Chapter 5

Quantification and characterization of extracellular mycotoxins

5.1 Introduction:

Mycotoxins are low molecular weight secondary metabolites produced by filamentous fungi which have no significant role in fungal growth and development but can cause damage to the plants, animals and humans and hence they are of high public health significance. Mycotoxins are produced by fungi but not all secondary compounds produced by fungi are considered as mycotoxins (Bennett and Klich, 2003; Moss, 1991). Mycotoxins have diverse chemical structure and have different biosynthetic pathway. The biological effects exerted by mycotoxins depends upon host and organs exposed to it. Base on their effects, they are classified as nephrotoxins, neurotoxins, haepathotoxins, immunotoxins etc. Mycotoxins are well known food contaminant. Mycotoxin production is greatly affected by environmental condition. 25% of the world crop is contaminated by mycotoxin producing fungi and molds (Pandya and Arade, 2016). Mycotoxicosis is referred as toxic effect or poisoning effect of mycotoxin on human and animal health (Bennett and Klich, 2003; Liew and Mohd-Redzwan, 2018). The effect of mycotoxins on human and animal health is depending upon type of mycotoxin, dosage and duration of exposure, age and gender of individual, immunological, nutritional and physiological status of individual and synergistic effect of mycotoxin with other chemicals (Bennett and Klich, 2003; Gajęcka *et al*., 2013). The mycotoxins reported to be produced by *Fusarium spp*. includes, Fumonisin B1, Deoxynivalenol, Zearalenone, T-2 and HT-2 toxins, beauvericin, Enniatin B, and Nivalenol (Bertero *et al*., 2018)

5.2 Materials and Methods:

5.2.1 Growth of fungi:

For mycotoxin production, *Fusarium* isolates were grown in 4 different types of media which were PDB, SDB, Rice media and Corn media. For Rice media, 10 gm of rice were taken in to 500 ml conical flask. 10 ml D/W was added to it and autoclaved. For corn media, 10 gm of corn was taken and broken in to pieces with help of mortar pestle. Cracked corn was also taken in 500 ml conical flask and 10 ml D/W was added to it and autoclaved. 100 ml of PDB and SDB were also taken in 500 ml conical flasks and autoclaved. The *Fusarium* isolates were first grown on PDA for active culture. After 7 days of

growth approximately 10 mm disc was cut from the PDA plate of actively growing culture and the disc was inoculated in all 4 different media and allowed to grow for 20 days at 30ºC without shaking in dark. At the end of 20th day the mycelial mass was separated from culture filtrate using autoclaved whatman no.1 filter paper from SDB and PDB.

5.2.2 Extraction of crude toxins:

Crude toxin extraction was carried out using method of (Cavaliere *et al*., 2005) from fungus grown on cracked corn and rice. It was pre-wetted with solvent system containing ACN (Merck, USA): water (3:1) (V/V) and taken in to mortar pestle, more solvent was added to it and grinded for 15-20 minutes. Grinded mixture was taken in to flask and supernatant was separated using whatman no. 1 filter paper. For crude toxin extraction from fungus grown in PDB and SDB media, culture filtrate was separated from fungal mycelia with help of whatman No. 1 filter paper and obtained culture filtrate was extracted with equal volumes of ACN: Water (3:1) (V/V). The obtained supernatant was passed through column of sodium sulphite (Merck, USA) to remove moisture and poured in to glass petri plates. The plates were kept in fume hood for vaporization of solvent. After vaporization, dried crystals were visible on glass petri plate were dissolved in HPLC grade acetonitrile and stored at 4˚C till further use.

5.2.3 Characterization of toxin:

Toxin characterization was primarily done with TLC, HPLC and MS/MS.

5.2.3.1 Thin Layer Chromatography:

Crude toxin extracted from *Fusarium* isolates was primarily characterized with respect to standard toxins: Zearalenone and T2 Toxin (Himedia, India). TLC plates (Kieselgel 60, E. Merck, USA) were cut in to piece of 10x6 cm, 10x10 cm and 10x14 cm. Plates were marked with pencil 1 cm from bottom end and 1 cm from upper end. Spots were marked with pencil at distance of 1 cm. TLC chamber was saturated with solvent system for 30 minutes. Toluene (Merck, USA): Ethyl acetate (Merck, USA): Formic acid (Merck, USA) (6:3:1) (V/V) solvent system was used for toxin characterization. Both the standard toxins were dissolved in HPLC grade acetonitrile at 1mg/ml

concentration. 10 µl of 1mg/ml standard ZEA and T2 toxin and 10 µl of extracted crude toxin were applied to marked spots on TLC plate with and TLC plate was placed carefully into the TLC chambers in vertical position. As soon as solvent reached to marked top line at a distance of 8 cm, TLC plate was removed from TLC chamber and air dried. For ZEA estimation, TLC plate was placed in Ultra violet light (UV) chamber and ZEA could be observed as fluorescent blue spot in long UV light. For T2 toxin estimation, TLC plate was sprayed with 20% H_2SO_4 and heated at 80˚C for 10 minutes. T2 toxin could be observed as brown spot in white light.

5.2.3.2 High Pressure Liquid Chromatography (HPLC):

Quantitative analysis of ZEA was done through high performance liquid chromatography. The analytical equipment for HPLC (Series 200 Perkin Elmer) consisted of a Quaternary gradient pump, manual injector system with a 20 µl sample loop, and variable length absorbance fluorescence detector was set at 274 nm excitation and 440 nm emission. The analytical column was RP C-18 (250x4 mm), (150x4.6 mm) (SS Wakosi/II 5) with 5 µm particle size (Merck/ Thermo scientific). Analysis of ZEA was done by using modified method of Scudamore and Patel (2000). The mobile phase consisted of ACN: water (55:45) (V/V) and was used at a flow rate of 0.5 ml/min. Injection volume was 20 µl. Standard ZEA concentration used was 10 ppm. 20 µl of crude extracted toxin samples were injected. Also few samples were spiked with 2 ppm standard for peak confirmation. Analysis was performed at room temperature (25-30°C) and quantification of ZEA was done by comparison of the retention time and peak area observed in the standard ZEA with those observed in samples.

5.2.3.3 LC-MS/MS (Liquid chromatography-Mass spectroscopy):

LC-MS analysis was outsourced (Performed at Sophisticated instrumentation centre for applied research and testing (SICART), Vallabh vidhyanagar, Anand, Gujarat). LC-MS was performed on an LCQ fleet ion trap mass spectrometer (Thermo scientific, USA) equipped with an electrospray ionization (ESI) source. Standard ZEA and crude extracted toxin samples were analysed in single reaction monitoring (SRM) mode. ACN was used as mobile

phase at flow rate of 5 µl/min. Sample was injected with syringe pump. Injection volume was 10 µl.

5.3 Results:

5.3.1 Characterization of Mycotoxins:

Detection of mycotoxins; Zearalenone and T2 toxin were focused in present study. The standard toxins were used to detect the presence of ZEA and T2 toxins in crude extracted toxins from fungal isolates.

5.3.1.1 Thin layer chromatography:

The presence of ZEA was detected as fluorescent blue spot in long UV light. For T2 toxin estimation TLC plate was sprayed with 20 % H_2SO_4 and heated at 80˚C for 10 minutes. T2 toxin was observed as brown spot in white light.

a. Zearalenone:

Figure 5.1 shows the extracted toxin from FSSC Cc240 on SDB and PDB medium from day 10 to 20. The fluorescent blue spot on TLC (Marked in red box) was tentatively characterised as zearalenone. The production fluorescent blue spot was increased from 12th day and was consistent till 20th day in SDB medium but the production was consistent throughout from 10th day to 20th day in PDB medium. Figure 5.2 shows the extracted toxin from *F. sacchari* Cc215 on SDB and PDB medium from day 10 to 20. The production of fluorescent blue spot was less at 10th, 12th and 14th day but increased at 16th day and was maximum on 16th day. At day 18th the production was decreased and was same at 20th day in PDB medium but no production was seen in SDB medium. The extraction of toxin was carried out on 20th day from all *Fusarium* isolates. The fungal growth for toxin production was carried out in four different media for all fungal isolates. The TLC was carried out with standard ZEA toxin for detection of ZEA from *Fusarium* isolates. Some of the isolates produce ZEA toxin. Production of ZEA was varying depending upon the medium (PDB, SDB, YMB, rice and corn media) and isolates.

Figure 5.3 shows the TLC plate of crude extracted toxins from FSSC (Cc50 and Cc240), *F. sacchari* (Cc52, Cc61, Cc215 andCc167) and *F. incarnatum-equiseti* Cc172 grown in corn and rice medium. Figure 5.4 and Figure 5.5 shows the TLC plate of crude extracted toxins from FSSC CSH1, CSH2,

CSH3, CSH4, CSH5, CSH6, CSH7, CSH8, CSH9, CSH10, CSH11, Cs1 and Cs2 grown in corn and rice medium. Figure 5.6 and Figure 5.7 shows the TLC plate of crude extracted toxins from FSSC (Cc240 and Cc50) and *F. sacchari* (Cc52, Cc215, Cc61 and Cc167) and *F. incarnatum-equiseti* Cc172 grown in YMB, PDB and SDB medium. None of the isolates grown in YMB gave band similar to ZEA. Hence, other isolates were not further grown in YMB medium. Figure 5.8, Figure 5.9 and Figure 5.10 shows the TLC plate of crude extracted toxins from FSSC and FDSC isolates, CSH1, CSH2, CSH3, CSH4, CSH5, CSH6, CSH7, CSH8, CSH9, CSH10, CSH11, Cs1 and Cs2 grown in PDB and SDB medium. Table 5.1 shows summarized table for presence and absence of ZEA toxin in all *Fusarium* isolates based on TLC. The isolates which were positive for ZEA, were further tested for presence of ZEA by HPLC.

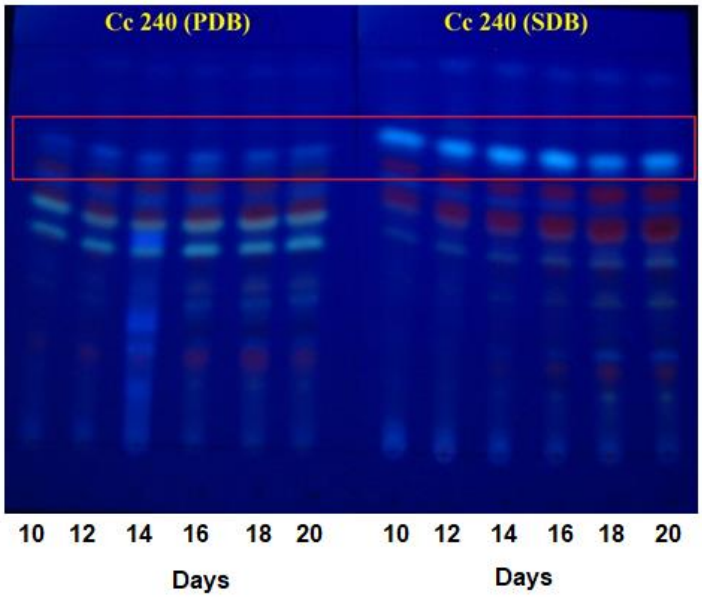

Figure 5.1: TLC plate of crude toxin extracted from FSSC Cc240 from day 10 to day 20 in PDB and SDB medium. The fluorescent blue spots marked in red box were tentatively characterised as ZEA.

