6. PRODUCTION OF SECONDARY METABOLITES FROM THE FUNGUS ALTERNARIA ALTERNATA

6.1. Introduction

Natural products are chemical compounds derived from living organisms throughout the six kingdoms Archaebacteria, Protista, Eubacteria, Fungi, Plantae and Animalia (Woese et al., 1977). As diverse as their sources, the compounds and their biological functions that are often highly specific. Natural product derived drugs are usually secondary metabolites and their derivatives. Secondary metabolites are not essential for normal growth, development or reproduction of an organism. Instead, their functions may be as diverse as competitive weapons against other bacteria, fungi, amoebae, plants, insects and large animals, metal transporting agents, agents of symbiosis between microbes and plants, nematodes, insects, higher animals and sexual hormones and its differentiation effectors.

Approximately 50,000 different secondary metabolites are discovered so far, but this is only a small fraction of the total existing in protists, bacteria, fungi and plants (Demain and Fang, 2000). Since decades secondary metabolites from different taxonomic groups provide a variety of molecules of pharmaceutical, medical and economic value (Fox and Howlett, 2008). Continuously, new metabolites of high industrial potential are discovered, with those produced by fungi being of paramount importance. Several thousands of secondary metabolites are known (Turner and Aldridge, 1983) sharing the following characteristics:

1. They are usually synthesized at the end of the exponential and during the stationary growth phases.
2. They derive from common metabolic intermediates but are encoded through specific genes in specific enzymatic pathways.

3. They are not essential for the survival of an organism.

4. The metabolite compositions from any genus or species are strain specific.

In 1950, Raistrick initiated the first systematic study of fungal metabolites, and made a fertile contribution to the recognition of fungi as a major source of natural products (Saleem et al., 2007) which have unique and unusual pathways. Genes involved in primary metabolism are generally scattered throughout the fungal genome, but genes of secondary metabolism are arranged in clusters similar to the bacterial secondary-metabolite operons (Calvo et al., 2002; Keller et al., 2005). Depending on their origin in the primary metabolism there are four main groups: polyketides with fatty acid derivates, non-ribosomal peptides, isoprenoids and alkaloids (Kueck et al., 2009). The most relevant pathway is the polyketide pathway, which has Acetyl-CoA as a precursor with a number of important end products, such as griseofulvin or aflatoxins (Payne and Brown, 1998; Bennett and Klich, 2003). Some of the secondary metabolites from these sources have significant economic and pharmaceutical value.

New lead compounds from fungi are often expected to be found in unusual habitats. These habitats may accommodate new species, which may be used as unique sources of enzymes or secondary metabolites of biotechnological or pharmaceutical potential. The species found in extreme environments may also produce excellent novel chemical compounds (Stierle et al., 2006; Vining, 1992).
Halocins (bacteriocins) produced by extremely halophilic members of the domain Archaea were first identified by Rodriguez Valera (1992). Out of 40 extreme halophiles screened for halocin production, seven strains inhibited a broad spectrum of pathogenic bacterial strains. Their bactericidal modes of action are wide ranging and include inhibition of transcription, translation, DNA and RNA nuclease activity, pore formation, bacteriolysis and disruption of cellular membranes (Barefoot et al., 1992).

The secondary metabolites does not occur randomly but is correlated with ecological factors (Dobler et al., 2002). There are less data on the possibility to find new extrolite producers in extreme environments, the general opinion being that extremophiles may need a smaller number of bioactive molecules to compete with fellow extremophiles. Solar salterns matured a surprisingly rich diversity and abundance of halophilic and halotolerent fungi showed that increasing in NaCl concentration to 3-5% in fungal growth media will often produce increase extrolite production (Masuma et al., 2001; Gunde Cimmerman et al., 2009) and some genes could be activated at high salt concentrations that lead to some new secondary metabolites. Some filamentous fungi are unusually halotolerant (Reed, 1986; Truper and Galinski, 1986) and among the filamentous fungi, a large number of species produce several extrolites.

The halotolerant fungi, *Aspergillus terreus* was identified in richest salinity at 10% compared with those at 0% and 3% salt showed the production of famous statins drug lovastatin, an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase), for lowering cholesterol to prevent cardiovascular disease (Alberts et al., 1980; Manzoni and Rollini, 2002). A series
of compounds such as terreineol (Macedo et al., 2004), terreulactone A (Kim et al., 2002), terrain (Ghisalberti et al., 1990), terreic acid (Yamamoto et al., 1980) and aspulvinones (Ojima et al., 1976) were also produced from this fungus.

