reduction of 5.80±0.35 5.67±0.045 and 4.70±0.00 were shown *by S. aromaticum*, *C. verum* and *E. cardamomum*. After 30 min exposure a log reduction of 5.71±0.004 was observed for *E. cardamomum* against *A. ochraceus* (*Aso-3*). After 60 and 90 min exposure time of *A. ochraceus* spores of all three isolates with essential oil, completely inactivated fungal spores and 6±0.00 log reduction was observed by three oils with non-significant differences.

The essential oil of cinnamon exhibited a more inhibitory effect than other essential oils in present study. The findings of this study are consistent with the results obtained by Hua and his coworkers and natural cinnamaldehyde (cinnamon oil) proved to be the most effective in reduction of *A. ochraceus* growth as compared to other essential oils. Complete growth inhibition of fungus was recorded at 150-250 µL/L by fumigation and 250-500 µL/L by using contact assays for cinnamon oil. A complete inhibition of ergosterol biosynthesis was obtained at approximately 100 µg/mL of natural cinnamaldehyde. The inhibition of OTA production by natural cinnamaldehyde is mainly due to the reduction in fungal biomass (Hua *et al.,* 2014). In another study antifungal activity of cinnamon, clove, anise, peppermint, citronella, camphor and pepper essential oils were investigated against three common fungi *Aspergillus niger*, *A. ochraceus*, and *Aspergillus oryzae*. Among all, the cinnamon EO showed highest antifungal activity for all three fungal species with the largest inhibition zone at concentration of 800 mg/mL and lowest MIC ranging from 0.0625 to 0.125 34 mg/mL, followed by clove EO in line with current study. The remaining EOs exerted moderate inhibitory effects (Hu *et al.,* 2019).

Plant essential oils successfully substituted synthetic fungicides and now a days essential oil based preservatives are available commercially because they are considered eco-friendly (Dwivedy *et al.,* 2016). Therefore, in our study antifungal effects of cinnamon oil were also evaluated against *A. ochraceus* and OTA production in maize, wheat and rice intact and broken grains during storage. Each type of grains was divided into un-inoculated (Sterile grains without *A. ochraceus*), Inoculated (Grains inoculated with *A. ochraceus*) and inoculated treated (Grains mixed with cinnamon oil and inoculated with *A. ochraceus*) having different moisture contents (10, 20, 30, 40, 50, 60 and 70%). OTA production by *A. ochraceus* was observed after 15th, 30th, 45th and 60th day post inoculation. At 10% moisture level, OTA production was low and increased with increasing moisture of grains up to 40%. After 40% moisture level, a decreasing pattern in OTA production was observed with increasing moisture. Cinnamon has showed antifungal activity against *A. ochraceus* at all moisture levels. This shows that cinnamon oil is effective to inhibit the growth of *A. ochraceus* in stored grains even at 60th day of experiment. In support of our study Kalagatur *et al.,* documented potent antifungal and anti-mycotoxin activities of *Cinnamomum zeylanicum*, *Z. officinale*, *C. longa* and *Cymbopogon martini* essential oils on *A. ochraceus* in maize grains (Kalagatur *et al.,* 2020b). Sumalan *et al.,* demonstrated the antifungal and mycotoxin inhibitory activities of *C. zeylanicum* on Aspergillus and Fusarium in wheat grains in line with our study (Sumalan *et al.,* 2013). As n=02 experimental group samples were positive for OTA production by TLC technique and further HPLC was applied for OTA standard followed by experimental samples. Present study as well as earlier studies showed that plant essential oils of cinnamon bark significantly appropriate for controlling the fungal growth and mycotoxins.

In conclusion, *C. verum* plant essential oil can be used as an effective antifungal and antitoxic agent for stored grains and food safety can be assured at large scale. So the EO of cinnamon bark could be a novel replacement of chemical agents used for controlling the fungal growth and mycotoxins.

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