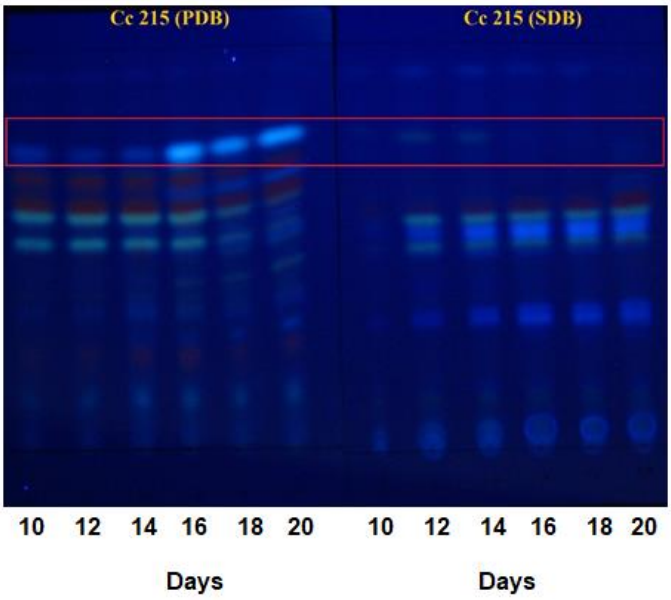

Figure 5.2: TLC plate of crude toxin extracted from *F. sacchari* Cc215 grown in PDB and SDB medium from day 10 to day 20. The fluorescent blue spots marked in red box were tentatively characterised as ZEA.

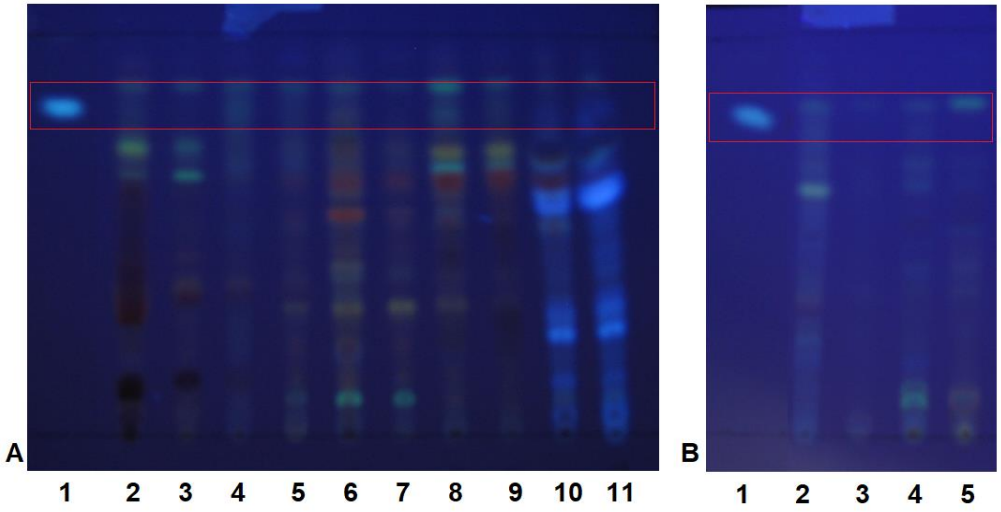

Figure 5.3: TLC plate of standard ZEA and crude extracted toxin from FSSC Cc50 and Cc240, *F. sacchari* Cc52, Cc61, Cc167 and Cc215, and *F. incarnatum-equiseti* Cc172. (A) Lane 1: Std. ZEA, lane 2: Cc52 (corn), lane 3: Cc52 (rice), lane 4: Cc61 (corn), lane 5: Cc61 (rice), lane 6: Cc50 (corn), lane 7: Cc50 (rice), lane 8: Cc240 (corn), lane 9: Cc240 (rice), lane 10: Cc215 (corn) and lane 11: Cc215 (rice), and **(B)** Lane 1: Std. ZEA, lane 2: Cc167 (corn), lane 3: Cc167 (rice), lane 4: Cc172 (corn) and lane 5: Cc172 (rice). Brackets represents media in which isolate was grown.

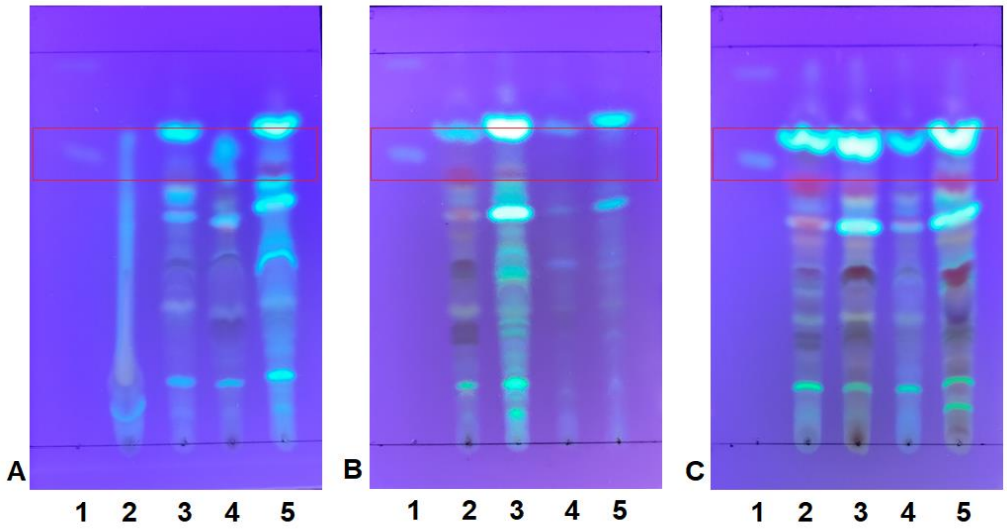

Figure 5.4: TLC plate of standard ZEA and crude extracted toxin from FSSC CSH1, CSH2, CSH3, CSH5 and CSH6, and FDSC CSH4. (A) Lane 1: Std. ZEA, lane 2: CSH1 (corn), lane 3: CSH1 (rice), lane 4: CSH2 (corn) and lane 5: CSH2 (rice), **(B)** Lane 1: Std. ZEA, lane 2: CSH3 (corn), lane 3: CSH3 (rice), lane 4: CSH4 (corn) and lane 5: CSH4 (rice), and **(C)** Lane 1: Std. ZEA, lane 2: CSH5 (corn), lane 3: CSH5 (rice), lane 4: CSH6 (corn) and lane 5: CSH6 (rice). Brackets represents media in which isolate was grown.

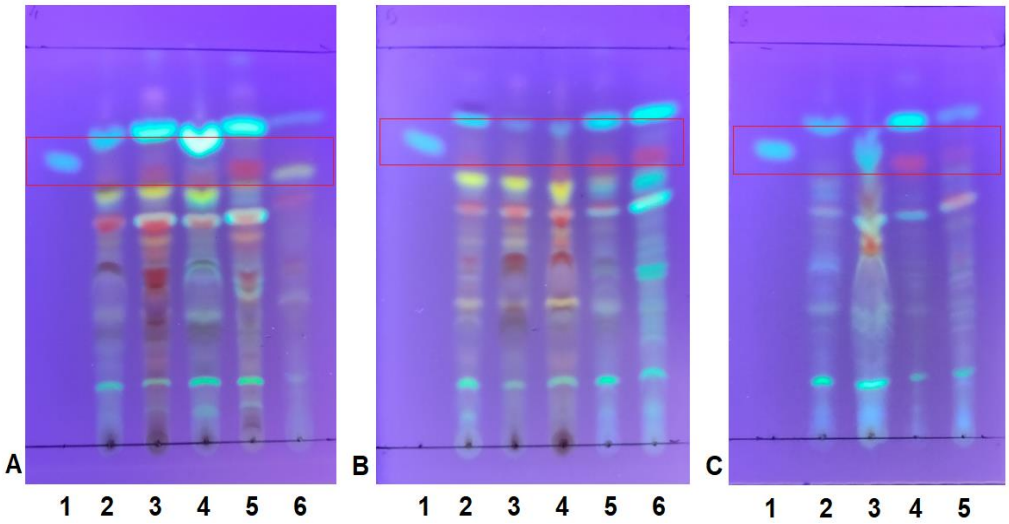

Figure 5.5: TLC plate of standard ZEA and crude extracted toxin from FSSC CSH7, CSH8, CSH9, CSH10, CSH11, Cs1 and Cs2. (A) Lane 1: Std. ZEA, lane 2: CSH7 (corn), lane 3: CSH7 (rice), lane 4: CSH8 (corn), lane 5: CSH8 (rice) and lane 6: CSH9 (corn), **(B)** Lane 1: Std. ZEA, lane 2: CSH9 (rice), lane 3: CSH10 (corn), lane 4: CSH10 (rice), lane 5: CSH11 (corn) and lane 6: CSH11 (rice), and **(C)** Lane 1: Std. ZEA, lane 2: Cs1 (corn), lane 3: Cs1 (rice), lane 4: Cs2 (corn) and lane 5: Cs2 (rice). Brackets represents media in which isolate was grown.

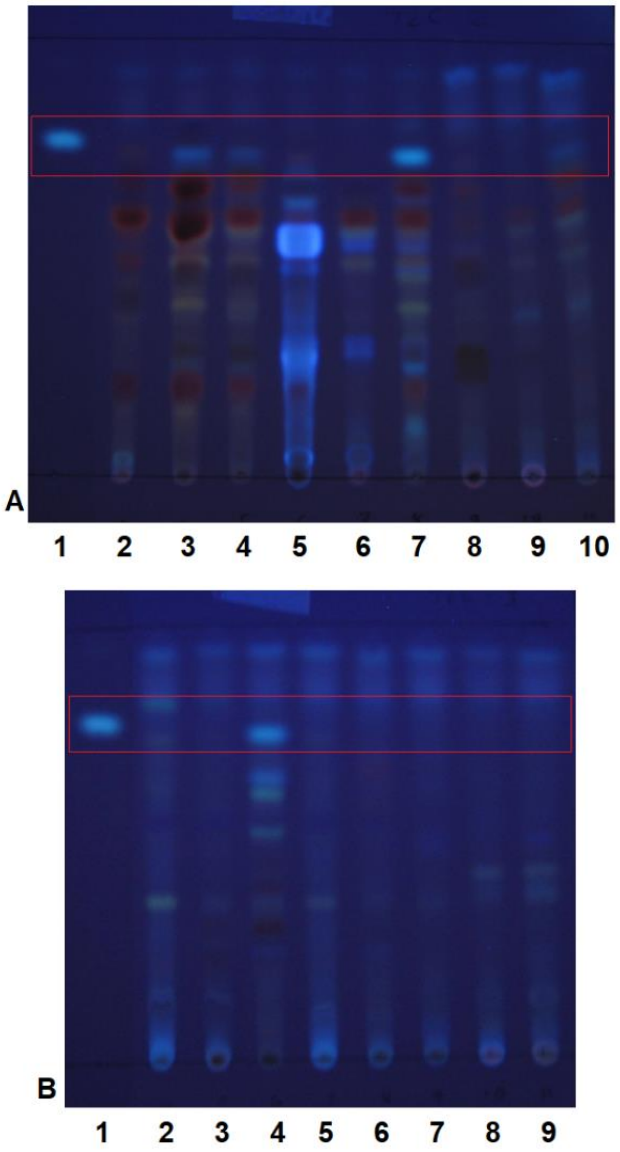

Figure 5.6: TLC plate of standard ZEA and crude extracted toxin from FSSC Cc50 and Cc240, *F. sacchari* Cc61, Cc167 and Cc215, and *F. incarnatum-equiseti* Cc172. **(A)** Lane 1: Std. ZEA, lane 2: Cc240 (YMB), lane 3: Cc240 (SDB), lane 4: Cc240 (PBD), lane 5: Cc215 (YMB), lane 6: CC215 (SDB), lane 7: Cc215 (PDB), lane 8: Cc50 (YMB), lane 9: Cc50 (SDB) and lane 10: Cc50 (PDB) and **(B)** Lane 1: Std. ZEA, lane 2: Cc61 (YMB), lane 3: Cc61 (SDB), lane 4: Cc61 (PBD), lane 5: Cc167 (YMB), lane 6: CC167 (SDB), lane 7: Cc167 (PDB), lane 8: Cc172 (SDB) and lane 9: Cc172 (PDB). Brackets represents media in which isolate was grown.