Secondary metabolites were consistently produced by six species of Eurotium. It produces echinulins, neoechinulins and cryptoechinulins, while other species produce physcion, emodin, flavoglaucin, auroglaucin, dihydroauroglaucin, tetrahydroauroglaucin and several other extrolites (Turner and Aldridge, 1983; Butinar et al., 2005). Some of these metabolites are also produced by Chaetosartorya, another moderate to extreme halophile, but both Eurotium and Chaetosartorya species have unique metabolites only found in that genus. Cladosporium cladosporioides (marine-derived fungi) produced many interesting secondary metabolites like peroxyergosterol and p- methyl benzoic acid (San-Martin et al., 2005).

*Alternaria* are a large group of molds generally occurring on dead or dying plant tissue, but are often parasitic on plants (Alexopoulous and Mims, 1979). It causes a range of economically important diseases like stem cancer, leaf blight or leaf spot on a large variety of crops. However, some species have acquired pathogenic capacities causing diseases over a broad host range. Molds of this genus require relatively high moisture content (28 to 34% moisture) for growth. Various species within the genus *Alternaria* produce large number of secondary metabolic products often with toxic properties against various cell types (King and Schade, 1984). Host-selective toxins produced by fungal plant pathogens are generally low molecular weight and are critical determinants of host-specific pathogenicity or virulence in several plant-pathogen interactions (Wolpert et al.,
Secondary Metabolites From Alternaria alternata

2002; Howlett, 2006). However, the pathogenic variants (pathotypes) which produce different host-selective toxins and cause necrotic diseases on different plants (Thomma, 2003). Most of the toxic substances from Alternaria belong to the dibenzopyrone or polyketide biosynthetic groups (Stinson, 1985). Brefeldin A (dehydro-) curvularin, tenuazonic acid, tentoxin and zinniol are examples of toxins that are produced by several Alternaria species. For instance, Tentoxin is produced by A. alternata acts as a photophosphorylation inhibitor through specific binding to chloroplast ATP synthase, causing the inhibition of ATP hydrolysis and ATP synthesis (Steele et al., 1978).

Alternaria species produce a wide variety of primary and secondary metabolites, e.g. phytotoxins and mycotoxins (Ichihara et al., 1983; Nishimura and Kohmoto, 1983; Robeson et al., 1984) which have an varied structure and unusual partially saturated compounds like perylene and ergosta-4,6,8 (14)-22-tetraen-3-one (ETO) (Seitz and Paukstelis, 1977). In some cases, the polyketides alternariol (AOH) and alternariol monomethyl ether (AME) during the stationary growth phase in the presence of dark environments were synthesized by A. alternata (Soderhall et al., 1978; Haggblom and Niehaus, 1986). Likely, three secondary metabolites, altertoxins I, II, and III, have been isolated and have hydroxyperylenequinone structures and a cyclic depsipeptide such as alternaramide was also isolated from the marine-derived halotolerant fungus Alternaria sp. (Kim et al., 2009).

Though several well known antimicrobial agents like fusidic acid (Godtfredsen et al., 1962) and griseofulvin (Grove et al., 1952), novel semisynthetic antifungal drugs like anidulafungin (Eraxis®) and caspafungin
(Cancidas®) derived from halophilic fungal metabolites (Butler, 2004). The increasing prevalence of multiple drug resistance has still shown in the development of new natural synthetic antimicrobial, anti-oxidative and anti-inflammatory drugs for an alternative source.

Halophilic fungi are poorly investigated group of microorganisms that represent an abundant and dependable source of bioactive and chemically novel compounds with potential for exploitation in wide variety of medical, agricultural and industrial areas. Hence, the present investigation was undertaken.
6.2. Materials and Methods

6.2.1. Halotolerance test on Fungi

During the present survey, *Hortaea werneckii*, *Alternaria alternata*, *Fusarium verticilliodes*, *Cladosporium herbarum* and *Cladosporium cladosporioides* were isolated frequently on high salinity. Thus, these strains were subjected for halotolerant test (Moubasher *et al.*, 1990) in the potato dextrose agar medium amended with the different NaCl concentrations (5, 10, 15 and 20%). All these isolates were inoculated in the centre of the plates and incubated at 27°C for 168 hrs. After the stipulated period, the diameter of the colony was measured and the high salinity tolerance strains were selected for secondary metabolites screening.

6.2.2. Small Scale fermentation

Based on the halotolerant test, halotolerant fungi were grown on small scale fermentation medium. A fungal disc (6 mm diameter) of 10 days old cultures was inoculated into 500 ml of flask containing 250 ml of the autoclaved Potato Dextrose Broth (PDB) amended with 20% of NaCl and incubated at room temperature for 10 days.

