Production and characterization of colored metabolites and pigments of microbial isolates

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By

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IN THE NAME OF

ALLAH

THE MOST

MERCIFUL

AND

MIGHTY

O my Lord! Open for me my chest
(Grant me Self-confidence,
Contentment and boldness)
And ease my task for me,
And loose the knot from my tongue that
they understand my speech (words)

Surah 20, Ta Ha (Al-Quran)

CERTIFICATE

This thesis, submitted by **Ms. Salma Gul Shah** to the Department of Microbiology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan, is accepted in its present form as satisfying the thesis requirement for the Degree of Doctor of Philosophy in Microbiology.

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CONTENTS

List of Figures

List of Tables

List of appendix

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Summary

Among different microbes, fungi have been found as an important source of various exogenous natural color metabolites and pigments of immense industrial importance in sectors of food, dyeing, pharmaceutical and cosmetic. Based upon these facts, the present study evaluated the potential of selected fungal isolates towards production of color metabolites. Besides, the color metabolites were biochemically characterized in addition to their biological activities.

Colored secondary metabolites production and their bioactive potential were evaluated in three different newly isolated fungi. Two of these fungi were isolated from Kala pani soil and identified as *Penicillium verruculosum* SG (KC698959) and *Aspergillus fumigatus* SG4 (JX863917) whereas, the endophytic fungus isolated from *Taxus baccata* was *Chaetomium strumarium*. The pigment production was initially screened out on solid state fermentation conditions using five different media however, Potato dextrose agar (PDA) proved to be the best culture medium. In liquid state fermentation conditions, Potato dextrose broth (PDB) was selected as the best culture medium. After selection of the culture media, different operational parameters viz, pH, temperature, carbon source and concentration, nitrogen source and C: N ratios were optimized. Overall, the production was most efficient at pH 5.5, with 20-40g/L glucose (C source) and yeast extract (N source), C: N ratio was 4:1 for *Penicillium verruculosum*; 10:1 for *Chaetomium strumarium* and 8:1 for *Aspergillus fumigatus* under mesophilic temperature (25-30°C) in PDB. Standard qualitative and quantitative screening test of the colored extracts of fungi indicated presence of important classes of compounds viz. phenols, flavonoids, anthraquinones, terpenoids, alkaloids and coumarins, showing some similarity between plant and fungal pigments whereas, tannins were absent in them. These compounds were considerably stable in a pH range of 5-8 and ≤100°C with red pigment of *Penicillium verruculosum* being stable at even acidic conditions i.e., pH 3. Pigmented filtrates of the fungi revealed a significant antimicrobial activity in terms of zone of inhibition (mm) against test pathogenic bacteria (0-24) and fungi (0-30). Besides, they showed varying antioxidant (EC_{50} = 7-21 µg/ml) and free radical scavenging activities [EC_{50} (DPPH) = 10-38, (ABTS) = 95-134 µg/mL]. Howover, the pigmented

filtrates of fungi showed a low level of phytotoxicity (Seed germination and shoot length) and cytotoxicity (EC_{50}). Such potential characteristics indicated the likelihood of their utilization in food and pharmaceutical industries.

Fungal isolates viz. *Penicillium verruculosum* SG, *Aspergillus fumigatus* SG4 and *Chaetomium strumarium* SG1 were evaluated for the production of bioactive color metabolites on optimized potato dextrose broth (25°C). Logical fragmentation pattern following column chromatography, thin layer chromatography (TLC) and liquid chromatography and mass spectrometry (LCMS) of crude culture filtrate of fungus *Penicillium verruculosum* SG demonstrated presence of polyketide pigments (Monascorubrin, Monascin, Glutamyl Monascorubrine and analogue of Monascorubrine) and other different bioactive compounds viz. Pyripyropene, Orevactaene, N-De methyl Calcimycin and Citrinadin. Cytotoxicity against five different cell lines i.e., KA3IT, MDCK, HSCT6, NIH3T3 and HEK293 of the selected colored fractions of fungal filtrate containing different compounds revealed IC_{50} (µg/mL) values ranging from 5-100. It was comparatively higher in case of Orevactaene (5+0.44) [(non-significantly differed with Monascorubrine)] followed by Pyripyropene (8+0.63) against cancer cell line KA3IT. Overall, these compounds significantly showed less toxicity towards normal cell lines. X ray crystallography of yellow crystals of a pure compound (224.21 m/z) confirmed its 3 dimensional structure and was identified as Phenazine 1 carboxylic acid ($C_{13}H_8N_2O_2$), which is previously known as a broad spectrum antibiotic, antifungal and bio-control agent. Some unknown compounds not found in libraries were also detected in the culture filtrate.

