Chapter 6

Identification and characterization of *Fusarium* pigments

6.1 Introduction:

Quinones are small, six member compound with α, β- dienonic ring which are found in plants, fungi, algae and many other microorganisms naturally. They have aromatic carbon skeleton and are divided in to main 4 class, benzoquinones, phenanthrenequinones, naphthoquinones and anthraquinones. Among all quinones, naphthoquinones are most commonly found in nature (Ferreira *et al*., 2018).

Naphthoquinone shows many biological activity, including phytotoxic, insecticidal, antibacterial and fungicidal properties (Albrecht *et al*., 1998; Baker *et al*., 1990). In addition, some of them produce cytotoxic and anticarcinogenic effects (Vogel *et al*., 1975). Isodiospyrin, a 1,2-binaphthoquinone inhibits human topoisomerase I by inhibiting its DNA relaxation activity (Ting *et al*., 2003). Hydroxynaphthoquinone interferes with electron transport chain by acting as ubiquinone antagonist in mammalian mitochondria (Matsuura *et al*., 1983).

Naphthoquinones act as cytotoxic compounds by inhibiting topoisomerase I and II, When enzyme was incubated with beta-lapachone the efficiency of enzyme to bind DNA was drastically reduced (Boothman *et al*., 1989; Coelho-Cerqueira *et al*., 2014; Karkare *et al*., 2013; Krishnan and Bastow, 2000). Similar studies was carried out with 5-amino-8-hydroxy-1,4-naphthoquinone (ANQ) and 5-amino-2,8-dihydroxy-1,4-naphthoquinone (ANQOH) on V79 Chinese hamster lung fibroblast cells using standard Comet assay and DNA damage was observed in all cells (Da Costa Medina *et al*., 2008).

So far more than 100 naphthoquinone metabolites have been elucidated (Medentsev et al., 2005), indicating the structural diversity of this group. The ability to produce naphthoquinones is widespread among fungal organisms, especially among members of the genus *Fusarium*. In the present work, we tried to identify two orange pigments majorly produced by all FSSC isolates.

6.2 Materials and Methods:

6.2.1 Growth of Fungi:

For pigment production, fungal isolates were grown in 4 different types of media which were PDB, SDB, YMB and NB (Himedia, India). 100 ml PDB, SDB, YMB and NB were taken in 500 ml conical flasks and autoclaved. All *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 till 20 days at 30ºC without shaking in dark.

6.2.2 Pigment extraction:

For pigment extraction, 10 ml of culture filtrate was taken in to sterile falcon tube at 2^{nd} day, 4^{th} day till 20^{th} day from fungus grown in all four types of media. Ethyl acetate (Merck, USA) was used to extract pigment. Equal volume of ethyl acetate and media was taken and shacked for 3-5 minutes and allowed to separate phases. The uppermost organic phase of ethyl acetate contained pigment and was taken in to another conical flask. This step was repeated three times. The obtained supernatant was passed from column of sodium sulphite to remove any moisture and poured into glass petri plates to vaporize the solvent in to fume hood. The dried crystals visible on glass petri plates were dissolved in HPLC grade acetonitrile and stored at 4˚C till further use. For large scale pigment extraction, *Fusarium* isolate was grown in 3 litters of media in dark at 30ºC without shacking for 20 days and extraction was carried out using glass separating funnel.

6.2.3 Pigment characterization by TLC:

Pigment characterization was carried out primarily using TLC. 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 pigment characterization. 10 µl of extracted crude pigment was applied to marked spots on TLC plate and TLC plate was placed carefully into

the TLC chambers in vertical position. As soon as the solvent reached to marked top line, TLC plate was removed from TLC chamber and air dried. Plates were observed for different types of pigment in white light, long and short UV light. Characterization was done by heating the plate at 160˚C for 30 minutes to observe change in pigment. The 2 orange bands were consistent which were named fast orange and slow orange. The lower band converted in to purple band which could be anhydrofusarubin.

6.2.4 Pigment purification and characterization using MS/MS:

After primarily characterising the pigment, large scale pigment production and extraction was carried out. For pigment purification, TLC was carried out. TLC plate was cut in to piece of 20x10 cm. and crude extracted pigment was applied on plate. TLC was carried out as mentioned above. The fast orange and slow orange bands were marked and scrapped with help of surgical blade and collected in to small beakers. The pigment was extracted from silica gel using chloroform (Merck, USA), as pigment was soluble in chloroform as well. The extracted pigment was passed through 0.2 µm syringe cellulose acetate filter (Merck, USA) to remove silica gel. The obtained fraction was collected in falcon tube and allowed to vaporise in fume hood. The obtained crude pigment was stored in dark at RT.