**Composition for Potato Dextrose Broth Medium (g/lit)**

- Potatoes infusion - 200 g
- Dextrose - 20 g
- Distilled water - 1000 ml
- pH - 6.5

200 gms of potato was smashed and boiled with distilled water and filtered using a clean muslin cloth. To this filtrate, 20 gms of dextrose and 20 gms of NaCl
was suspended in 1000 ml of distilled water and the pH was adjusted to 6.5. The medium was boiled to dissolve completely and sterilized by autoclaving at 15 lbs (121°C) for 15 minutes.

6.2.2.1. Crude Extraction

After the incubation periods, the mycelium was separated from the fermented culture broth of three isolates by filtration. The culture filtrates was extracted three times with 300 ml of ethyl acetate. The resulting layers were combined and the solvent was eliminated by evaporation using a rotator vacuum distilling apparatus to get the crude extracts. It was then dissolved in various solvents like methanol, chloroform, hexane, petroleum ether and ethanol, in order to determine the solubility of the crude compounds.

6.2.3. Antibacterial activity

6.2.3.1. Pathogens used for the antibacterial assays

Human bacterial pathogens like *Escherichia coli*, *Salmonella typhi*, *Klebsiella pneumoniae*, *Vibrio parahaemolyticus* and *Klebsiella oxytoca* were used for the antibacterial assay. The bacterial pathogens were maintained on Nutrient Agar (NA) medium.

6.2.3.2. Preparation of test micro organisms

A loopful of test organisms was transferred to 10 ml of sterilized Nutrient Broth (NB) and incubated overnight at 37°C.

**Composition of Nutrient Broth (g/lit)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>5 g</td>
</tr>
<tr>
<td>Beef extract</td>
<td>5 g</td>
</tr>
<tr>
<td>Peptone</td>
<td>5 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5 g</td>
</tr>
</tbody>
</table>
20 g of nutrient agar was suspended in 1000ml of distilled water and the pH was adjusted to 6.8 to 7.0. The medium was boiled to dissolve completely and sterilized by autoclaving at 15 lbs (121°C) for 15 minutes.

### 6.2.3.3. Disc Diffusion Technique

25 ml of sterilized Muller-Hinton Agar (MH) (Hi Media, Mumbai, India) was poured in petriplates and allowed to solidify at room temperature. The pathogenic bacterial strains were then seeded on the MH agar media in the petri dish by streaking the plate with the help of sterile swabs. The concentrate crude extracts was then impregnated on the sterilized whatman (GF/F) filter paper discs of 6 mm diameter and the antibacterial activity was assayed against *E. coli, Salmonella typhi, Klebsiella pneumoniae, Vibrio parahaemolyticus, Klebsiella oxytoca*. The plates were then incubated at 37°C for 24 hrs. The result was examined by presence or absence of zone of inhibition. Streptomycin was used as standard antibacterial agents for positive inhibitory controls.

**Composition of Muller-Hinton Agar (g/lit) medium**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef infusion</td>
<td>30</td>
</tr>
<tr>
<td>Casein acid hydrolysate</td>
<td>17</td>
</tr>
<tr>
<td>Starch</td>
<td>1.5</td>
</tr>
<tr>
<td>Agar</td>
<td>17.0</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000</td>
</tr>
<tr>
<td>pH</td>
<td>7.3 ± 0.2</td>
</tr>
</tbody>
</table>
Based on the results of best antibacterial screening, the crude extracts of potent strain were chosen for further work.

6.2.4. Mass Scale Production and extraction

A fungal disc (6 mm diameter) of 10 days old cultures of potent strain was inoculated into a 500 ml conical flask containing of 300 ml autoclaved PDB medium amended with 20% of NaCl to the medium. The unchanged procedure was followed as like in small scale productions until crude extracts were obtained.

6.2.4.1. Analytical methods

6.2.4.1.1. Column chromatography

Crude extract was subjected to silica gel column (mesh size 60-120) and eluted with Petroleum ether: CHCl$_3$ (0-100%) and CHCl$_3$:CH$_3$OH (0-100%) respectively. 50 ml of each fractions were collected based on the below tabulated solvent system (Table 17).

**Table 17. Fractionation of the crude extracts**

<table>
<thead>
<tr>
<th>ELUENTS</th>
<th>RATIOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petroleum Ether: CHCl$_3$</td>
<td>(50:0)</td>
</tr>
<tr>
<td>Petroleum Ether: CHCl$_3$</td>
<td>(45:5)</td>
</tr>
<tr>
<td>Petroleum Ether: CHCl$_3$</td>
<td>(40:10)</td>
</tr>
<tr>
<td>Petroleum Ether: CHCl$_3$</td>
<td>(35:15)</td>
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<td>(30:20)</td>
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<td>(25:25)</td>
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<td>Petroleum Ether: CHCl$_3$</td>
<td>(15:35)</td>
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<td>(10:40)</td>
</tr>
<tr>
<td>Petroleum Ether: CHCl$_3$</td>
<td>(5:45)</td>
</tr>
<tr>
<td>Petroleum Ether: CHCl$_3$</td>
<td>(0:50)</td>
</tr>
<tr>
<td>CHCl$_3$: CH$_3$OH</td>
<td>(45:5)</td>
</tr>
</tbody>
</table>
Secondary Metabolites From Alternaria alternata