In case of *Chaetomium strumarium*, logical fragmentation pattern following column chromatography, TLC and LCMS of crude culture filtrate revealed the presence of colored compounds i.e., Monascorubramine (purple red), Alizarin and Cochliodinol (Purple). Besides, bioactive colorless compounds Dihydromaltophytin, Nivalenol and Paxilline were also detected. Overall, cytotoxicity against all five cell lines i.e., KA3IT, MDCK, HSCT6, NIH3T3 and HEK293 of the selected colored fractions of fungal filtrate containing different compounds revealed IC_{50} (µg/ml) values ranging from 1-73 µg/ml. It

was comparatively higher in case of Nivalenol/Paxilline (10.6±9.76) followed by crude filtrate (28± 20.4) [(Non-significantly differed with Cochliodinol, Monascorubramine and Alizarin)] > Dihydromaltopytin (45.2±24.4). Overall, these compounds significantly showed less toxicity towards normal cell lines. Whereas in case of cancer cell line KA3IT, Cochliodinol containing fraction was most effective ($1IC_{50}$=0±0.44) [(Non significantly differed from crude culture filtrate)] followed by Nivalenol/Paxilline containing fraction (20±0.34) [(Non significantly different from Dihydromaltophytin and Alizarin)] >Monascorubramine (50±0.89). A pure orange compound was isolated and its proton NMR and LCMS confirmed it to be anthraquinone compound. Besides, many unknown compounds were also detected in LCMS profile of the selected fungi.

In *Aspergillus fumigatus*, logical fragmentation pattern following column chromatography, TLC and LCMS of crude culture filtrate revealed the presence of colored compounds i.e., PPR Monascorubramine and Fusarubin. Other colorless bioactive compounds were also detected in the fungal filtrate i.e., Pyrrocidine b, Communosine g, Teritrem and Tryptoquialanine A and B. Overall, the cytotoxicity against the five cell lines viz. KA3IT, MDCK, HSCT6, NIH3T3 and HEK293 shown by these compounds ranged from 1-200 µg/ml. All the fractions of this fungus showed quite low cytotoxicity (IC_{50}) value i.e., 1-1.5 µg/ml against cancer cell line (KA3IT) which differed significantly from crude culture filtrate (2.8±0.44). Overall, the pigmented filtrate of this fungus was more cytotoxic as compared to the other 2 fungi showing its higher anticancer potential.

Fungi proved to be an important source of colored metabolites of polyketide nature. Production of these metabolites helped under mesophilic temperature, pH (5.5-7), glucose and yeast extract. Stability of the metabolites was considerably high at temperature $\leq$ 100°C though it varied a little with varying pH clearly predicting their industrial applications. Furthermore, detailed evaluation of different compounds in various fractions of fungal filtrates typically indicated their non-toxic nature and role as biomedical agent in treating cancer. The study concludes that optimized yield of aforementioned compounds by manipulating different biotechnological procedures can

further help their large scale application in pharmaceutical, cosmetic and food industries. However, large scale production and application is still needed to be explored.

INTRODUCTION

1. Introduction

Since the beginning of human civilization, the use of colored compounds and pigments derived from various biological sources has been commonly practiced as dietary products, drugs and cosmetics (Anthony, 2009). Besides, they have been used in different commercial applications as dyeing, printing and painting agents (Hernandez et al., 2002; Kamel et al., 2009; Rekaby et al., 2009; Chengaiah et al., 2010). Earliest written record of the use of dyestuffs goes back to 2600 BC in China. Natural colorants have been used for dyeing candies and Egyptian pyramids. By 715 BC, wool dyeing was established as craft in Rome (Welham, 2000). In 400 BC, they were also used to impart color to wines. In 327 BC, Alexander the Great mentions "beautiful printed cottons" in India. People used them for wall paintings as evident from Ajanta, Ellora, Mithila, and Vedas. The ancient craftsmen used to produce dyes like blue from indigo, yellow from turmeric and saffron, red from lac, madder and safflower, brown from cutch and other dyes such as cochineal and logwood have been used traditionally (http://nptel.iitm.ac.in/courses/116104046/1.pdf). The angkak (Red yeast rice) has been used for Chinese cheese preparation and beverages, coloring of bagoong, atsike salted fish (Palo et al., 1960) and was described as a medication in Chinese pharmacopoeia of medicinal foods and herbs useful for improving digestion and revitalizing the blood (Heber et al., 1999).