6.3 Results:

6.3.1 Pigment Extraction:

Initially pigment extraction was carried out with two different methods (a) using Ethyl acetate extraction method and (b) using Methylene chloride extraction method. From both methods, better results were obtained in ethyl acetate extraction method and hence this method was used later for all the isolates. This extracted pigment was used for pigment characterization.

6.3.2 Pigment characterization using TLC:

Primarily pigment characterization was carried out using TLC. Several methods were used for standardization of solvent system for pigment separation which are mentioned in Table 6.1. Separation was better in solvent system 4 and 6 mentioned in the Table 6.1 but solvent system 7 was used for further experiments. Figure 6.1 shows the representative image of FSSC Cc240 grown

in different media. Visually no pigmentation was seen in NB media. The production of pigment was very less or negligible in YMB as well and hence, pigment extraction from YMB was not carried out further for other *Fusarium* isolates. Also FDSC groups did not produce any pigment in any of the medium. Isolates of FSSC and *F. sacchari* group were able to produce pigment.

Table 6.1: Different solvent systems were used to separate and to characterize the naphthoquinone pigment.

No.	Solvent system	V/V
1	Butanol: Ethyl acetate: Acetic acid : Water	3:2:2:2
2	Isopropyl alcohol: Butanol: Water: NH_4OH	6:2:1.5:0.5
3	Isopropyl alcohol: Ethyl acetate: Acetic acid : Water	4:3.8:0.2:2
4	Toluene: Ethyl acetate: Formic acid	6:3:1
5	Toluene: Ethyl acetate: Formic acid	5:4:2
6	Toluene: Ethyl acetate: Formic acid	7:3:1
7	Toluene: Ethyl acetate: Formic acid	8:1:1
8	Toluene: Ethyl acetate: Formic acid	9:0.5:0.5

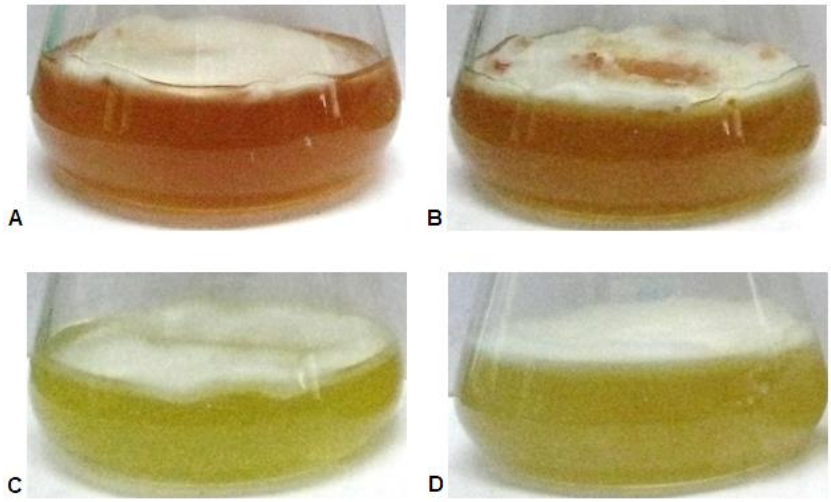

Figure 6.1: Growth of FSSC Cc240 in different media. (A) SDB, **(B)** PDB, **(C)** NB and **(D)** YMB. No pigmentation was seen visually in NB and YMB.

Figure 6.2 and 6.3 shows the TLC plate of the pigment extracted at alternate days from 10th day to 20th day from one representative isolate from FSSC (Cc240) and *F. sacchari* (Cc215) group which were grown in SDB and PDB medium. The maximum pigment production was seen on 20th day in both the media in both the isolates. From other isolates, pigment extraction was carried out on 20th day. In Cc215 more pigment production was seen in PDB medium while in Cc240 more pigment production was seen in SDB which can be seen in Figure 6.1 also. In TLC plate it can be seen that there are two prominent orange bands in crude pigment extracted from both SDB and PDB medium in both the isolates. Tentatively, they were characterised as napthoquinone. The upper and lower bands were named as fast orange (F.O.) and slow orange (S.O.), respectively.