<table>
<thead>
<tr>
<th>CHCl₃: CH₃OH</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>(40:10)</td>
<td></td>
</tr>
<tr>
<td>(35:15)</td>
<td></td>
</tr>
<tr>
<td>(30:20)</td>
<td></td>
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<tr>
<td>(25:25)</td>
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<td>(20:30)</td>
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<td>(15:35)</td>
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<tr>
<td>(10:40)</td>
<td></td>
</tr>
<tr>
<td>(5:45)</td>
<td></td>
</tr>
<tr>
<td>(0:50)</td>
<td></td>
</tr>
</tbody>
</table>

6.2.4.1.2. Thin Layer Chromatography

Fractions were concentrated by rotator evaporator and the fractions were spotted onto the analytical TLC plate (pre-coated with silica gel GF-254) and then placed in the air tight beaker with an appropriate solvent system. The TLC plates were removed from the solvent before they reach to the end of the plate. The reaching point of the solvent marked and allowed to air dry for few minutes. The fractions were combined according to their composition as revealed by analytical TLC. The TLC plates was visualized by UV lamp (\( \lambda = 254 \) nm) or under vapors of iodine crystals. Retention factor (Rf) value was calculated according to the following equation

\[
Rf = \frac{\text{Distance traveled by solute}}{\text{Distance traveled by solvent}}
\]

**Note:** The fractions were pooled together containing the similar Rf value. Rechromatographed on fresh silica gel with different ratios of eluents if, two or more bands were observed in TLC plates.
6.2.5. Antibacterial activities of each fraction

The bacterial pathogens such as *Escherichia coli*, *Salmonella typhi*, *Klebsiella oxytoca* and *Vibrio parahaemolyticus* were used for antibacterial activity against all eleven fractions with similar procedure as described earlier.

6.2.6. Gas Chromatography-Mass Spectroscopy (GC-MS)

The best active fraction was then subjected to GC-MS instrument, GC-MS-5975C (AGILENT) to find out the active compounds. Chromatography was performed on a DB-Wax capillary column (30 m×0.25 mm ID and 0.25 µm film thickness). The electron impact technique (70 eV) was used. The carrier gas was helium at a flow rate of 1.0 ml/min and 1 µl of sample was injected. The injector and detector temperatures were 230ºC and 200ºC, respectively. The column oven was programmed as follows: initial temperature 60ºC, initial time 2.0 min, program rate 10ºC/min; final temperature 250ºC; final time 10 min. The sample was dissolved in CH$_2$Cl$_2$ and a split injection technique was used. The identification of the compounds was based on comparison of their retention indexes (RI), obtained using n-alkanes (C$_{11}$-C$_{31}$) and retention time. They can also be confirmed by comparison of their mass spectra with the NIST/NBS - Wiley library spectra and literature data. Relative percentage amounts were calculated from TIC (Total Ion Chromatogram) by the computer.

6.2.7. Analytical and Preparative HPLC analysis

The highly active fraction was subjected for purity which was assessed by Shimatzu 9A apparatus employing a reversed-phase C18 column with a UV detector. A portion (20 µl) of the sample was injected each time and detected at the range of 240 nm. The mobile phase was methanol/acetonitrile/water (25:35:40
by volume) at 1.0 ml/min. The sample and the mobile phase were filtered through a 0.2 μm-pore-size PVDF filter before placing them on the column. Further, the impurities were removed using preparative HPLC. Preparative HPLC was performed using an ODS column (YMC- pack ODS-A, 10× 250 mm, 5 μM, 4 ml/min). The spectral data (UV, FT-IR, $^1$H NMR and $^{13}$C NMR) obtained for the compound were used to establish the structure of compound.

6.2.8. UV-visible spectrophotometer

The purified sample was dissolved in 100% methanol, analyzed by Beckman DU-40 UV spectrophotometer.

6.2.9. Fourier Transform-Infrared spectroscopy

FT-IR used to investigate the vibrations of molecules and polar bonds between the different atoms. Functional groups can be analyzed using FT-IR spectroscopy. Samples (2 mg) were mixed with KBr and pressed into pellets of 13 mm size and infrared spectrum was recorded using Perkin - Elmer IR spectrophotometer (Model IR 577).