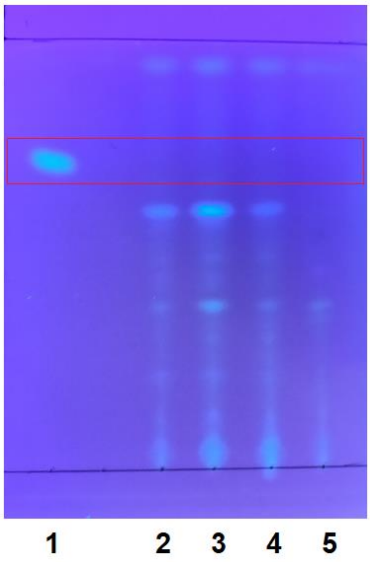

Figure 5.7: TLC plate of standard ZEA and crude extracted toxin from *F. sacchari* Cc52 and *F. incarnatum-equiseti* Cc172. Lane 1: Std. ZEA, lane 2: Cc52 (YMB), lane 3: Cc52 (SDB) lane 4: Cc52 (PBD) and lane 5: Cc172 (YMB). Brackets represents media in which isolate was grown.

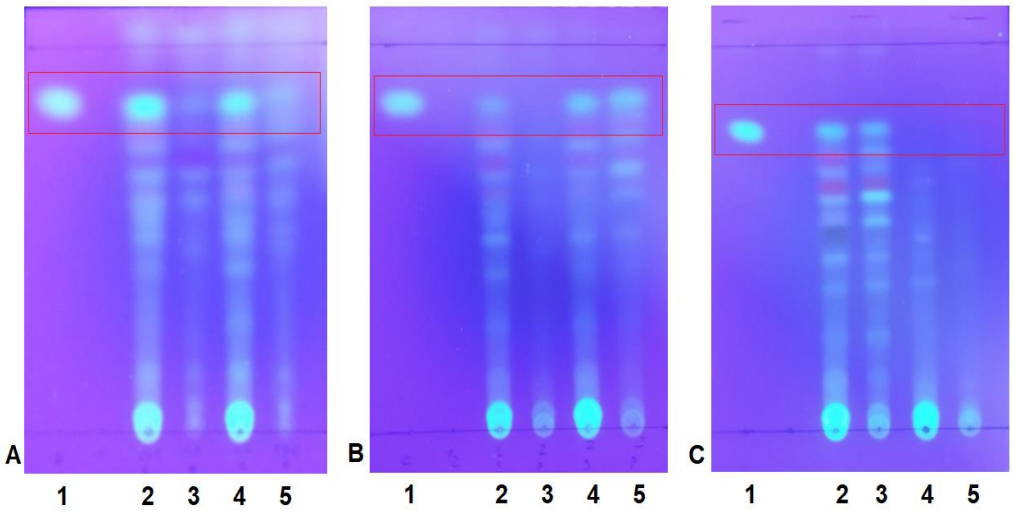

Figure 5.8: TLC plate of standard ZEA and crude extracted toxin from FSSC Cs1, Cs2, CSH1, CSH2 and CSH3, and FDSC CSH4. (A) Lane 1: Std. ZEA, Lane 2: Cs1 (SDB), lane 3: Cs1 (PDB), lane 4: Cs2 (SDB) and lane 5: Cs2 (SDB), **(B)** Lane 1: Std. ZEA, lane 2: CSH1 (SDB), lane 3: CSH1 (PDB), lane 4: CSH2 (SDB) and lane 5: CSH2 (PDB), and **(C)** Lane 1: Std. ZEA, lane 2: CSH3 (SDB), lane 3: CSH3 (PDB), lane 4: CSH4 (SDB) and lane 5: CSH4 (PDB). Brackets represents media in which isolate was grown.

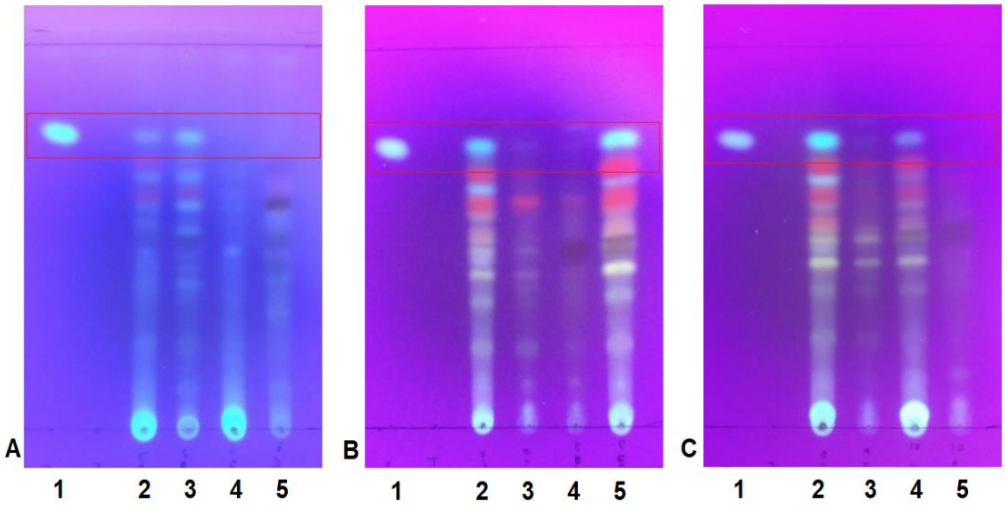

Figure 5.9: TLC plate of standard ZEA and crude extracted toxin from FSSC CSH5, CSH6, CSH7, CSH8, CSH9 and CSH10. (A) Lane 1: Std. ZEA, Lane 2: CSH5 (SDB), lane 3: CSH5 (PDB), lane 4: CSH6 (SDB) and lane 5: CSH6 (PDB), **(B)** Lane 1: Std. ZEA, lane 2: CSH7 (SDB), lane 3: CSH7 (PDB), lane 4: CSH8 (SDB) and lane 5: CSH8 (PDB), and **(C)** Lane 1: Std. ZEA, Lane 2: CSH9 (SDB), lane 3: CSH9 (PDB), lane 4: CSH10 (SDB) and lane 5: CSH10 (PDB). Brackets represents media in which isolate was grown.

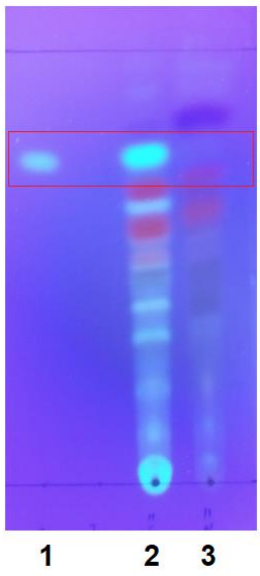

Figure 5.10: TLC plate of standard ZEA and crude extracted toxin from FSSC CSH11. Lane 1: Std. ZEA, Lane 2: CSH11 (SDB) and lane 3: CSH11 (PDB). Brackets represents media in which isolate was grown.

b. T2 toxin:

Figure 5.11 shows the TLC plate of crude extracted toxins from FSSC (Cc50 and Cc240), *F. sacchari* (Cc52, Cc61, Cc215 andCc167) and *F. incarnatum-equiseti* Cc172 grown in corn and rice medium. The standard T2 toxin after spraying with 20 % H_2So4 and heating, appears as brown spot in visible light. Figure 5.12 and 5.13 shows the TLC plate of crude extracted toxin from FSSC and FDSC, CSH1, CSH2, CSH3, CSH4, CSH5, CSH6, CSH7, CSH8, CSH9, CSH10, CSH11, Cs1 and Cs2 grown in corn and rice medium. Figure 5.14 and 5.15 shows the TLC plate of crude extracted toxins from FSSC (Cc240 and Cc50) and *F. sacchari* (Cc52, Cc215, Cc61 and Cc167) and *F. incarnatum-equiseti* Cc172 grown in YMB, PDB and SDB medium. Figure 5.16 and Figure 5.17 shows the TLC plate of crude extracted toxins from FSSC and FDSC, CSH1, CSH2, CSH3, CSH4, CSH5, CSH6, CSH7, CSH8, CSH9, CSH10, CSH11, Cs1 and Cs2 grown in PDB and SDB medium. Similar to isolates grown in corn and rice medium. Table 5.1 shows summarized table for presence and absence of T2 toxin in all *Fusarium* isolates based on TLC. The isolates which were positive were further tested for presence of T2 toxin by HPLC. None of the isolates were positive in HPLC.

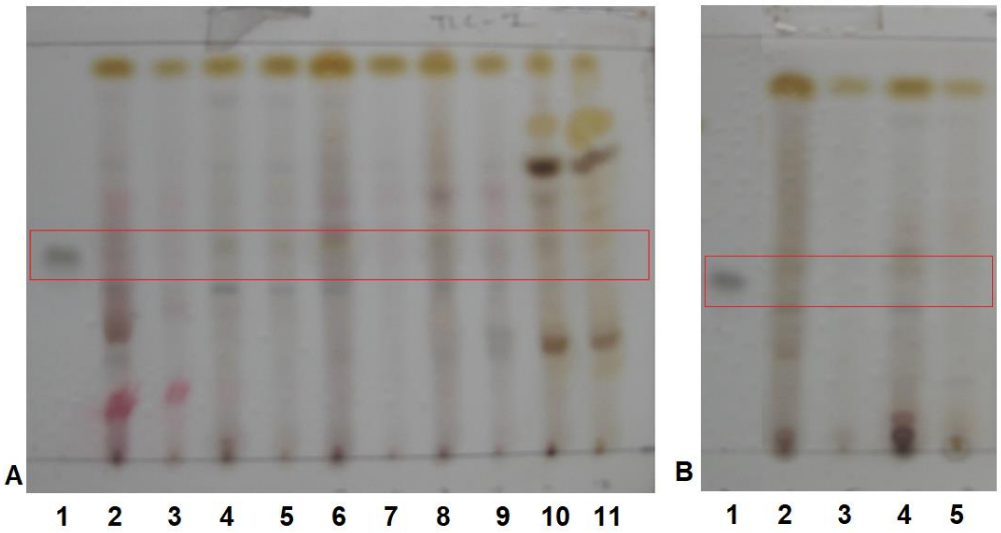

Figure 5.11: TLC plate of standard T2 toxin and crude extracted toxin from FSSC Cc50 and Cc240, *F. sacchari* Cc52, Cc61, Cc167 and Cc215, and *F. incarnatum-equiseti* Cc172. (A) Lane 1: Std. T2 toxin, lane 2: Cc52 (corn), lane 3: Cc52 (rice), lane 4: Cc61 (corn), lane 5: Cc61 (rice), lane 6: Cc50 (corn), lane 7: Cc50 (rice), lane 8: Cc240 (corn), lane 9: Cc240 (rice), lane 10: Cc215 (corn) and lane 11: Cc215 (rice), and **(B)** Lane 1: Std. T2 toxin, lane 2: Cc167 (corn), lane 3: Cc167 (rice), lane 4: Cc172 (corn) and lane 5: Cc172 (rice). Brackets represents media in which isolate was grown.