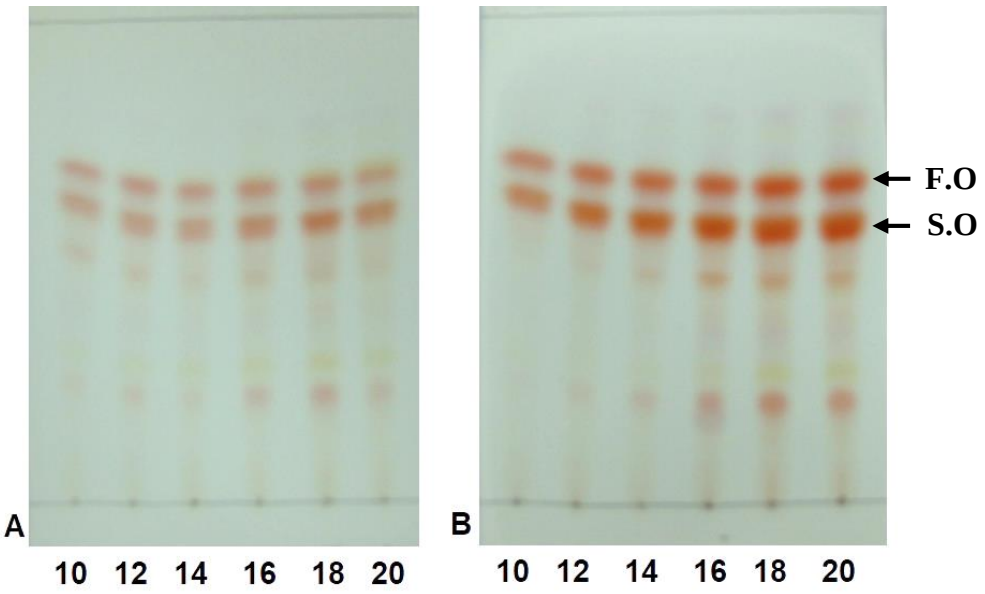

Figure 6.2: TLC plate of extracted pigment from Cc240 from 10th to 20th day. (A) PDB and **(B)** SDB.

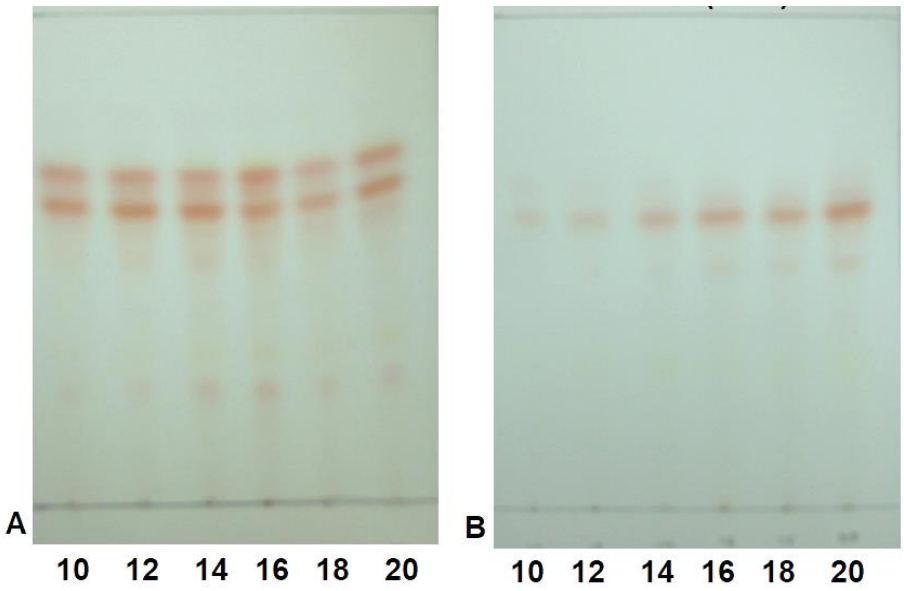

Figure 6.3: TLC plate of extracted pigment from Cc215 from 10th to 20th day. (A) PDB and **(B)** SDB.