6.2.10. Nuclear Magnetic Resonance (NMR) spectroscopy

To identify the types of compounds in the bioactive fraction of the methanol in ethyl acetate extracts of fungus extracts, the bioactive fraction was analyzed using Nuclear Magnetic Resonance (NMR) Spectroscopy. The proton spectra at 400 MHZ and proton decoupled $^{13}$C NMR spectra at 100 MHZ were recorded at room temperature on DRx 400 NMR spectrometer using 10 mm sample tube. Sample were prepared by dissolving about 10 mg in 0.5 ml of dimethyl sulfoxide d6 (DMSO-d6) as solvent containing 1% TMS for $^1$H and
0.5 ml of sample in 2.5 ml of chloroform-d a few drop as TMS for $^{13}$C. The solvent chloroform-d also provided the integral field frequency lock signal.
6.3. Results

6.3.1. Halotolerance test on fungi

Results of the halotolerance test on fungi showed that the colony diameter were maximum in *Hortaea werneckii* (>2.1 cm) followed by *Alternaria alternata* (>2.0 cm) and *Cladosporium cladosporioides* (>1.9 cm) which observed at 20% of NaCl concentration whereas, minimum colony diameter in *Fusarium verticilliodes* (>1.0 cm) and *Cladosporium herbarum* (>1.0 cm). Thus, the strains yield maximum colony diameters were chosen for the screening of secondary metabolite production.

6.3.2. Small scale fermentation and extraction

The halotolerant strains were inoculated in small scale fermentation broth and the culture filtrates was extracted with ethyl acetate (Fig. 48). Result shows that the highest percentage of crude extracts (1.7 g) was obtained in *Alternaria alternata* followed by 1.4 g in *Cladosporium cladosporioides* and 1 gm in *Hortaea werneckii*. Further, crude extracts showed the best solubility in methanol followed by chloroform and petroleum ether.

![Fig. 48. Production of crude extracts from halophilic fungi](image)
6.3.3. Antibacterial activity

The results of the antibacterial activity in crude extract of three halotolerant strains were tested against five human bacterial pathogens were shown in the photoplate 4. The maximum antibacterial activity were observed in the extracts of *Alternaria alternata* against *E. coli* (17 mm), *V. parahaemolyticus* (14 mm), *Salmonella typhi* (12 mm) and minimum in *Klebsiella oxytoca* (10 mm) whereas, no activity were observed in *Klebsiella pneumoniae*. On the other hand, *Cladosporium cladosporioides* showed maximum activity against *Salmonella typhi* (15 mm) and *Klebsiella pneumoniae* (12 mm), and the extracts showed no activity against *E. coli*, *V. parahaemolyticus* and *Klebsiella oxytoca*. In case of *Hortaea werneckii*, least activity were observed against *E. coli* (10 mm) and *Vibrio parahaemolyticus* (9 mm) and no activity was observed against *Klebsiella pneumoniae*, *Salmonella typhi* and *Klebsiella oxytoca*. As a result, *Alternaria alternata* extracts showed wide range of activity against all the tested human pathogens and the strain *A. alternata* were selected for the mass scale production and characterization of its bioactive metabolites.

6.3.4. Fractionation

15.7 g of crude extracts from *Alternaria alternata* were obtained by mass scale production, which were fractionized by column chromatography. As a result, 21 fractions were collected. In analytical TLC, bands in 5-15 fractions were visualized under vapors of iodine crystals among 21 fractions. These 11 fractions were used for antibacterial assay.
6.3.4.1. Antibacterial activities of fractions

The fractionized extracts from *Alternaria alternata* showed maximum antibacterial activity against all four pathogens (Fig. 49). In fifth fraction, the maximum activity were observed in *Vibrio parahaemolyticus* (17 mm), followed by *Escherichia coli* (9 mm) *Klebsiella pneumoniae* (7 mm) and no activity were observed in *Salmonella typhi*. Apparently, in sixth fractions *Escherichia coli* (20 mm) showed maximum activity followed by *Klebsiella pneumoniae* (7 mm) and no activity were observed in both *Salmonella typhi* and *Vibrio parahaemolyticus*. In seventh fraction, the maximum activity were observed against the pathogens of *Klebsiella pneumoniae* (80 mm), *V. parahaemolyticus* (80 mm) and minimum in *E. coli* (77 mm) and no activity was observed in *Salmonella typhi*. There is no activity was observed in fraction eight against all four pathogens. In fraction nine, maximum activity were observed in *Salmonella typhi* (53 mm) followed by *Escherichia coli* (33 mm), *Vibrio parahaemolyticus* (30 mm) and minimum in *Klebsiella pneumoniae* (18 mm). In fraction ten, higher activity was observed in *Salmonella typhi* (9 mm), *Klebsiella pneumoniae* (7 mm) and least activity in *Escherichia coli* (1 mm) and no activity in *Vibrio parahaemolyticus* was noted. In case of eleventh fraction, maximum was observed in *Vibrio parahaemolyticus* (40 mm) followed by *Escherichia coli* (33 mm), *Klebsiella pneumoniae* (25 mm) and minimum in *Salmonella typhi* (18 mm). In 12th fraction, maximum was observed in *Klebsiella pneumoniae* (15mm), *Escherichia coli* (12 mm), *Salmonella typhi* (11 mm) and minimum activity was observed in *Vibrio parahaemolyticus* (10 mm). In fourteenth fraction, maximum activity was recorded in *Salmonella typhi* (7 mm) and no activity was recorded in
other three pathogens. Finally, in fifteenth fraction, maximum activity was recorded in *Klebsiella pneumoniae* (7 mm) and no activity was observed in *Escherichia coli, Salmonella typhi* and *Vibrio parahaemolyticus* (Photoplates 5-8).