Currently, a vast array of both synthetic dyes and natural pigments (>10,000) are extensively being utilized in textile (Gupta et al., 1992, Shukla and Gupta, 1992, Sokolowska-Gajda et al., 1996), leather tanning (Tünay et al., 1999 and Kabadasil et al., 1999), paper (Ivanov et al., 1996), food (Bhat and Mathur, 1998; Slampova et al., 2001), pharmaceuticals and cosmetics (Dziezak, 1987; Francis, 1989; Kim et al., 1995). A major shift from natural to synthetic dyes and pigments started with the invention of first synthetic dye by William Henry Perkin in 1856. Generally, there are six different classes of synthetic dyes (Table 1.1). Most widely used dyes on industrial scale are azo lead by anthraquinones, triarylamines, and phthalocyanines (Easton, 1995; Aberoumand, 2011; http://www.understandingfoodadditives.org/pages/Ch2p1-2.htm#Natural).

Generally, synthetic dyes are broadly aromatic compounds which are made color fast and chemically strong to persist in oxidative environmental conditions in water and soil (Saranaik & Kanekar, 1995; Banat et al., 1996; Modi et al., 2010). Moreover, the reduction by-products of dyes with substituents like halogen (Cl), Sulfo, nitro, methyl or methoxy are comparatively more toxic than intact dyes (Chung & Cerniglia, 1992). The harmful effects of dyes have been reported at all trophic levels i.e., from microbes to vertebrates. Typically, they proved to be toxic, carcinogenic and mutagenic in nature (Spadaro et al., 1992; Lu et al., 2010; Modi et al., 2010) besides causing allergic, skin and reproductive problems (Kučerová et al., 1987; Collier et al., 1993). The Ecological and Toxicological Association of the Dyestuffs Manufacturing Industry (ETAD) reported more than 90% dyes with LD_{50} values above 2 x 10^3 mg/kg, so they are banned by many western countries (Shore, 1996; Robinson et al., 2001).

Recently, there is growing concern in environmental circle to mitigate hazardous effects of synthetic dyes in the ecological health perspective. In this reference, various scientists are promoting the use of natural pigments procured from natural sources like plant, animals and different microbes (Duran et al., 2002). The natural colorants considered for industrial level applications are safe and biodegradable regenerating the basic elements to the nature. The reason for their non-toxic nature has been related to absence of harmful substitutions which normally constituted in synthetic dyes (Duran et al., 2002; Raisanen, 2002).

1.1 Natural colorants (dyes and pigments)

The pigments of biological origin contain Nitrogen and Oxygen. They are secondary metabolites of different living sources synthesized, accumulated or excreted by them. Pigments are compounds that absorb light in the wavelength range of the visible region (400-700nm). The color of the biological molecules is due to its size, structure, electron's energy state, elemental composition and solubility. They can also be artificially synthesized and the examples include Riboflavins, Canthaxanthins and beta Carotenes. Unlike dyes or secondary colored metabolites, pigments are generally water insoluble, retain their crystalline and particulate nature and require additional

fixatives to attach to the substrate. (Andrews et al., 1976; Baranyovits, 1978; Bosevska et al., 1993; Boyles and Wrolstad, 1993; Barth et al., 1995; Alexandra et al, 2001; Beyer et al., 2002).

1.2 Sources of natural colorants

Natural pigments and colored metabolites can be procured from natural sources like plants (indigo and saffron), animals (mollusks or shellfish), insects (cochineal beetles), minerals (ferrous sulfate, ochre and clay) and some microbes (bacteria and fungi) (Johns and Stuart 1991; Hanagata et al. 1993; Delgado-Vargas et al., 2000; Kadolph, 2008). Comparatively, microbial sources are currently more preferred in terms of continuous mode production of colored compounds under laboratory setups (Duran et al., 2002). Microbial pigments are broadly classified as polyketides and carotenoids (Mapari et al., 2006). Different bacterial (*Serratia, Chromobacter*) (Williams, 1973; Rettori & Duran, 1998; Kim et al., 1999; Ahmad et al., 2012), Streptomyces (Bystrykh et al., 1996; Kelemen et al., 1998) and fungal species (*Monascus, Paecilomyces, Cordyceps, Penicillium*) have been reported to produce industrially important colored metabolites (Hajjaj et al., 1997; 2000; Cho et al., 2002; Unagul et al., 2005).

1.3 Fungal pigments

Fungi, specifically filamentous (Ascomycota) are preferred over other microbes (Berry, 1988; Hejazi and Wijffels, 2004) in terms of scope, production and stability of colored metabolites under ambient conditions (Zollinger, 2003). The classes of stable colored metabolites of microbes can be represented as carotenoids (Nielsen and Holst, 2002; Adrio and Demain, 2003), flavonoids, quinones, rubramines, polyketides (Hejaji et al., 2000), alkaloids and terpenoids (Mapari et al., 2006). The most important and widely studied fungi with reference to high yield and stable pigment production are *Monascus* (Mak et al., 1990; Johns and Stuart, 1991; Yongsmith et al., 1993; Hamdi et al., 1997; 2000). Moreover, ascomycete fungi (*Penicillium* sp., *Epicoccum nigrum, Fusarium* sp.) have been studies thoroughly (Hajjaj et al., 2000).