Figure 6.4 shows the TLC plates of pigment extracted from FSSC (Cc50), FDSC (Cc26 and Cc119), *F. sacchari* (Cc52, Cc61 and Cc167) and *F. incarnatum-equiseti* Cc172 grown in PDB and SDB medium. Figure 6.5 shows the TLC plates of pigment extracted from FSSC (Cs1, Cs2, CSH1, CSH2, CSH3, CSH5 and CSH6) and FDSC CSH4 grown in PDB and SDB medium. Figure 6.6 shows the TLC plates of pigment extracted from FSSC (CSH7, CSH8, CSH9, CSH10 and CSH11) grown in PDB and SDB medium. All the isolates show pigment except Cc52, Cc26, Cc119, Cc167, Cc172 and CSH4 which does not produce any pigment in PDB and SDB medium. Cc61 showed pigment production but it is not at the position where other isolates gave fast orange and slow orange bands.

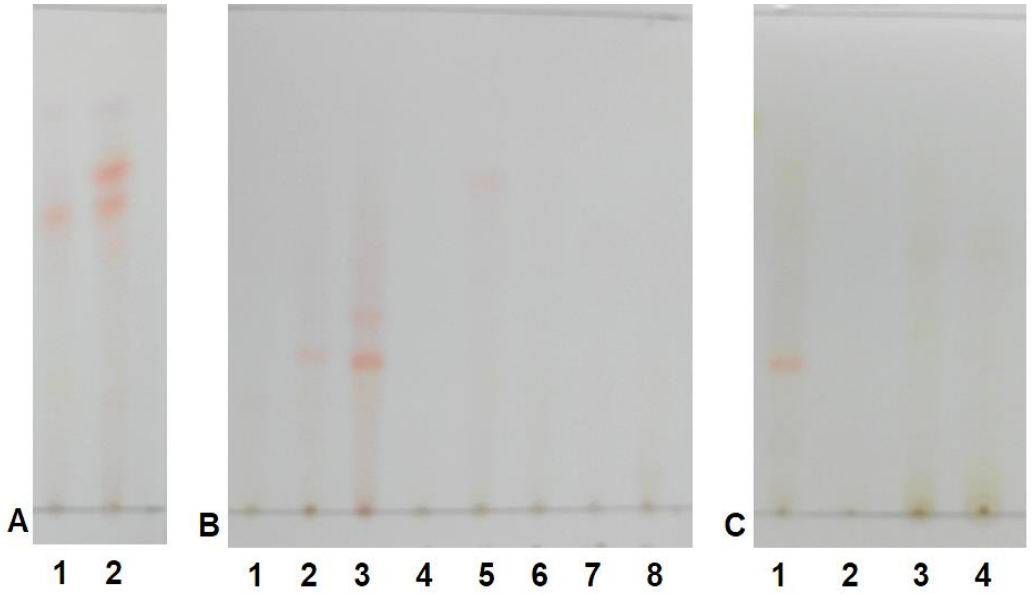

Figure 6.4: TLC plate of extracted pigment from FSSC Cc50, *F. sacchari* Cc52, Cc61 and Cc167, FDSC Cc26 and Cc119, and *F. incarnatum-equiseti* Cc172. (A) Lane 1: Cc50 (SDB), lane 2: Cc50 (PDB), **(B)** Lane 1: Cc52 (PDB), lane 2: Cc61 (PDB), lane 3: Cc61 (SDB), lane 4: Cc52 (SDB), lane 5: Cc167 (PDB), lane 6: Cc167 (SDB), lane 7: Cc172 (SDB) and lane 8: Cc172 (PDB), and **(C)** Lane 1: Cc26 (SDB), lane 2: Cc26 (PDB), lane 3: Cc119 (SDB) and lane 4: Cc119 (PDB). Brackets represents media in which isolate was grown.