As a result, maximum antibacterial activity was observed in seventh fraction against three bacterial pathogens. Thus, further purification and characterization of compounds in this fraction was carried out.
Fig. 49. Antibacterial activities of 11 fractions from *Alternaria alternata*
6.3.4. Gas Chromatography-Mass Spectroscopy (GC-MS)

Fraction 7 was exhibited strong antibacterial activity and it was subjected to GC-MS analysis. The chromatogram results showed that F7 was a mixture of 3 different compounds (Fig. 50 and 51). However, there was only one major component which accounted for 90% of the total mass. Composition of the remaining 10% could not be ascertained due to their low abundance. The compounds of F7 were identified as 1- Hexanol, 2-ethyl-, 5-Oxa-6-azaspiro [3.4] oct-6-ene and 2,5-Di (trifluoromethyl) benzoic acid are presented in Figure 52.

**Fig. 50.** GC-MS analysis of purified compound

**Fig. 51.** GC-MS Library search report
Fig. 52. GC-MS library match report

6.3.5. Analytical HPLC analysis

In HPLC analysis, the peaks strike at two retention time of 1.961 (Area=2.05%) and 3.005 (Area=90.42%) which was shown in the Figure 53. High abundance area (90.42%) with retention time of 3.005 was selected for purification in semi-preparative HPLC.

Fig. 53. Analytical HPLC analysis of purified compounds
6.3.6. Semi-preparative HPLC analysis

Further, purification of compound was performed in semi-preparative HPLC at the retention time at 3.005 with 100% purity (Fig. 54).

![HPLC Analysis](image1)

**Fig. 54.** Preparative HPLC analysis of purified compound

6.3.7. UV-Visible spectrum

The unknown compounds were subjected in UV absorbance in the random range of 290-700nm, UV/vis (MeOH) $\lambda_{\text{max}}$ 239.60 nm (Fig. 55)

![UV-Visible Spectrum](image2)

**Fig. 55.** UV-vis spectrum of unknown compound
6.3.8. FT-IR

Infra red spectroscopic (IR) revealed a peak at 3531 and 3415cm⁻¹ due to the presence of OH and NH group. The peak at 2954 and 2854cm⁻¹ denoted the aliphatic CH stretch and the peak at 1217 and 1024cm⁻¹ are due to the presence of C-O stretch. The peak at 921, 840cm⁻¹ was due to the presence of methylene and C-O-O stretch. The weak band at 769 indicated the presence of C-H bend. The peak at 669cm⁻¹ denoted the presence of alkyne, C-H bend (Fig. 56).

![FT-IR spectrum of unknown compound](image)

**Fig. 56.** FT-IR spectrum of unknown compound

6.3.9. Nuclear Magnetic Resonance (NMR)

¹H NMR indicated the presence of methylene protons and the compound strikes a peak at 2.503 ppm. The methane protons attached to the nitrogen shifted and their signal to the downfield region and formed the peak at 2.863 ppm. The electronegative element attached to the pentane ring formed a peak at 6.641 ppm (Fig. 57).
Fig. 57. $^1$H NMR spectrum of 5-Oxa-6-azaspiro [3.4] oct-6-ene

In $^{13}$C NMR, the solvent generated strong absorbance at 41.75 ppm and the signal recorded at 28.96 ppm denoted the presence of methylene protons. Likewise, the nitrogen element attached with methane protons showed signal at 38.69 ppm. The signal recorded at 76.63 ppm signified the presence of electronegative atom in ring protons (Fig. 58).