1.4 Bio-processes used for production of colored metaboites

Generally, the bio-process used to produce color metabolites or pigments are batch and continuous mode. Moreover, they can be harvested in optimized artificial laboratory condition in solid or liquid state fermentation (Submerged fermentation) using packed bed columns, rotating drum fermenters and open trays reactors (Han and Mudget, 1992; Lee et al., 2002; Rosenblitt et al., 2002). Basal production media used are; minimal salt media (MSM), sabouraud dextrose broth, potato dextrose broth, czapek dox broth and yeast malt broth (Mapari et al., 2009; Pradeep et al., 2013). Moreover, cheap nutrients sources like (rice, wheat straw, grape waste and other raw materials) can also be used in order to make the bio-process cost-effective (Smedsgaard, 1997; Frisvad and Thrane, 2004, Samson et al., 2004). Biosynthesis of colored secondary metabolites, their chemical nature, and their stability has been found to be regulated through factors like carbon source, nitrogen source, pH, temperature and light (Lee et al., 1995; Gunasekaran and Poorniammal, 2008; Quereshi et al, 2010). Therefore, it is of utmost importance to consider these parameters to procure efficient pigments from fungi.

1.5 Applications of fungal pigments in different industries

The colored metabolites are reported to have antibacterial, antifungal, antioxidant (Frisvad, 1989) and anticancer activities. The use of medicinally important natural colorants can be an additional advantage of using them in food and dyeing industries (Gupta and Shukla, 1992). Previously, the application of these fungal pigments like anthraquinones and pre-anthraquinones in cloth dyeing has also been reported (De Santis et al., 2005; Nagia & EL-Mohamedy, 2007). In Japan, the annual consumption of *Monascus* pigments in Japan's food industry alone moved to 600 tonnes at the end of the 1990s, valued about $1.5 million (Duffose, 2006). One can predict that fungal colorants might replace the other synthetic and natural pigments in near future (Nielsen et al., 2004).

1.6 Limitations with reference to production of colored metabolites

Natural pigment production and exploitation at large scale is still falling some serious shortfall in terms of efficient, cost effective production, characterization and valued application. An effective biotechnology solution that is sustainable and environmentally friendly is needed for successful usage of pigments from fungi. Long term optimized batch fermentation procedures can lead to possibly higher yields and easier extraction (hobson and wales, 1998; Aberoumand, 2011). Isolation of new fungal strain is still of particular interest with suitable characteristics for submerged cultivation (Rasheva et al., 1998).

1.7 Aims and objectives of study

The basic aim of the study was to screen out the ability of different fungi for bioactive pigments or colored secondary metabolites production under solid and liquid state fermentation conditions. It evaluated the role of different operational parameters on the enhanced production and stability of colored metabolites. Detailed characterization procedures were adopted in order to classify and identify diverse array of the fungal compounds of varying significance. Biochemical nature of the compounds in fungal extracts was inferred by using different biological, antioxidant/reducing, cytotoxic, phytotoxic and mammalian cell lines assays.

The specific objectives of the research work are as follows;

- Isolation, identification (molecular) and screening out of pigment producing (solid and liquid state fermentation) fungi
- Production of colored metabolites of fungi under liquid state fermentation under varying conditions of pH, temperature, culture media, C and N source and their ratios (C/N).
- Biochemical stability of colored metabolites of fungi at different temperatures and pH.

- Qualitative and quantitative screening of different classes of compounds exogenously produced by different fungi
- Biological screening of colored extracts of fungi for antimicrobial, antioxidant (DPPH), free radical scavenging (DPPH, ABTS) and reducing activities.
- Bulk production of colored metabolites (under solid state fermentation) and their extraction and purification using different chromatographic techniques.
- Structural elucidation of compounds after using techniques like LCMS, LCMS-MS, proton nuclear magnetic resonance (NMR) and 3 dimensional X Ray crystallography (3D XRD) techniques.
- Cytotoxicity (using normal and cancerous mammalian cell lines) studies of colored fractions obtained from different fungi.