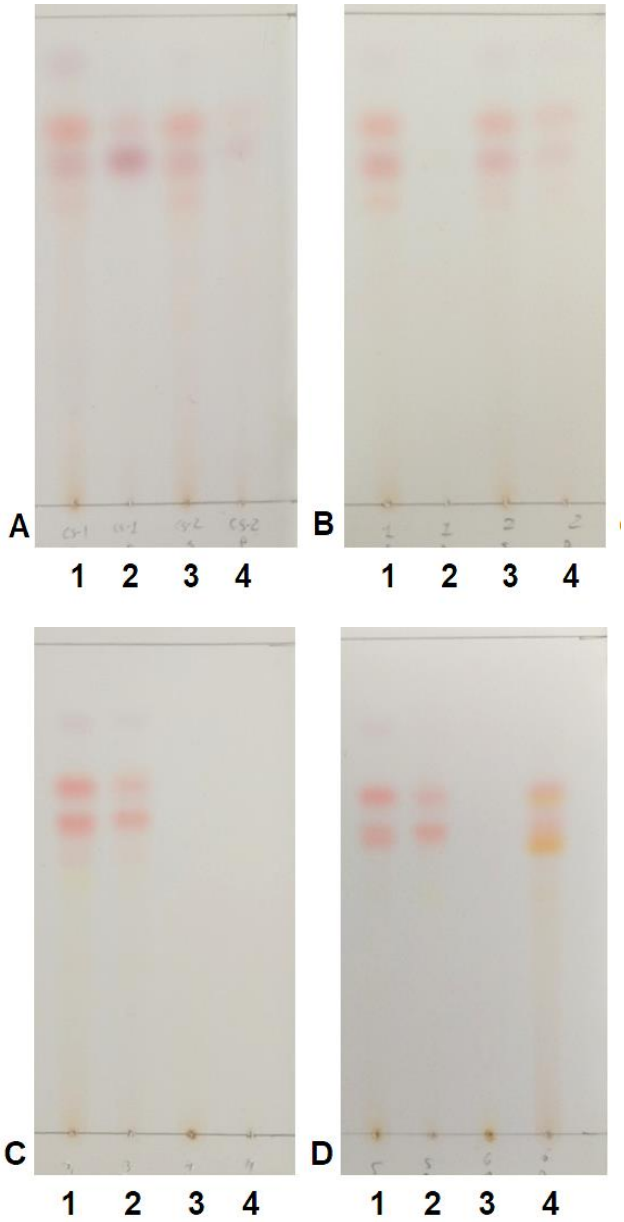

Figure 6.5: TLC plate of extracted pigment from FSSC Cs1, Cs2, CSH1, CSH2, CSH3, CSH5 and CSH6. (A) Lane 1: Cs1 (SDB), lane 2: Cs1 (PDB), lane 3: Cs2 (SDB) and lane 4: Cs2 (PDB), **(B)** Lane 1: CSH1 (SDB), lane 2: CSH1 (PDB), lane 3: CSH2 (SDB) and lane 4: CSH2 (PDB), **(C)** Lane 1: CSH3 (SDB), lane 2: CSH3 (PDB), lane 3: CSH4 (SDB) and lane 4: CSH4 (PDB), and **(D)** Lane 1: CSH5 (SDB), lane 2: CSH5 (PDB), lane 3: CSH6 (SDB) and lane 4: CSH6 (PDB). Brackets represents media in which isolate was grown.

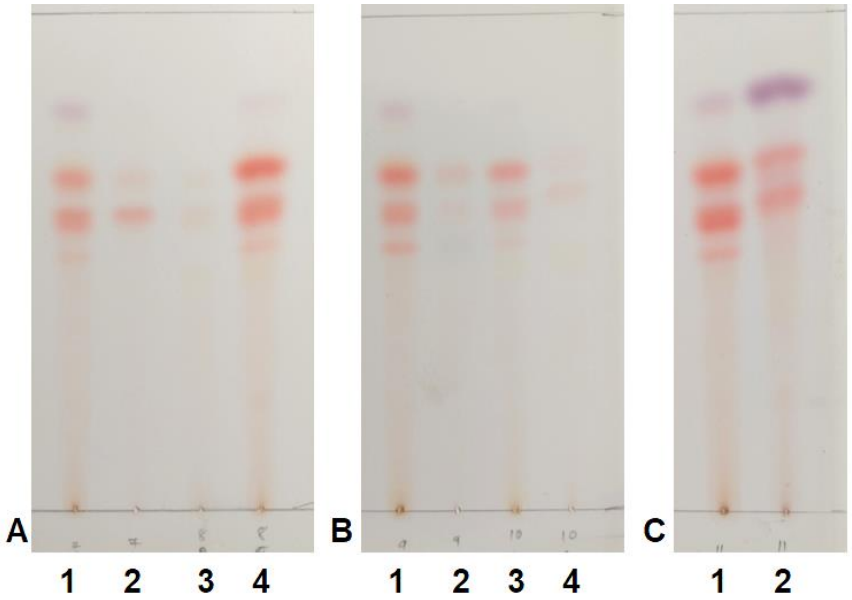

Figure 6.6: TLC plate of extracted pigment from FSSC CSH7, CSH8, CSH9, CSH10 and CSH11. (A) Lane 1: CSH7 (SDB), lane 2: CSH7 (PDB), lane 3: CSH8 (SDB) and lane 4: CSH8 (PDB), **(B)** Lane 1: CSH9 (SDB), lane 2: CSH9 (PDB), lane 3: CSH10 (SDB) and lane 4: CSH10 (PDB), and **(C)** Lane 1: CSH11 (SDB) and lane 2: CSH11 (PDB). Brackets represents media in which isolate was grown.