Fig. 58. $^{13}$C NMR spectrum of 5-Oxa-6-azaspiro [3.4] oct-6-ene

According to NMR derivation, results of colorless compound were supposed to be 5-Oxa-6-azaspiro [3.4] oct-6-ene.
6.4. Discussion

The study of halophilic organisms is not only for the ecology of hypersaline habitats but also for million applications in various fields of medicine. These organisms offer a multitude of actual or potential applications in various fields of science (Galinski and Tindall, 1992; Ventosa and Nieto, 1995). Salt-tolerant fungi belong to extremophiles which can survive under the conditions of high salinity that can produce new and unique secondary metabolites at high salinity was rarely reported (Lu et al., 2008; Zheng et al., 2009; Zheng et al., 2010).

Novel alkaloids were identified as secondary metabolites of halotolerant microorganisms (Wang et al., 2009). In this present study, unique with remarkable characters of secondary metabolites were synthesized from extreme marine derived halotolerant fungi *Alternaria alternata* isolated at high salt concentration of 400‰ in Marakkanam saltpan. Xiao et al. (2013) reported some new compounds (Cytochalasin E and Roellichalasin) from *Aspergillus* sp. in marine solar saltern, China. Peng et al. (2011) reported a new cyclopentanopyridine alkaloid, 3-hydroxy-5-methyl-5, 6-dihydro-7\textit{H}-cyclopenta [\textit{b}] pyridin-7-one, together with 11 known aromatic compounds from the halotolerant fungal strain *Wallemia sebi* in 10% NaCl.

The secondary metabolites from halotolerant fungi *Wallemia* for extrolites (wallemninol and wallemia A and B) and its derivatives auroglaucins, flavoglaucins, and different anthraquinones such as catenarin, questin and questinol were reported by Turner, 1971; Cole and Cox, 1981; Turner and Aldridge, 1983; Cole et al., 2003; Cole and Schweikert, 2003.
Zheng et al. (2009) reported two novel cyclic hexapeptides, sclerotides A and B, with antibiotic activity from the marine-derived halotolerant fungus *Aspergillus sclerotiorum*. Malmstrom et al. (2002) reported the marine derived fungus *Emericella variecolor* yielded varitriol, varioxirane, dihydroterrein and varixanthone from Caribbean Sea off Venezuela. Wang et al. (2011) also investigated the structurally novel and bioactive natural compounds from marine-derived fungus, *Aspergillus terreus* which was isolated from the sediment of Putian Sea Saltern, China. Allen (1972); Ishikawa et al. (1984) reported the metabolites like Echinuline and flavoglaucin were only produced in the ascomata and ascospores of *E. amstelodami* cultures have the properties of antioxidants.

In this present investigation, methanolic extracts of three halophilic fungi (*Alternaria alternata*, *Hortaea werneckii* and *Cladosporium cladosporioides*) were tested against both Gram positive and negative bacterial pathogens. Among these, *Alternaria alternata* extract showed promising antibacterial activity against both tested gram positive and negative bacteria. This may be due to the presence of effective secondary metabolites in halophilic fungus *A. alternata* strain that may inhibit the growth of bacterial pathogens. Brauers et al. (2001) reported that a phenolic natural product known as hortein, which is the only compound that has been isolated from *H. werneckii* to date, did not show any inhibitory activity against *B. subtilis, E. coli* and other bacterial and fungal strains tested.

In this study, *Hortaea werneckii, Alternaria alternata* and *Cladosporium cladosporioides* showed maximum colony diameter at 20% of NaCl concentration and minimum in *Fusarium verticilliodes* and *Cladosporium herbarum* this may be due to the weakly halotolerant that yield small colonies compared to obligate.
Cantrell *et al.* (2006) reported that the weakly halotolerant species can growth rate up to <2 cm in 25% and also at 15% of NaCl concentration at Cabo Rojo solar salterns.

In this study, *A. alternata* synthesized the notable secondary metabolites which have the multitude properties. It produced 15.7 g of crude extracts by ethyl acetate extraction at the period of 10 days using mass scale production but Wang *et al.* (2007) reported 97.9 g of crude extracts by ethyl acetate extraction at the period of 45 days from the halotolerant fungus *Aspergillus versicolor*. This may be due to the varied fermentation period.

Asami *et al.* (2002) reported the compound 1-oxa-7-azaspiro [4.4] non-2-ene-4, 6-dione which showed the IR spectrum peak at 3570, 1735, 1715, 1705, and 1675 cm$^{-1}$ indicated the presence of a hydroxyl, amide and unsaturated carbonyl group. In this study, FT-IR spectrum showed peaks at 3576, 1217, 1024 cm$^{-1}$ that indicates the presence of hydroxyl and carbonyl groups and shows the absence of amide groups. Instead, the stretch observed in 921, 840, 769 cm$^{-1}$ indicates that contains cyclohexane ring vibrations (Methylene group) and alkyne bend.