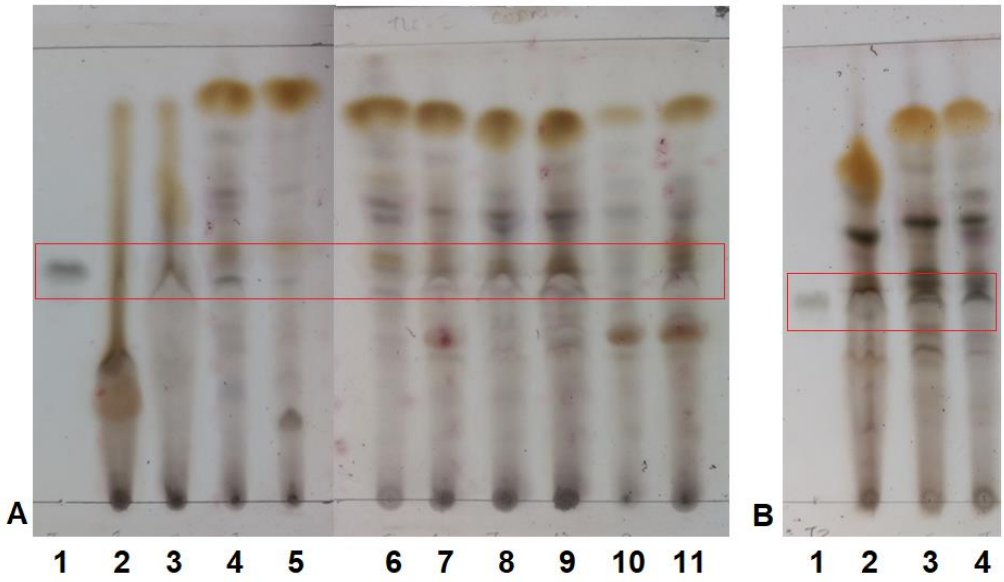

Figure 5.12: TLC plate of standard T2 toxin and crude extracted toxin from FSSC and FDSC isolates grown on corn medium. (A) Lane 1: Std. T2 toxin, lane 2: CSH1, lane 3: CSH2, lane 4: CSH3, lane 5: CSH4, lane 6: CSH5, lane 7: CSH6, lane 8: CSH7, lane 9: CSH8, lane 10: CSH9 and lane 11: CSH10, and **(B)** Lane 1: Std. T2 toxin, lane 2: CSH11, lane 3: Cs1 and lane 4: Cs2.

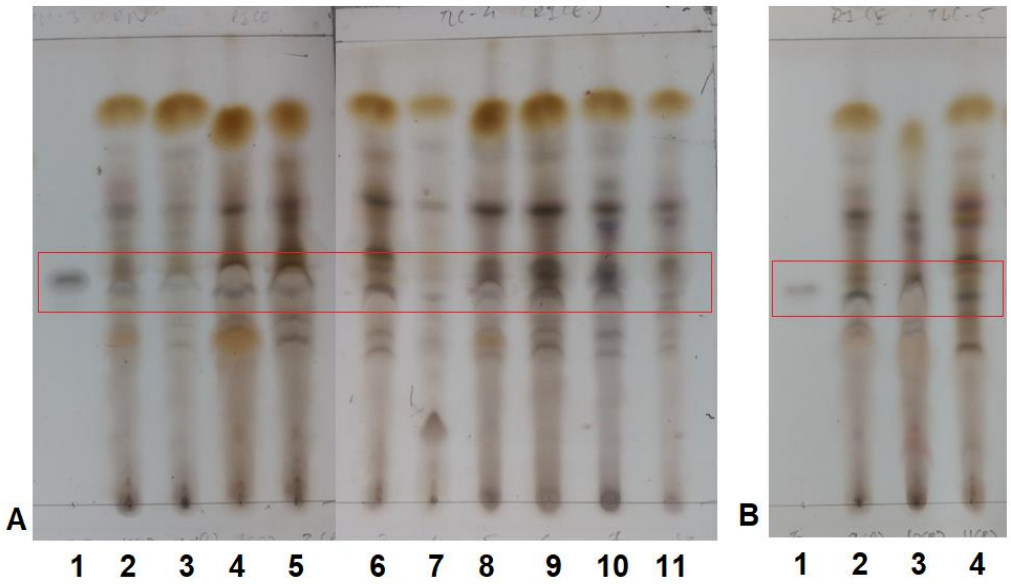

Figure 5.13: TLC plate of standard T2 toxin and crude extracted toxin from FSSC and FDSC isolates grown on rice medium. (A) Lane 1: Std. T2 toxin, lane 2: CSH1, lane 3: CSH2, lane 4: CSH3, lane 5: CSH4, lane 6: CSH5, lane 7: CSH6, lane 8: CSH7, lane 9: CSH8, lane 10: CSH9 and lane 11: CSH10, and **(B)** Lane 1: Std. T2 toxin, lane 2: CSH11, lane 3: Cs1 and lane 4: Cs2.

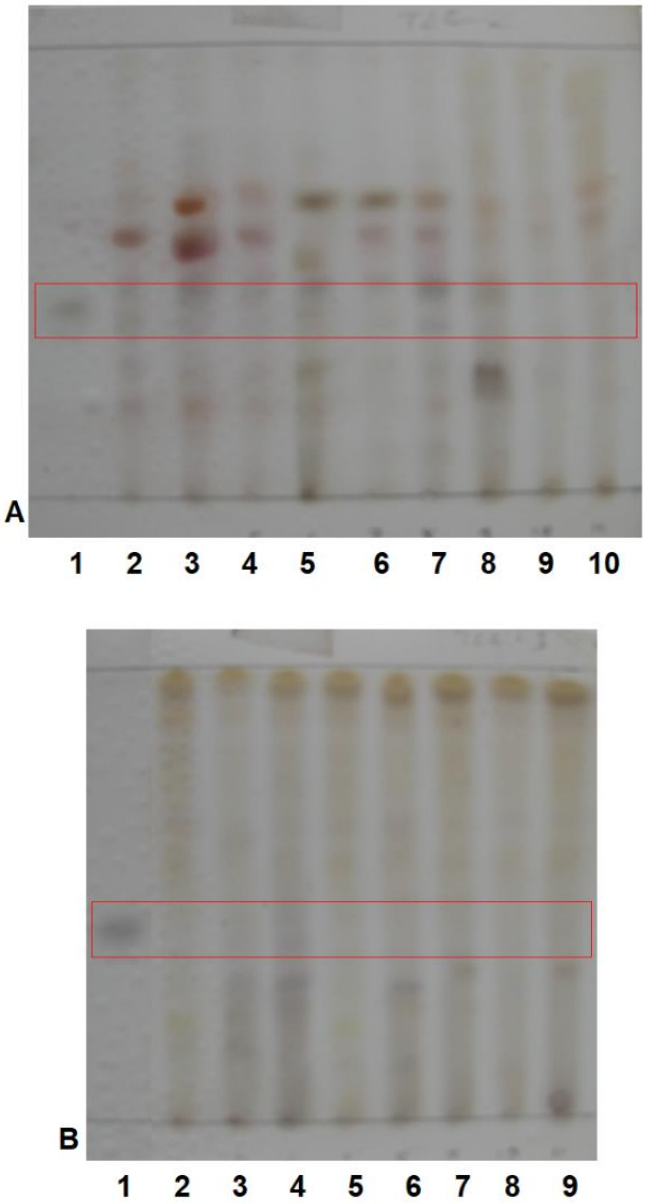

Figure 5.14: TLC plate of standard T2 toxin and crude extracted toxin from FSSC Cc50 and Cc240, *F. sacchari* Cc61, Cc167 and Cc215, and *F. incarnatum-equiseti* Cc172. **(A)** Lane 1: Std. T2 toxin, lane 2: Cc240 (YMB), lane 3: Cc240 (SDB), lane 4: Cc240 (PBD), lane 5: Cc215 (YMB), lane 6: CC215 (SDB), lane 7: Cc215 (PDB), lane 8: Cc50 (YMB), lane 9: Cc50 (SDB) and lane 10: Cc50 (PDB), and **(B)** Lane 1: Std. T2 toxin, lane 2: Cc61 (YMB), lane 3: Cc61 (SDB), lane 4: Cc61 (PBD), lane 5: Cc167 (YMB), lane 6: CC167 (SDB), lane 7: Cc167 (PDB), lane 8: Cc172 (SDB) and lane 9: Cc172 (PDB). Brackets represents media in which isolate was grown.

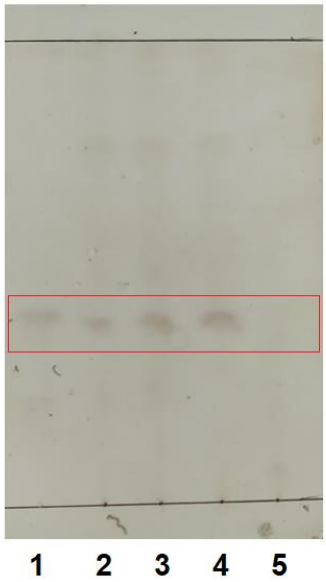

Figure 5.15: TLC plate of standard T2 toxin and crude extracted toxin from *F. sacchari* Cc52 and *F. incarnatum-equiseti* Cc172. Lane 1: Std. T2 toxin, lane 2: Cc52 (YMB), lane 3: Cc52 (SDB) lane 4: Cc52 (PBD) and lane 5: Cc172 (YMB). Brackets represents media in which isolate was grown.