Further characterization was carried out using different treatments on separated pigments on TLC plate. The TLC plate was sprayed with methanolic KOH (20%) and ethanolic KOH (20%). After spraying plate was heated in an oven at 80˚C for 10 minutes. Figure 6.7 shows the TLC plate after heating. It can be seen that from two orange band the lower band was converted in to purple band and the upper orange band turned slight pink but not purple. Which indicates that the lower band was anhydrofusarubin and upper band was of fusarubin. Both of these pigments are naphthoquinone.

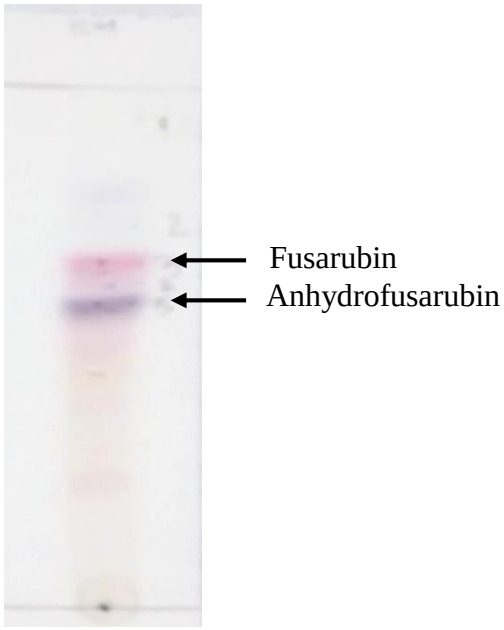

Figure 6.7: TLC plate of extracted pigment from FSSC Cc240 at 20th day after treatment with methanolic KOH followed by heating at 80˚C.

6.3.3 Pigment characterization using MS/MS:

For further characterization large amount of pigment production was carried out from Cc240 and pigment was purified as mentioned in method section. This purified F.O pigment was subjected to Mass spectroscopy. Two napthoquinones were identified in mass spectroscopy, 19-oxoandrost-4-ene-3,17-dione and 3-hydroxy-3-methyl-2-(3,7,11,15-tetramethylhexadec-2-enyl)-2H-naphthalene-1,4-dione. Figure 6.8 shows the mass spectrum and Figure 6.9 shows structure of 19-oxoandrost-4-ene-3,17-dione compound. The molecular formula was $C_{19}H_{24}O_3$ and the molecular mass was 300.17 Da. Figure 6.10 shows shows the mass spectrum and Figure 6.11 shows structure of 3-hydroxy-3-methyl-2-(3,7,11,15-tetramethylhexadec-2-enyl)-2H-naphthalene-1,4-dione compound. The molecular formula was $C_{31}H_{78}O_3$ and the molecular mass was 468.36 Da.

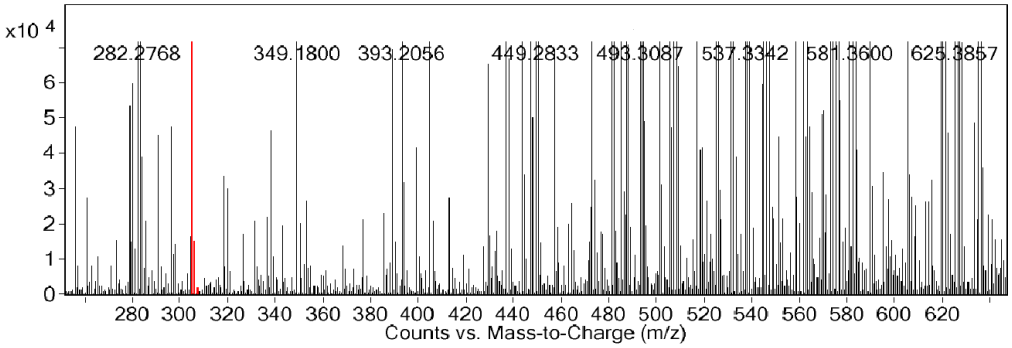

Figure 6.8: Mass spectrum of fast orange compound in ESI positive mode. Peak in red was identified as 19-oxoandrost-4-ene-3,17- dione. (Molecular formula: $C_{19}H_{24}O_3$ and Molecular mass: 300.17 Da).