LITERATURE REVIEW

2. Review of Literature

Colors are molecules that absorb specific wavelengths of visible light and transmit or reflect others. Whereas, colorant is the activity of this molecule that is displayed when applied to a substance/substrate (Hari et al., 1994). A colorant can be both a pigment and a dye depending on mode of application. The bio-colorants are coloring agents whose source is natural or nature identical. They can be obtained from different biological sources which can be unicellular like bacteria or multicellular like plants, animals and fungi. Certain bio-pigments like anthocyanin are water soluble, whereas, most of them are insoluble in water (resulting in a suspension). They are mostly used in ink, paper and textiles. Dyes on the other hand are generally easily miscible in organic solvents. Certain colored compounds like carotenoids can act as pigments in water while dyes in oil. Natural colorants are pigments produced by living organisms. Colorants such as Caramel, Cu-chlorophyllin and vegetable carbon do not exist naturally but they are still considered as natural (Mortensen, 2006). Nature-identical colorants such as b-carotene and riboflavin are artificially manufactured; however, they also exist in nature. Moreover, natural bio-colorants are different from artificial ones not only because of their sources, but also in terms of composition, related biochemical and physiological activities. Generally, impurities are much more in natural bio-colorants. Yet, natural bio-colorants are much bioactive as compared to their chemical counterparts (Mapari et al., 2010). Recently, the trend is increasing towards natural pigments because of consumer's demand for clean label and safe ingredients. Researchers can foresee that natural pigments can replace harmful synthetic pigments in upcoming times at industrial level i.e., food, leather, textile and pharmaceutical.

2.1. History of bio colorants

Dyes or colorants are being used by man for various purposes and the earliest record of their use dates back to 2600 BC in china. Dyes were in use in Europe since Bronze Age. In Mohenjodaro and Harappa civilizations (3500 BC), colorants (madder) were used in dyeing clothes. In India, dyes were used since Indus valley civilization in 2500 BC. In Egypt, the red clothes found in the tomb of King Tutankhamen were colored with Alizarin, a natural pigment of madder. Dyes such as indigo and madder

were known in 4th century AD and use of saffron has also been mentioned in Bible. The use of biocolorants in food has been practiced in Japan since 8th century as evident in shosoin text of the Nara period where they were used to color soybean and adzuki-bean cakes. The natural colorants from plants and wines were used as food colorant as early as 1500 BC (Downham and Collins, 2000). The increasing demand of various industries for colorants has led to development of various synthetic dyes in the late 19th century and it continued till 20th century in the form of the coal-tar dyes.

2.2. Need of bio-colorants

The growing application of synthetic dyes or colorants in dyeing industries is because of their easier and more economical synthesis. Moreover, the efficient coloring properties of chemically manufactured colors are because of their aromatic structures which make them more color fast. However, due to poor exhaustion rates of dyes, a significant proportion (10–50%) of them is wasted in dye-bath and ultimately finds their way into the environment (Rai et al., 2005). Moreover, the hazards associated with synthetic colorants i.e., toxic, carcinogenic (Osman et al., 2004), mutagenic (Spadaro et al., 1992; Modi et al., 2010), allergenic and intolerance reactions (Blenford, 1995; Reyes et al., 1996), polluting nature, resulting in infertility and chromosomal aberrations is a serious threat (Kucerova et al., 1987; Collier et al., 1993). Synthetic dyes generally contain benzene backbone moiety (aromatic) that makes them more recalcitrant in nature. Their toxicity is increased by substituents like nitro, methyl, methoxy or halogen groups. One example of the synthetic colorant is Sudan series which are banned due to their carcinogenicity. In addition, under the new legislation approved by the European Parliament, foodstuffs containing artificial colorants (sunset yellow carmoisine, quinoline yellow, tartrazine, ponceau 4R and allura red) must be labeled as "may have an adverse effect on activity and attention in children" (Fruend et al., 1988; Francis, 1989). Food and drug administration (FDA) reported (1993) that dyes containing metals such as lead chromate and copper sulphate possess the potential risk of causing serious health and environmental hazards (Francis, 1989). In US during 1960's, diverse environmental activities were made pointing out hazardous effects of synthetic colorants and showing the benefits of natural colorants especially emphasizing their dietetic characteristics as sales tools.

Currently, natural colorants are again overtaking synthetic counter parts in market (Carvalho et al., 2003; Joshi et al., 2003).

The chemistry of natural colorants cannot fail to attract the present day consumers and in turn different industries thus making them the most important part of any product (Clydesdale, 1993). In the last few years, the accessibility and utilization of natural colorants has significantly increased (Dweck, 2009) as an outcome of legislative action which has sustained the delisting of approved artificial colorants (Garcia and Cruz-Remes, 1993). The current consumer inclination towards naturally derived colorants is largely due to consumer's awareness over the link between diet and health as well as environmental concerns. So, there is an increasing trend especially in food industry towards clean label ingredients in form of food colorants obtained from natural sources. The value of the international colorant market for food industry was estimated at around $1.07 billion USD in 2004 and it rose to $1.15 billion USD in 2007 i.e., a 4.6% increase in 3 years (www.foodnavigator-usa.com). Moreover, trials are being carried out at laboratory scale in order to use these bio-colorants in industries like leather and textile (Velmurugan et al., 2010).