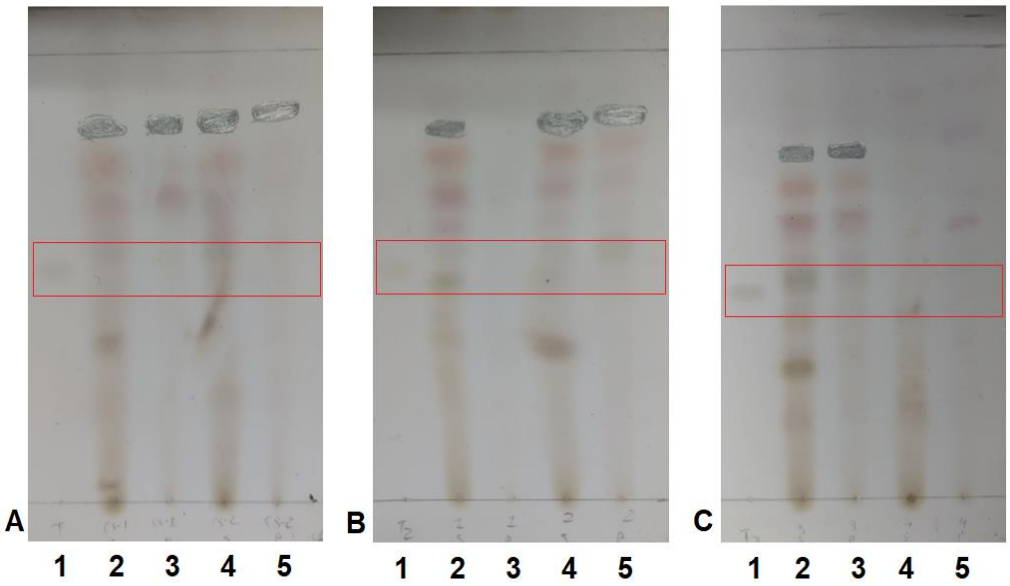

Figure 5.16: TLC plate of standard T2 toxin and crude extracted toxin from FSSC Cs1, Cs2, CSH1, CSH2 and CSH3, and FDSC CSH4. (A) Lane 1: Std. T2 toxin, Lane 2: Cs1 (SDB), lane 3: Cs1 (PDB), lane 4: Cs2 (SDB) and lane 5: Cs2 (SDB), **(B)** Lane 1: Std. T2 toxin, lane 2: CSH1 (SDB), lane 3: CSH1 (PDB), lane 4: CSH2 (SDB) and lane 5: CSH2 (PDB), and **(C)** Lane 1: Std. T2 toxin, lane 2: CSH3 (SDB), lane 3: CSH3 (PDB), lane 4: CSH4 (SDB) and lane 5: CSH4 (PDB). Brackets represents media in which isolate was grown.

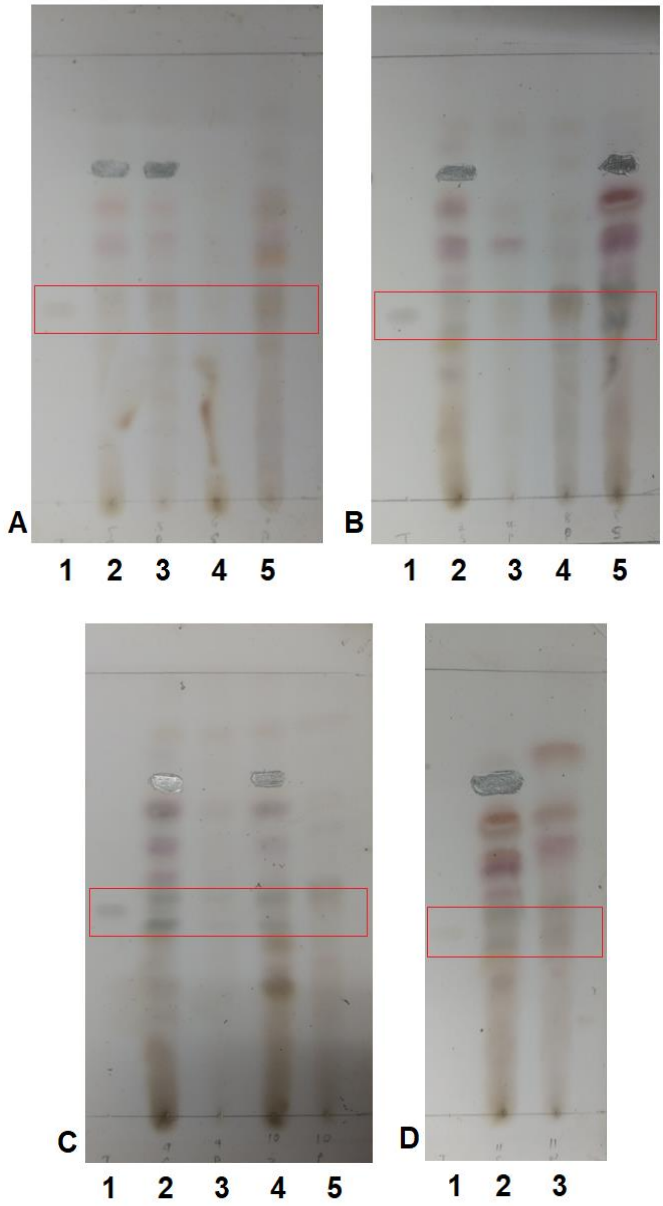

Figure 5.17: TLC plate of standard T2 toxin and crude extracted toxin from FSSC CSH5, CSH6, CSH7, CSH8, CSH9, CSH10 and CSH11. (A) Lane 1: Std. T2 toxin, Lane 2: CSH5 (SDB), lane 3: CSH5 (PDB), lane 4: CSH6 (SDB) and lane 5: CSH6 (PDB), **(B)** Lane 1: Std. T2 toxin, lane 2: CSH7 (SDB), lane 3: CSH7 (PDB), lane 4: CSH8 (SDB) and lane 5: CSH8 (PDB), **(C)** Lane 1: Std. T2 toxin, Lane 2: CSH9 (SDB), lane 3: CSH9 (PDB), lane 4: CSH10 (SDB) and lane 5: CSH10 (PDB), and **(D)** Lane 1: Std. T2 toxin, Lane 2: CSH11 (SDB) and lane 3: CSH11 (PDB). Brackets represents media in which isolate was grown.

Table 5.1: Summarised table for presence and absence of ZEA and T2 toxin in *Fusarium* isolates based on TLC.

Strain	T2 TOXIN					ZEARALENONE				
	PDB	SDB	YMB	Corn	Rice	PDB	SDB	YMB	Corn	Rice
FSSC isolates										
CSH1	-	+	NA	-	+	+	-	NA	-	-
CSH2	-	-	NA	-	+	+	+	NA	-	-
CSH3	+	+	NA	+	+	+	+	NA	-	-
CSH5	+	+	NA	+	+	+	+	NA	-	-
CSH6	+	-	NA	+	+	-	-	NA	-	-
CSH7	+	-	NA	+	+	-	+	NA	-	-
CSH8	+	+	NA	+	+	+	-	NA	-	-
CSH9	-	+	NA	+	+	-	+	NA	-	-
CSH10	-	+	NA	-	+	-	+	NA	-	-
CSH11	+	+	NA	+	+	-	+	NA	-	-
Cs1	-	-	NA	+	+	+	+	NA	-	-
Cs2	-	-	NA	+	+	+	+	NA	-	-
Cc 50	-	-	-	+	-	-	-	-	-	-
Cc 240	-	-	-	+	-	+	+	-	+	-
FDSC isolates										
Cc 26	-	-	-	-	-	-	-	-	-	-
Cc 119	-	-	-	-	-	-	-	-	-	-
CSH4	-	-	NA	+	+	-	-	NA	-	-
***F. sacchari* isolates**										
Cc52	+	+	+	-	-	-	-	-	-	-
Cc 61	+	-	-	+	+	+	-	-	-	-
Cc 167	-	-	-	-	-	-	-	-	-	-
Cc 215	+	-	-	+	+	+	-	-	+	+
***F. incarnatum-equiseti* isolate**										
Cc 172	-	-	-	-	-	-	-	-	-	-

(-): absence, (+): presence and (NA): not attempted

5.3.1.2 High performance liquid chromatography (HPLC):

The isolates which gave fluorescent blue spot similar to ZEA on TLC were further tested for ZEA presence using HPLC. HPLC was performed with two different C18 RP columns and in both the columns standardization of solvent system was carried out. Columns had diameters of 250x4mm and 150x4.6mm. Hence, the retention time of standard ZEA was different in both column. C18 RP Column with diameter 250x4mm was used for crude toxin extracted from Cc26, Cc119, Cc50, Cc240, Cc52, Cc61, Cc167, Cc215 and Cc172. C18 RP Column with diameter 150x4.6mm was used for crude toxin extracted from Cs1, Cs2 and CSH1 to CSH11.

Figure 5.18 shows the HPLC chromatogram of standard ZEA (10ppm) using column of diameter 250x4mm. Run was carried out for 20 minutes. The retention time was 14.9 minute. Figure 5.19 shows the HPLC chromatogram of toxin extracted from Cc61 PDB. The peak at r.t. 14.9 minutes was not found but peak at r.t. 13.6 minutes was found which is nearest peak to r.t. 14.9. To confirm that this peak was of ZEA, the same sample was spiked with 2 ppm standard ZEA, the height of peak at r.t. 13.6 minutes was increased. This indicated that peak at r.t. 13.6 is of ZEA (Figure 5.20). Figure 5.21, Figure 5.22 and Figure 5.23 shows HPLC chromatogram of Cc215 (PDB), Cc240 (PDB) and Cc240 (SDB). The peak at r.t. 13.58, 13.55 and 13.52 was considered as peak of ZEA in Cc215 (PDB), Cc240 (PDB) and Cc240 (SDB), respectively.

Figure 5.24 shows the HPLC chromatogram of standard ZEA toxin using column of diameter 150x4.6mm. Run was carried out for 16 minutes. The r.t. for standard ZEA was 9.78 minutes. Figure 5.25 shows the HPLC chromatogram of toxin extracted from CSH11 grown in SDB. A very small peak was present at r.t. 10.0 which is nearest to r.t. 9.78 and was assumed to be peak of ZEA. To confirm that this peak was of ZEA, the extracted toxin sample of CSH11 (SDB) was spiked with 2 ppm standard ZEA and HPLC was carried out. The height of the peak at 10.0 was increased which can be seen in Figure 5.26. The peak at this position was considered to be of ZEA. Figure 5.27 and 5.28 shows HPLC chromatogram of CSH5 (PDB) and CSH9 (SDB)

with r.t. of 10.0 and 10.2, respectively for ZEA. From all the samples positive for ZEA on TLC, only Cc61 (PDB), Cc215 (PDB), Cc240 (PDB), Cc240 (SDB), CSH11 (SDB), CSH5 (PDB) and CSH9 (SDB) were able to produce ZEA toxin.

The quantification of toxin was carried out from HPLC. Table 5.2 shows the concentration of ZEA toxin in respective samples after HPLC. ZEA production was seen in both *F. sacchari* and FSSC isolates but not in FDSC and *F. incarnatum-equiseti* isolates. Also, not all the isolates of FSSC and F. sacchari produces ZEA. There is variability in toxin production among isolates of same species and toxin production also varies depending upon medium.

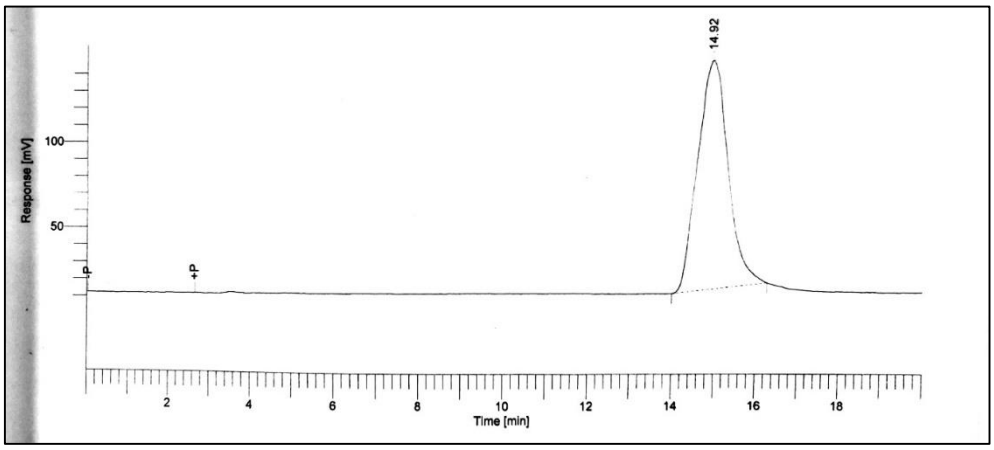

Figure 5.18: HPLC chromatogram of standard ZEA toxin.