In this study, a unique compound 5-oxa-6-azaspiro [3.4] oct-6-ene was identified by NMR structural characterization. Asami *et al.* (2002) reported the novel angiogenesis inhibitor, azaspirene, that inhibits the endothelial migration induced by vascular endothelial growth factor and its structure was determined to consist of a 1-oxa-7-azaspiro [4.4] non-2-ene-4, 6-dione moiety was isolated from the fungus *Neosartorya* sp. The structural characters has unique such as the presence of benzyl group instead of benzoyl and three conjugated double bonds coupled to the carbonyl group and a vicinal diol. The fact that it lacks an oxygen
atom at the benzyl position suggests a more convenient route for identification. Igarashi et al. (2004) reported the anti-angiogenic activity of fluorosynerazol, which possesses a 1-oxa-7-azaspiro [4.4] non-2-ene-4, 6-dione skeleton identical to that of azaspirene. Su and Tamm (1995) reported this structural frame is similar to that of pseurotins and synerazol.

The structure of Azaspirene is similar to Pseurotin A as reported by Bloch et al. (1976); Weber et al. (1976); Bloch and Tamm (1981). Ando et al. (1991) also reported that its structure resembles like synerazol isolated from the fungal strains Pseudeurotium ovalis (Ascomycetes) and Aspergillus fumigatus showed the antifungal activity against dermatophytic fungi. Komagata et al. (1996) investigated the compound 1-oxa-7-azaspiro [4.4] non-2-ene-4, 6-dione have the inhibitory activity in VEGF induced intracellular signal transduction nor anti-angiogenic activity and the similar compounds like Pseurotin A and synerazol have a neuritogenic activity in rat pheochromocytoma PC12 cells.

In $^1$H NMR spectrum, Asami et al. (2002) revealed that the compound 1-oxa-7-azaspiro [4.4] non-2-ene-4, 6-dione had four exchangeable protons in the downfield region at 4.96, 5.75, 6.07 and 9.94 ppm owing to the presence of hydroxyl or amide protons and in case of $^{13}$C NMR chemical shifts consisting of 1-oxa-7-azaspiro [4.4] non-2-ene skeleton in DMSO agree that indicating a stereochemical resemblance of Azaspirene. The reports that have building blocks of arise from 1 unit of propionate, 4 units of malonate, 1 unit of phenylalanine, and 2 units of methionine. In particular, the benzyl group indicates that oxidation at the benzyl position occurs after the incorporation of phenylalanine. The spectra of 1-oxa-7-azaspiro[4.4]non-2-ene-4,6-dione revealed the presence of 21 carbons,
including three methyls, one methylene, seven methines, two quaternary carbons, three quaternary carbons (one oxygenated) and three carbonyls.

In this present investigation, the $^1$H NMR spectrum showed the compound 5-oxa-6-azaspiro [3.4] oct-6-ene showed the attachment of pentane ring and methane protons which further attach to nitrogen shifts reflects the signal to the downfield region forms the peak at 2.86, 4.89 and 6.64ppm. Likely, in the $^{13}$C NMR comparison, the signal recorded at 28.96 ppm indicates the presence of methylene protons that attached the nitrogens element. The presence of electronegative atom in ring protons indicates with the signal recorded at 76.63 ppm. From the outcome of the NMR structural results, the meager dissimilarity may be due to the presence or absence of some benzyl group or carbonyl group and the shift of protons in 5-oxa-6-azaspiro [3.4] oct-6-ene.

It can be concluded that these halophilic mycological assemblages represents an extremely rich source for the isolation of new strains producing novel bioactive metabolites.
Photoplate 4. Preliminary antibacterial activity of halophilic fungal crude extracts

**Bacterial pathogens:** A- Vibrio parahaemolyticus, B- Salmonella typhi, C- Escherichia coli, D- Klebsiella pneumonia, E- Klebsiella oxytoca.

**Compounds:** A- Alternaria alternata, H- Hortaea werneckii, C- Cladosporium cladosporioides, N- Negative control, P- Positive control
**Photoplate 5.** Antibacterial activity of fractions (5-15) from *Alternaria alternata* against *Escherichia coli*  
(P-Positive control,  
N- Negative control)
Photoplate 6. Antibacterial activity of fractions (5-15) from *Alternaria alternata* against *Salmonella typhi* (P-Positive control, N- Negative control)
Photoplate 7. Antibacterial activity of fractions (5-15) from *Alternaria alternata* against *Klebsiella pneumonia* (P-Positive control, N- Negative control)
Photoplate 8. Antibacterial activity of fractions (5-15) from Alternaria alternata against Klebsiella oxytoca (P-Positive control, N- Negative control)