2.3. Source of bio colorants

Colorants are considered as natural pigments/colored metabolites when they have biological origin i.e., plants, animals or microorganisms (Joshi et al., 2003). A few of plant origin are accessible in market at adequate quantities for commercial usage in food, beverages, cosmetics and pharmaceuticals (Chengaiah et al., 2010). The details about source of natural colorants from different biological sources are mentioned in the following text.

2.3.1 Animal pigments/colored metabolites

Among animals, vertebrates (Amphibians, reptiles, birds, and mammals) have been reported to produce pigments like melanins, carotenoids, haem proteins and riboflavin. Similarly, invertebrates (Echinoderms, insects and molluscs) are known to produce carotenoids, quinones, melanins, heme and flavonoids (Hendry, 1996). The main pigment carminic acid was first isolated from invertebrates in 1858 and its

structural elucidation was done in 1913 (Allevi et al., 1998). Its biosynthesis in insects starts with an aliphatic heptaketide and is supposed to proceed through a five-stage process (Dawson, 2009). Scale insects (family Coccoidea) have been used to procure colorants (anthraquinones) for dyeing clothes. These include; Kermes vermilio, *Kerria lacca* and cochineal like *Porphyrophora hamelii*, *Dactylopius coccus* and *Porphyrophora polonica*. Cochineal is allowed in the EU and the United States (Santamaría et al., 1994; Schul, 1994).

2.3.2 Plant Pigments/colored metabolites

Natural colorants are obtained from plants like red beet and saffron in addition to grapes and pepper (Bridle and Timberlake, 1997; FDA/IFIC, 2000). The plant pigments are classified on the basis of their chromophores into the following types.

2.3.2.1 Carotenoids

Carotenoids are lipid-soluble found in some animals and all higher plants (Klaui, 1967; Bartley and Scolnik, 1995). They are most important group of pigments (Zeb and Mehmood, 2004) and their color range is yellow, orange to red. **ß-Carotene** is orange-yellow pigment which is soluble in oil but can be made into a water dispersible emulsion. The source of β-carotene is carrot (Barth et al., 1995) but most ß-carotene for commercial usage is currently extracted from algae. **Lycopene** is found in plants containing β-carotene as it is a precursor in the biosynthesis of ß-carotene. Though lycopene is found in plenty quantity in tomatoes (Giovannucci et al., 2002), this pigment is also found in other plants species i.e., Kapia pepper, red pepper, onion, *Rosa rubiginosa*, *Taxus baccata*, *Calendula officinalis* and *Citrullus lanatus* (Giusti et al., 1998; Dweck, 2009). **Xanthophylls** are oxygenated carotenes and orange to yellow in color. They are found in *Taxus baccata* (rhodoxanthin) (Dweck, 2009) whereas, yellow color in dog rose, *Rosa canina* is also due to Rubixanthin (Siwa, 2007). **Lutein** is also a very common carotenoid. Marigold flowers are by far the most copious natural source for commercial lutein (Vernon-Carter et al., 1996; Jothi, 2008). Whereas, other sources of lutein are Zucchini, different green vegetables (cabbage, spinach) and some fruits (Muntean, 2005). Lutein is not approved as a food colorant in the USA and allowed only for chicken feed (Mortensen, 2006). **Annatto** imparts

yellow and orange color to dairy products and has been used for over two centuries. The source of this pigment is outer layer of seeds of the tropical tree *Bixa orellana* (Haila et al., 1996), red carotenoids in paprika (*Capsicum annuum*), crocin obtained from dried styles and stigmas of the saffron plant, *Crocus sativa* (Farrell, 1985).

2.3.2.2 Flavonoids

The flavonoids are widespread group of polyphenolic compounds resulting in the yellow color of agricultural products. They are broadly distributed in the plant kingdom reaching about over 4000 structurally unique flavonoids (Patel, 2008).