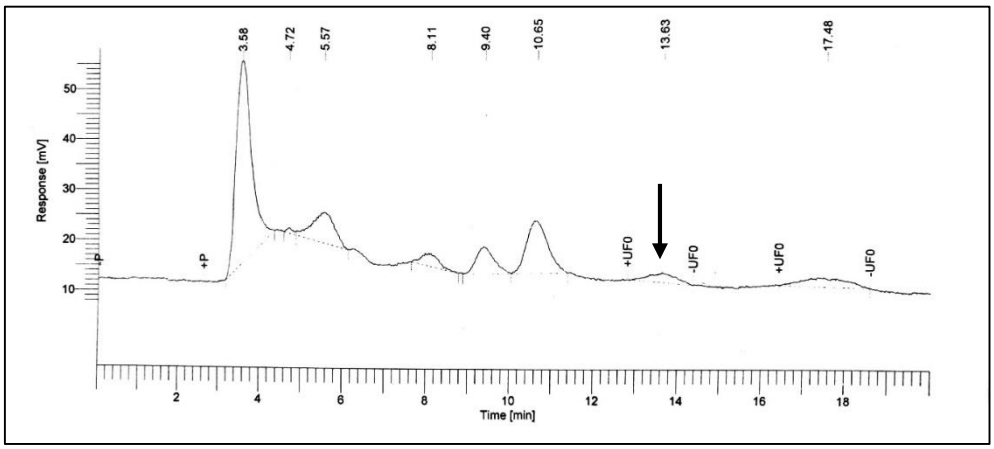

Figure 5.19: HPLC chromatogram of crude toxin extracted from *F. sacchari* Cc61 grown in PDB.

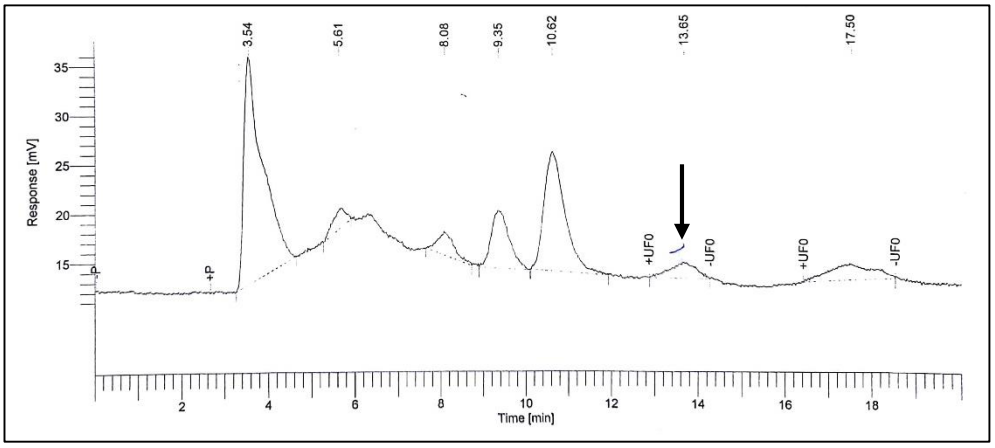

Figure 5.20: HPLC chromatogram of crude toxin extracted from *F. sacchari* Cc61 grown in PDB spiked with 2 ppm std. ZEA toxin.

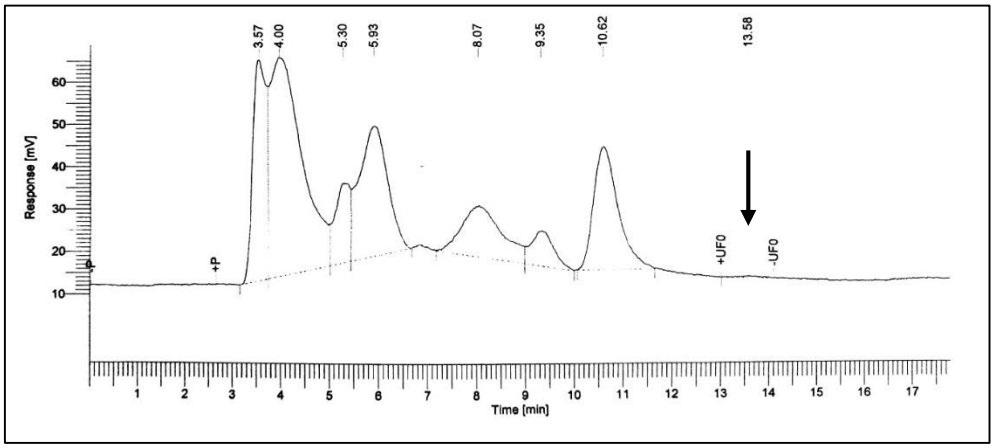

Figure 5.21: HPLC chromatogram of crude toxin extracted from Cc215 grown in SDB.

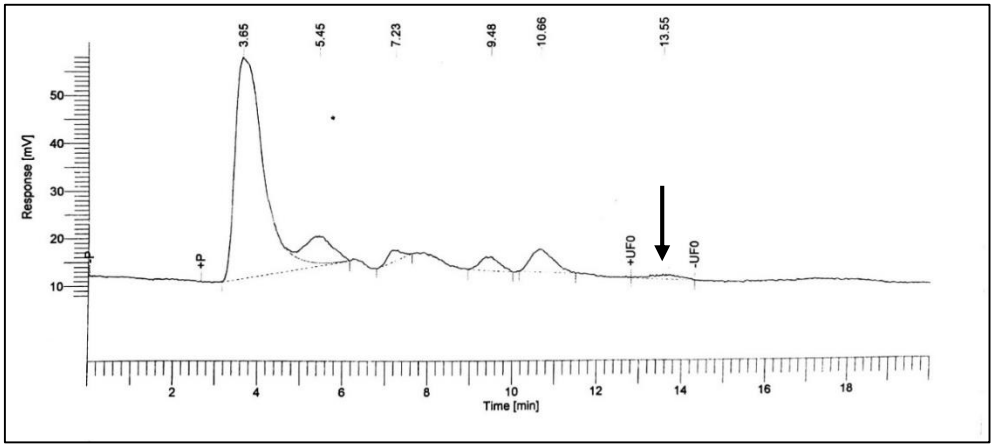

Figure 5.22: HPLC chromatogram of crude toxin extracted from Cc240 grown in PDB.

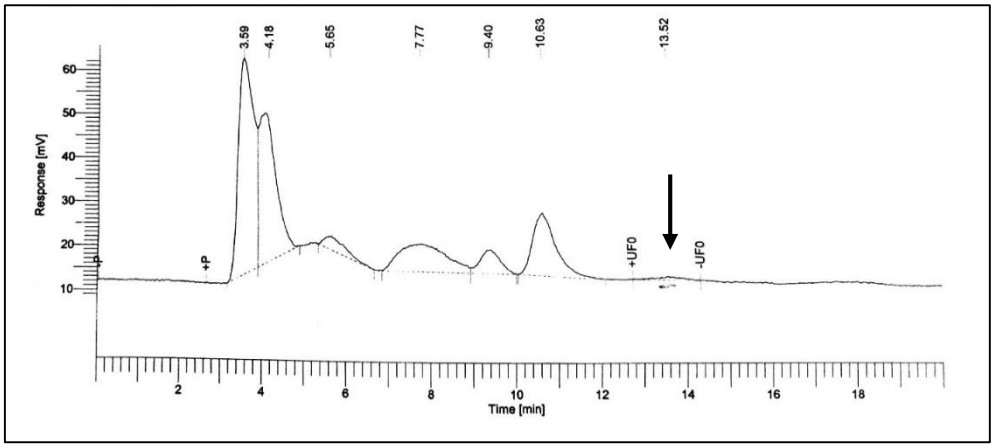

Figure 5.23: HPLC chromatogram of crude toxin extracted from Cc240 grown in SDB.

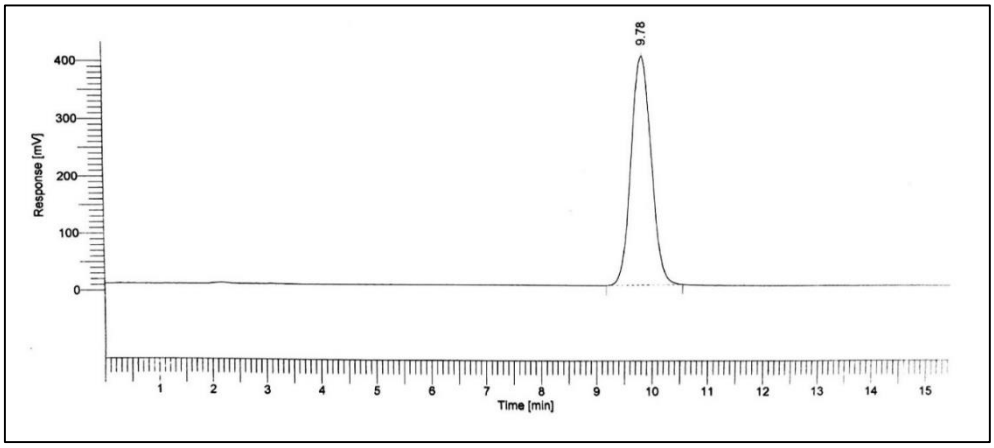

Figure 5.24: HPLC chromatogram of standard ZEA toxin.

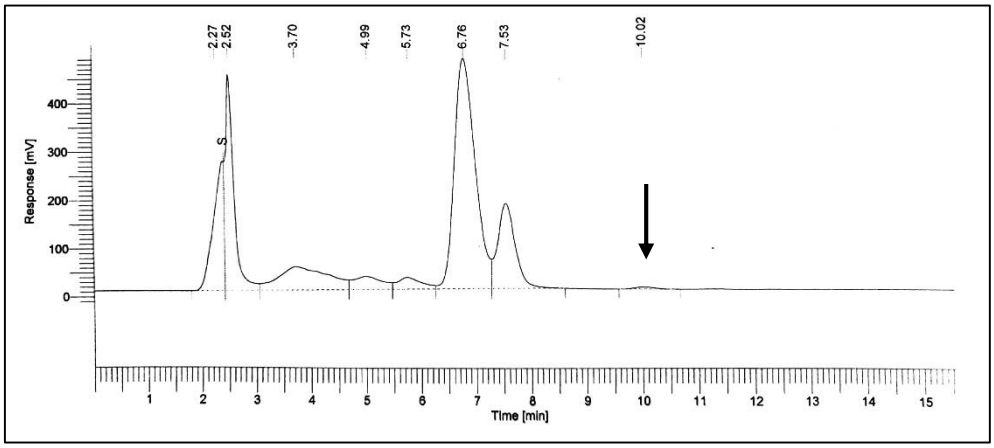

Figure 5.25: HPLC chromatogram of crude toxin extracted from CSH11 grown in SDB.