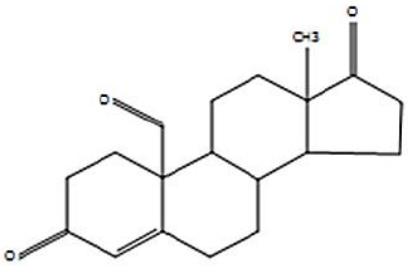

Figure 6.9: Structure of 19-oxoandrost-4-ene-3,17- dione.

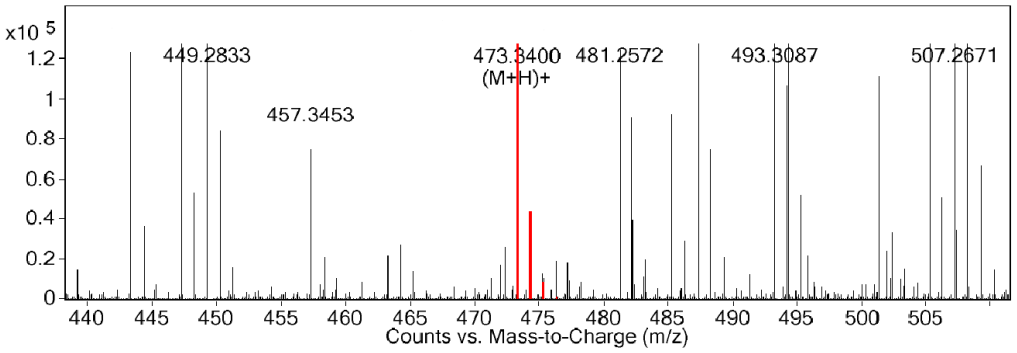

Figure 6.10: Mass spectrum of fast orange compound in ESI positive mode. Peak in red was identified as 3-hydroxy-3-methyl-2-(3,7,11,15-tetramethylhexadec-2-enyl)-2H-naphthalene-1,4-dione. (Molecular formula: $C_{31}H_{78}O_3$ and Molecular mass: 468.36 Da).

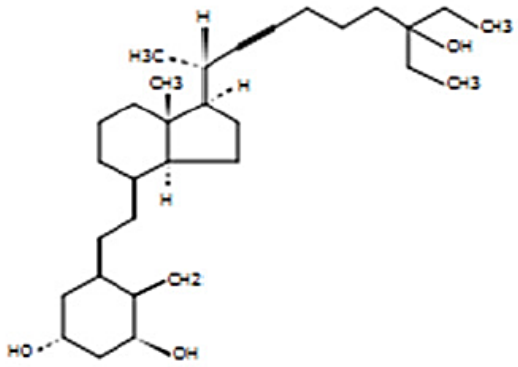

Figure 6.11: Structure of 3-hydroxy-3-methyl-2-(3,7,11,15-tetramethylhexadec-2-enyl)-2H-naphthalene-1,4-dione.

6.4 Discussion:

Microbial pigments play role in disease pathogenesis by manifesting cytotoxic or proinflmatory properties as well as by interference with host immune clearance mechanism (Liu and Nizet, 2009). Initial observation in the present study regarding distinctive colour of isolates lead us to choose napthoquinones as virulence factors in Fusarium spp. The orange to red pigmentation in *F. solani* is well studied and is largely due to the presence of naphthoquinone group of pigments. Fusarubin, Javanicin, Bikaverin, aurofusarin, anhydrofusarubin, solaninapthoquinone (Cambaza, 2018; Kumar *et al*., 2017; Medentsev and Akimenko, 1992; Spraker *et al*., 2018; Tadpetch *et al*., 2015) are reported to be present in *Fusarium* spp.

In present study, naphthoquinone pigment production was carried out in PDB, SDB, YMB and NB. Pigment production visually was higher in PDB and SDB and lower in YMB and NB. The extraction of pigment initially was carried out using method of (Medentsev *et al*., 2005) using chloroform. Also extraction was carried out using method of (Trisuwan *et al*., 2010) using ethyl acetate. Compared chloroform extraction, ethyl acetate extraction yielded more pigment. The pigment was extracted day wise and subjected to TLC. It was found that upon 20^{th} day pigment production was maximum. The chromatographic separation showed several compounds but two compounds were most prominent in ethyl acetate extract of all pathogenic isolates grown in

PDB and SDB medium. These two compounds were named as fast orange (F.O.) and slow orange (S.O.) depending upon migration on TLC plate.