Quercetin is the most important flavonoid and different plants including apples, onions, Cruciferae family, *Sambucus nigra* (Filimon, 2009), *Quercustinctoria* (Gilbert and cooke, 2010) and horse chestnuts are its richest source. **Luteolin** is found in perennial plant saw-wort, *Serratula tinctoria* L. and *Reseda luteola* was also a yellow dye yielding plant. **Anthocyanidins** are the enormously colored flavonoids (Hancock, 1997). Anthocyanins are a class of compounds fitting into phenols and are mostly present in vegetables in addition to flowers and fruits giving different color hues (blue, purple, red and orange) (Delgado vargas et al., 2000). So far, more than 540 anthocyanin pigments have been identified in nature (Anderson and Francis, 2004), with structural variations arising from glycosidic substitution at the 3 and 5 positions. Garden Huckleberry (*Solanum scabrum*) contains petunidin (Lehmann et al., 2007), Pelargonidin is found in Red radish roots (Giusti et al., 1998), onion and solid wastes (Makris, 2010) whereas, Cyanidin glucoside and cyanidin 3-rutinoside is found in mulberry. **Chlorophyll** is the green pigment of higher plants involved in their photosynthesis. It is used in several other foodstuffs i.e., jelly, candy and ice cream but chlorophyll finds narrow use as a colorant because of the liability of the coordinated magnesium and the limited color change (Mortensen, 2006). Chlorophyll is obtained from edible plants, grass, nettle and mulberry leaves. **Anthracenes**; Anthraquinones are the leading group of quinones, best recognized for their usage as mordant dyes. Various anthraquinones includes alizarin, purpurin, mungistin from Madder family; emodin from Persian berries, kermes, lac and Napthoquinones, e.g., alkanin, juglone (walnut) and hypericin. Other source of anthraquinone red dyes are madder plants i.e., Naga madder (*R. sikkimensis*) and Indian madder (*R. Cordifolia*

Linn) (Patel, 2011). Noni plant (*Morinda citrifolia*) is also a potential source of anthraquinones (Kamiya et al., 2010) and various flavonoids (Ramamoorthy and Bono, 2007; Deshmukh et al., 2011). **Betacyanins (betalains)** are acquired from the red beet (*Beta vulgaris*) plant used as a food coloring agents (Im et al., 1990) and *Chenopodiacae* family. It has wide application in diverse food commodies (Beverages, candy, dairy and cattle products) (Counsell et al., 1979). Bixin obtained from seeds of Sinduri (*Bixa orella* Linn.) imparts orange to yellow color to the products. Portulaca and goosefoot families also yield noteworthy quantity of betacyanins (Hancock, 1997). Indigo blue is also well-known to be present in few plants like woad (*Isatis tinctoria)*, *Nerium tinctorium*, knotweed *(Polygonum tinctorium; P. aviculare*) and *Lonchocarpus cyanescens* (Hancock, 1997).

2.3.3 Microbial Pigments/colored metabolites

Microorganisms possess capability to produce various bio-molecules such as antibiotics, vitamins, enzymes, texturizing agents and pigments (Suay et al., 2000; Visalakch and Muthumary, 2009; Zhang et al., 2009). Fungi, bacteria and algae are characterized based upon the production of diverse array of intra and extra-cellular pigments. In nature, there is abundant pigment producing organisms. Some of the microbial pigments includes carotenoids, melanins, quinones, prodigiosins, flavins, and more specifically Monascins and indigo (Joshi et al., 2003). These organisms are envisaged as the most expected marketable sources of novel pigments using different cell culture techniques.

Microbial colors are being used in different applications. For example, they are utilized in fish food industry to enhance the pink color of farmed salmon (Anderson, 2001). Some natural pigments are also being used as antioxidant (Yang et al., 2006). Microbial pigments perform diverse functions. β-Carotene and xanthophylls like Astaxanthin play central role in metabolism of eye's macula and retina and help maintaining a healthy vision. β-Carotene also plays a vital part in cancer prevention (Peto et al., 1981; Nakano et al., 1999; Siems et al., 2005). Xanthophyll functions as chemo-protective and may act as nutraceuticals (Adonirubin and Astaxanthin) that prevent carcinogenesis through anti-oxidative, free radical scavenging or other mechanisms (Young and Lowe, 2001) with additional advantages of heart attack and

strokes prevention (Arab and Steck, 2000). Microbial production of carotenoids and other pigments appears to be predominating as compared to the extraction from vegetables or chemical synthesis because of the seasonal problems in the production of several pigments of plant origin. The second reason is the economic advantage of the microbial processes that employs natural low cost substrates as carbohydrate source.