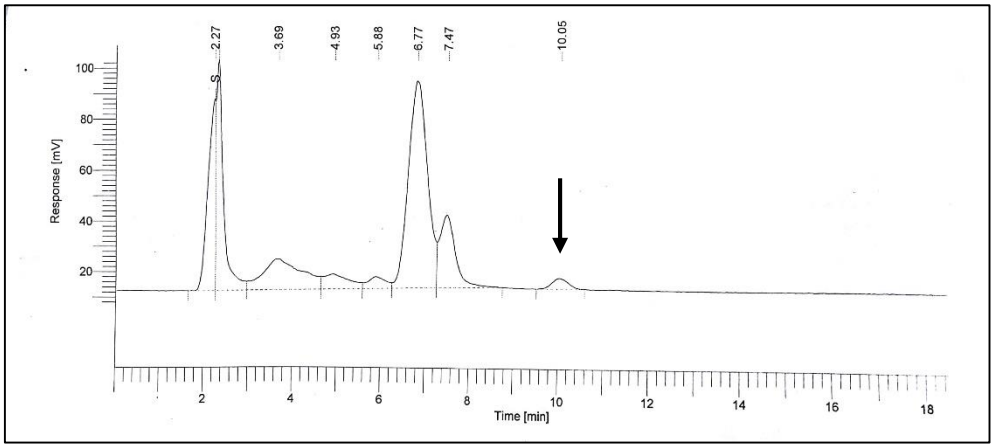

Figure 5.26: HPLC chromatogram of crude toxin extracted from CSH11 grown in SDB spiked with 2 ppm standard ZEA.

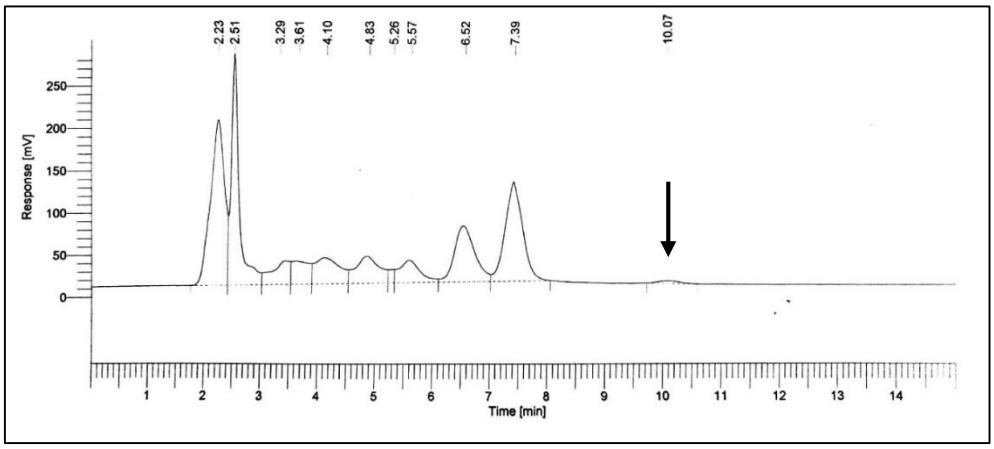

Figure 5.27: HPLC chromatogram of crude toxin extracted from CSH5 grown in PDB.

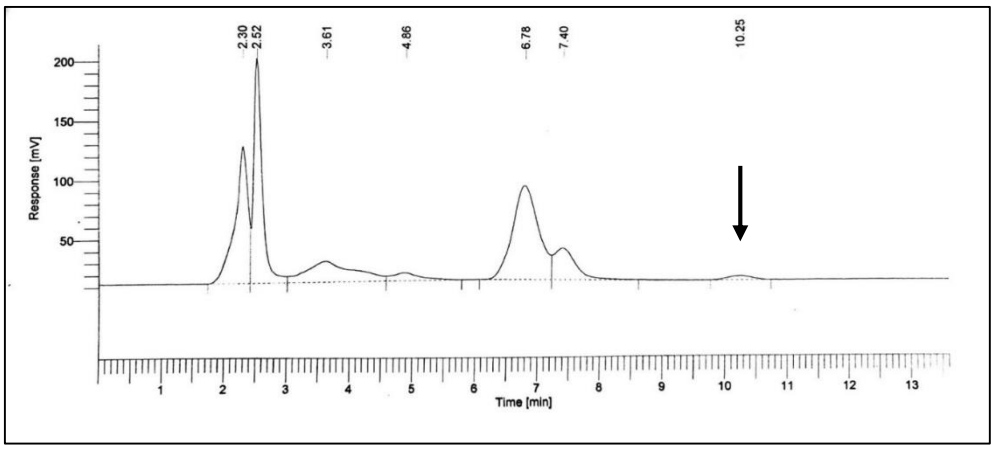

Figure 5.28: HPLC chromatogram of crude toxin extracted from CSH9 grown in SDB.

Table 5.2: Concentration of ZEA toxin in crude toxin extracted from pathogenic isolates.

Sample	Concentration (µg/L)
Cc61 PDB	39.8
Cc215 PDB	2.3
Cc240 PDB	16.3
Cc240 SDB	10.4
CSH5 PDB	5.7
CSH9 SDB	11.8
CSH11 SDB	38

5.3.1.3 LC-MS/MS:

After confirmation of the presence of ZEA in pathogenic *Fusarium* isolates by HPLC further confirmation was done with MS/MS. The standard ZEA gave peak at 317.15 m/z (Figure 5.29) and molecular weight of ZEA is 317. The sample CSH11 gave several peak in MS/MS but peak at 317.19 m/z was present in chromatogram (Figure 5.30) which indicates that the ZEA is present in *Fusarium* isolates.

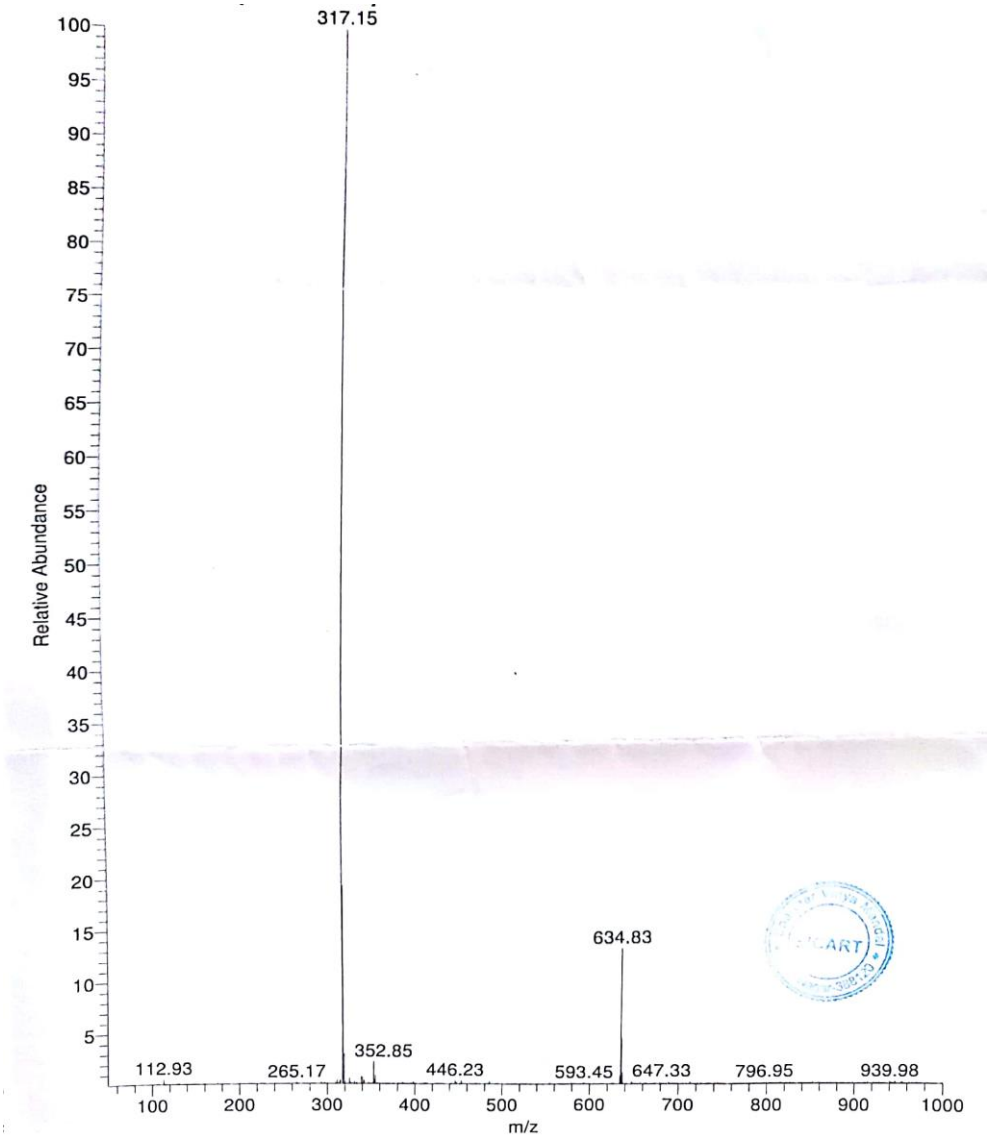

Figure 5.29: Mass spectrum of standard ZEA. The m/z of ZEA was 317.15.