Preliminary characterization in current study revealed 2 major pigments (named fast and slow orange) which were tentatively identified as Fusarubin and anhydrofusarubin based on brontraeger reaction. In one study, pigment from *F. solani* has been characterised where two orange pigments were also labelled as fast orange and slow orange. It was reported that when slow orange compound was heated at 160˚C for 30 minutes, it converts in to purple anhydrofusarubin (Ammar *et al.*, 1979). Further structural characterization was carried out with MS/MS of fast orange compound. The mass spectroscopy of fast orange compound yielded two naphthoquinone compounds which were 19-oxoandrost-4-ene-3,17- dione or 2-Hydroxy-3-(2-methyloctyl)-1,4-naphthoquinone and 19-oxoandrost-4-ene-3,17- dione or 2-Hydroxy-3-(2-methyloctyl)-1,4-naphthoquinone. Both have not been reported earlier. Hence, further structural characterization and studies on properties of these naphthoquinones need to be carried out.

Extraction of naphthoquinone from *F. decemcellulare, F. bulbigenum,* and *F. graminearum* was done with chloroform after dehydrating medium with acetone and ethanol. The pigments produced by *F. decemcellulare* were naphthoquinone pigments having napthazerin structure (fusarubin, javanicin, anhydrofusarubin, anhydrojavanicin, novarubin and bostrycoidin), *F. graminearum* and *F. bulbigenum* produced aurofusarin and bikaverin, respectively (Medentsev *et al.*, 2005). *F. oxysporum* f sp *vasinfectum* was reported to produce bikaverin, norbikaverin, nonaketide naphthazarin quinones, anhydrofusarubin lactol, 5-*O*-methyljavanicin and nectriafurone on PDA, PDB and DMD (Minimal dextrose broth) medium. Characterization was done using TLC, HPLC and NMR (Bell *et al.*, 2003; Lebeau *et al.*, 2019). In one study, *Fusarium* spp. was grown in PDB for pigment production and pigment extraction was carried out using 3 equal volumes of ethyl acetate and dried over anhydrous Na_2So_4 followed by evaporation to get dark red solid and the pigments produced were fusaron, fusarnaphthoquinone and fusaranthraquinone. Complete characterization and structure is established on the basis of results

from NMR and MS-MS (Trisuwan *et al*., 2010). *F. keratoplasticum*, *F. petroliphilum* and *F. falciforme* were reported to produce bikaverin, solaniol, fusarubin and anhydrofusarubin on PDA medium, characterization was done using HPLC and mass spectrometry (Short *et al*., 2013).

Naphthoquinone pigments exhibit cytotoxic property. Due to their cytotoxicity, most naphthoquinones were explored for cytotoxicity, antimicrobial and phytotoxicity (Khan *et al*., 2016). Cytotoxicity effects of napthoquinones were studied widely *in vitro* as well as *in vivo*. The effect has been studied on Human leukemia cells (K-562) (Khan *et al*., 2016), breast cancer cells (MCF-7) and oral human carcinoma (KB) cells (Tadpetch *et al*., 2015), erythrocyte (Munday *et al*., 1995a), human lymphoblast (Sasaki *et al*., 1997), V79 chinese hamster cells (Da Costa Medina *et al*., 2008; Ludewig *et al*., 1989) and B16F1 murine melanoma cells (Kumar *et al*., 2009). *In vivo* cytotoxicity was shown in guinea pigs (Schulz *et al*., 1977), rats (Munday *et al*., 1995b), swiss albino mice (Sivakumar and Devaraj, 2006) and BLAB/c mice (Garrido, 2016). Further, napthoquinones from plants and fungi are being tested as antifungal, antimalarial and chemotherapeutic agents (Da Costa Medina *et al*., 2008; Kumar *et al*., 2017; Lara *et al*., 2018; Liu *et al*., 2018; Riffel *et al*., 2002; Tadpetch *et al*., 2015).

In the present work, presence of pigments was predominantly seen during *in vitro* growth condition and not detected during the explant corneal infection. Hence, the role of nathoquinones during corneal infection is still not clear.