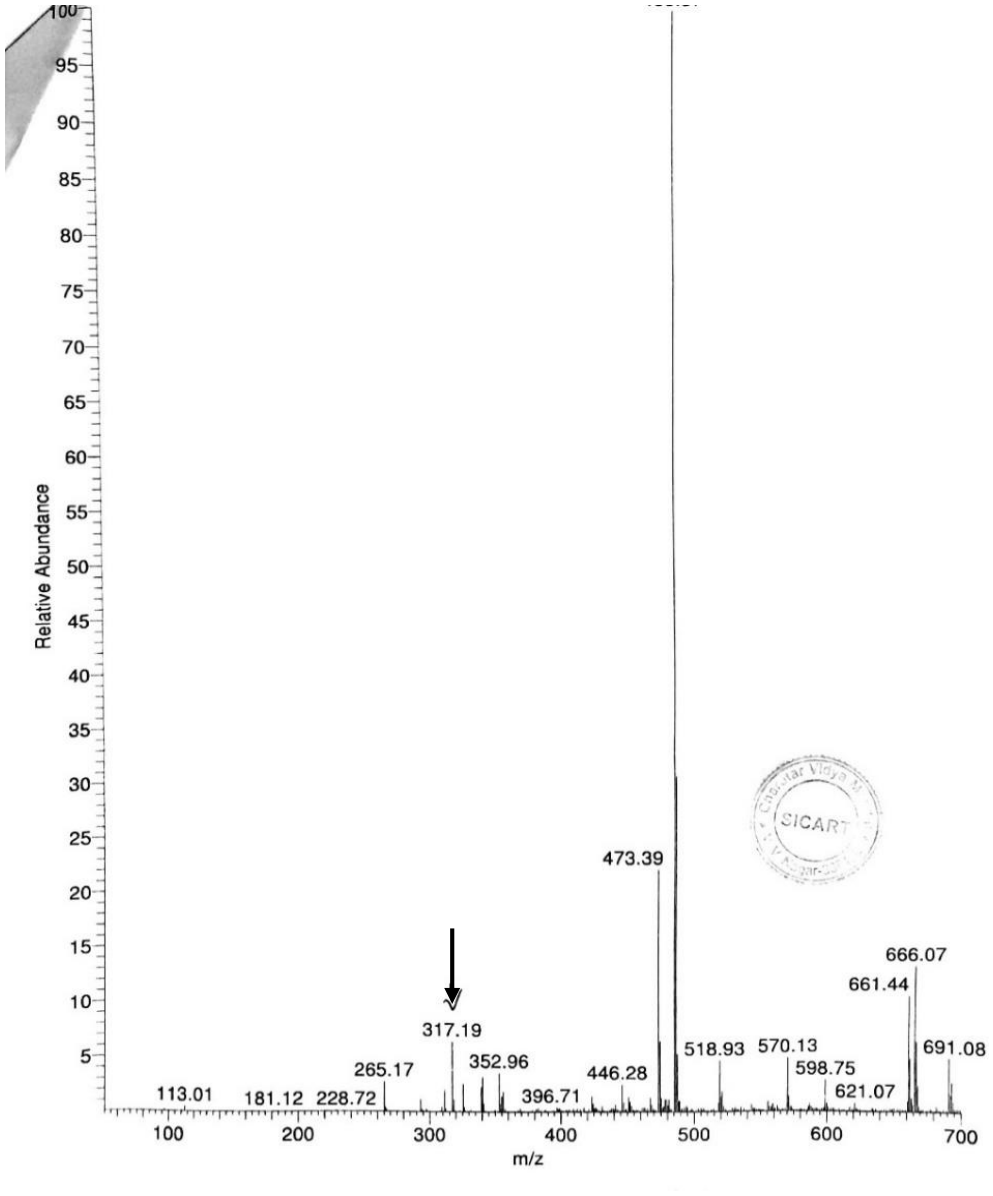

Figure 5.30: Mass spectrum of crude toxin extracted from CSH11 grown in SDB. The m/z of 317.19 was found which indicated presence of ZEA.

5.4 Discussion:

The genus *Fusarium* is known to produce several toxins and toxins that have a major impact on human and animal health are zearalenone, trichothecene and fumonisin. Other toxins produced by *Fusarium* includes, beuvericin, enniatin, fusaproliferin, moliniformin, aurofusarin, culmorin, fusarin and fusaric acid. (Ferrigo *et al*., 2016; Summerell and Leslie, 2011). Most studies on toxin production have measured the amount of toxins produced during plant infections. The toxic effects on mammals occurs only after consumption of infected food stuff. Role of toxin as a virulence factor during active fungal infection in mammals is less explored. Few studies on toxin production in keratitis causing isolates is discussed below. Further, studies on plant pathogenic *Fusarium* and regulation of toxin production are also discussed.

In the current study, TLC, HPLC and MS/MS were used which are well known and widely used methods for toxin detection. The method for toxin detection in food and feed includes primary screening using TLC which is cheap and easy method. In TLC ZEA can be seen as fluorescent blue under UV light (Makun *et al*., 2011; Njobeh *et al*., 2010). For detection of TC, sulphuric acid and paranaisaldehyde reagents are used (Köppen *et al*., 2010; Krska *et al*., 2008). Other high resolution methods includes, HPLC using fluorescence detector (Drzymala *et al*., 2015; Golge and Kabak, 2020; Pajewska *et al*., 2018), HPLC-MS (Asam *et al*., 2017), LC-MS/MS (Habler *et al*., 2017) and GC-MS (Rodríguez-Carrasco *et al*., 2014; Buśko *et al*., 2016; Escrivá *et al*., 2016; Habler *et al*., 2017).

In present study, initial experiments were done to detect fumonicin, fusaric acid, ZEA and T2 however, our isolates were positive for the presence ZEA and T2. Our results show that only 6 isolates (4 of FSSC and 2 of *F. sacchari*) produced ZEA while FDSC isolates did not produce ZEA. Also, T2 was not detected in any of the isolates. Initial studies showed that keratitis causing *Fusarium* isolates produced T2 toxin, DON, DAS and nivalenol (Raza *et al*., 1994). Later, it was reported that 76% of the keratitis isolates produced fumonicin, fusaric acid and moniliformin and among all, *F. dimerum* did not produce any of these toxins, FSSC isolates produced only fusaric acid with

variation in concentration, and *F. verticilliodes* produced all 3 toxins. (Naiker and Odhav, 2004). In another report, mycotoxin production from keratitis causing *Aspergillus* and *Fusarium* showed that *Aspergillus* spp. produced aflatoxins while *Fusarium* spp. produced DAS, ZEA and T2 toxin. The production of ZEA by FSSC isolates was more than 10 mg/ml (Gharamah *et al*., 2014). However, none of these findings show any correlation with disease progression and severity of disease. Among clinical isolates of *F. oxysporum*, *F. moliniforme, F. proliferatum, F. chlamydosporum* and *F. solani*; *F. solani* was the only species which produced cyclosporine A, which acts as an immunosuppressive agent and may influence on pathogenic potential of *F. solani* (Sugiura *et al*., 1999). The same study showed that none of the other *Fusarium* isolates produced trichothecene. Our results corroborate with these findings that production of each toxin is highly variable even within the same species. Moreover, the clinical significance of toxins is still unclear.

Few studies evaluate the genetic variation in the toxin genes in order to understand the disparity in toxin presence. Anutarapongpan and group has reported that among clinical *Fusarium* isolates, fumonisin biosynthetic gene 1 (*FUM1*) is present in majority of clinical isolates but *FUM8*, *Tri13DON*, *Tri13NIV* were not detected in clinical isolates. Further, *FUM1* positive isolates contributed to decreased visual acuity (Anutarapongpan *et al*., 2018). A study on *Aspergillus* toxins showed that Aflatoxin B1 production was higher in clinical isolates compared to environmental isolates and expression of *alfJ*, *nor-1* and *pks-A* was higher in clinical isolates (Leema *et al*., 2011, 2010).

Similar studies on plant pathogenic and environmental *Fusarium* isolates have also showed huge dissimilarity in toxin production. NIV, Don and 15-ADON toxin production from eleven *F. asiaticum* isolates were studied and only 5 isolates produced NIV, other 5 isolates produced DON and 2 isolates produced 15-ADON (Wang *et al*., 2012). Production of DON and ZEA were studied from *F. graminerum, F. avenaceum* and *F. culmorum* and production was not only variable among three species but also in the same species. Similar variation was seen among *Fusarium* spp. as well as intra-species variation was

seen (Pasquali *et al*., 2016). No ZEA was found in *F. graminerum* and *F. culmorum* (Walker *et al*., 2001). Pasquali and group had shown toxin production from *F. graminerum*, *F. poae* and *F. culmorum* on two different media and one of them was supporting the production of ZEA, DON, NIV, Fusaron-X, 3-ADON and 15-ADON. Similarly, variation in fusaric acid and fumonisin production was reported from *Fusarium* spp., grown on corn, rice and PDA media, where production of both toxins varied depending upon media and isolate, and production of fusaric acid was higher in PDB than corn and rice media (Shi *et al*., 2017). Our data comply with the above findings.

Initial reports on different strains of *Fusarium* for ZEA showed that *F. solani* did not produce ZEA toxin (Bilgrami *et al*., 1990; Bosch and Mirocha, 1992; Caldwell *et al*., 1970; Chandra and Sarbhoy, 1997; Ishii *et al*., 1974). Richardson and group was the first to ZEA production in *F. solani* (Richardson *et al*., 1985). Later, Other reports of ZEA production by *F. solani* also published (Abdel-Hafez *et al*., 1986; El-Hassan *et al*., 2007; Najim, 2013). These reports suggests that some strains of *F. solani* are capable of production of ZEA while others are not. Our results also are in accordance with literature.

To understand the variation in toxin production by pathogenic fungi, several genetic studies were conducted to evaluate the role of toxin biosynthesis genes in such variations. Mycotoxin producing genes are arranged in specific location of genome as biosynthetic gene cluster (BGC) (Medema *et al*., 2014). The genes which encodes for non-ribosomal peptide synthetases (NRPS), polyketide synthases (PKS), PKS-NRPS hybrid, prenyltransferase and terpene cyclase along with the genes for proteins which transport metabolites out of cell and genes involved in regulation of gene expression are usually present in BGC (Inglis *et al*., 2013; Lazarus *et al*., 2014). The genes which are involved in ZEA biosynthesis are *PKS* and for TC production several *TRI* genes are reported. The mechanism of toxin production is dependent upon diversity of strain (Pasquali *et al*., 2016).

ZEA is polyketide, synthesised by PKS. It has been reported that *PKS4* and *PKS13* are important for ZEA production in *Fusarium.* Two types of PKS are

present in fungi, reducing and non-reducing PKS (NRPKS). ZEA is synthesised by NRPKS (Kim *et al*., 2005). The mechanism of ZEA biosynthesis is not completely understood but it was found that *PKS4* gene product catalyse initial traction and product is transferred to *PKS13* gene product. Hence, *PKS4* is crucial for ZEA biosynthesis (Gaffoor and Trail, 2006). In one study, ZEA production in *F. graminerum* was correlated with presence of the gene PKS4 using PCR amplification and surprisingly isolates that did not show PKS4 also produced ZEA (Misiewicz *et al*., 2016). Similar studies were reported in *F. equiseti* and authors argue that polymorphism in *PKS4* could be the possible reason for such diversity and variation (Stępień *et al*., 2012). Horizontal gene transfer, gene duplication, gene loss and gene divergence could be responsible for distribution and diversity of PKS (Brown *et al*., 2012; Kroken *et al*., 2003).

More than 200 types of TC derivatives have been isolated and identified (Eriksen and Pettersson, 2004). The variation in TC structures is widely reported which can be because of two reasons, one is variation in assembly in core structure and second is types of different functional groups attached to core structure (Chappell *et al*., 2010). It has reported that loss or gain of *TRI* gene can affect the type of TC produced (Kimura *et al*., 2003; Rep and Kistler, 2010). *Tri13* gene disruption alter NIV-producing chemotypes to DON-producing chemotypes (Lee *et al*., 2002). Similarly, *TRI8* gene product, esterase enzyme exhibit differential activity and produce two different TC (3-ADON and 15-ADON) in *F. graminerum* (Alexander *et al*., 2011). Relocation of *TRI1* gene in telomere region in *Fusarium* is responsible for structure diversification of TC (Rep and Kistler, 2010). It has been reported that diversity in TC structure is dependent not only upon number of TRI gene loci and number of TRI genes but also upon other evolutionary processes. Also the difference in TC production by particular strain is dependent upon host availability which is driving force for diversity of TC production (Proctor *et al*., 2018). None of our *Fusarium* isolates showed the production of T2 toxin, hence it would be interesting to look at the diversity of TRI genes in our isolates.

To summarize, role of toxins in active fungal infections is still unclear. Huge variation in toxin production in *Fusarium* species warrants further study on biosynthesis and regulation of toxin producing genes as well as more investigation of genetic variation among *Fusarium* spp. at both inter-species and intra-species level.