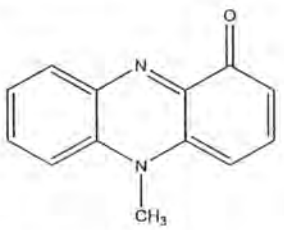
2.3.3.1 Bacterial Pigments/colored metabolites

Chlorophylls and carotenoids are present in photosynthetic bacteria whereas in non-photosynthetic bacteria, β-carotene has been recognized; yet, quinones, melanins, and flavonoids are infrequently found in this group. **Carotenes** are polyunsaturated hydrocarbons that comprise 40 carbon atoms per molecule. A rare halophilic bacterium, which necessitates 15–25% salt for its normal growth, produces extensive variety of isoprenoid compounds (phytoene, lycopene, phytofluene and *β*-carotene). The **violacein** is violet colored indole derivative, largely isolated from bacterial genera *Chromobacterium* (Rettori and Duran, 1998). **Phenazines** are small nitrogen-containing aromatic compounds that are redox active produced by a diverse array of bacterial genera (Turner and Messenger, 1986; Maskey et al., 2003; Pierson and Pierson, 2010). **Quinones** are colored compounds with an aromatic ring structure that range in color from yellow to orange red having many biological activities and they are used commercially (Akagawa-Matsushita, 1992; Maskey et al., 2003). **Prodiginines** share a mutual pyrrolyl dipyrromethene core structure having antimicrobial, antimalarial, immunosuppressive and anticancer activities (Kim et al., 1999; Bennett and Bentley, 2000; Williamson et al., 2007).

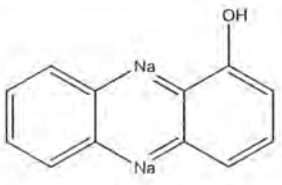
Table 2.1: Examples of bacteria with their specific pigments and related applications are shown.

Bacterial strains	Pigment	Activity	References
Streptomyces rubber	Undecylprodigiosin	Anticancer	Gerber, 1975
Pseudoalteromonas denitrificans	Cycloprodigiosin	Immunosuppressant; Anticancer; Antimalarial	Kawauchi et al., 1997; Kim et al., 1999; Yamamoto et al., 2001
α-Proteobacteria	Heptyl prodigiosin	Antiplasmodial	Lazaro et al., 2002
Pseudoalteromonas rubra, Hahella chejuensis	Prodigiosin	Antibacterial; Anticancer; Algicidal	Gerber and Gauthier, 1979; Kim et al., 2007
Agrobacterium aurantiacum	Astaxanthin (carotene)	Antioxidation	Gauthier, 1976; McCarthy et al., 1985; Novick and Tyler, 1985; Misawa et al., 1995
Pseudoalteromonas tunicate *Pseudoalteromonas* sp. 520P1 *Pseudoalteromonas luteoviolacea* *Collimonas* CT	Violacein	Antibiotic; Antiprotozoan; Anticancer	Matz et al., 2004; Yada et al., 2008; Hakvag et al., 2009
Pseudonocardia sp. B6273	Methyl saphenate (Phenazine derivative)	Antibiotic	Maskey et al., 2003
Bacillus sp	Phenazine derivatives	Cytotoxic	Lee et al., 2007
Pseudomonas aeruginosa	Pyocyanin and pyorubrin	Antibacterial	Saha et al., 2008
Pseudomonas aeruginosa	Phenazine-1-carboxylic acid	Antibiotic	Nansathit et al., 2009
Streptomycete sp	5,10-dihydrophencomycin	Antibiotic	Pusecker et al., 1997
Streptomycete sp. B6921	Fridamycin D, Himalomycin A, Himalomycin B	Antibacterial	Maskey et al., 2003
Streptomycete sp. M045	Chinikomycin A and Chinikomycin B, Manumycin A	Anticancer	Li et al., 2005
Pseudoalteromonas tunicate	Tambjamines (BE-18591 pyrrole and their synthetic analog	Antibiotic, Anticancer	Kotob et al., 1995; Ruzafa et al., 1995; Franks et al., 2005; Pinkerton et al., 2010

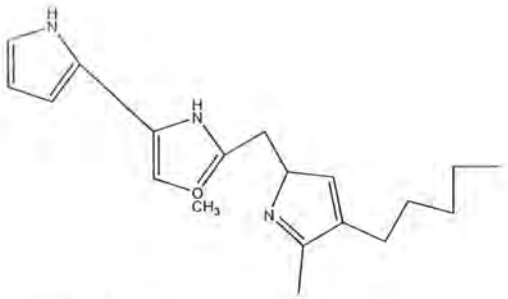
Bacterial strains	Pigment	Activity	References
Vibrio cholera *Shewanella colwelliana* *Alteromonas nigrifaciens* *Cellulophaga tyrosinoxydans*	Melanins	Protection from UV irradiation	Fuqua and Weiner, 1993; Kotob et al., 1995; Ivanova et al., 1996; Kahng et al., 2009
Cyanobacteria	Scytonemin	Protection from UV Anti-inflammatory, Antiproliferative	Stevenson et al., 2002
Cytophaga/Flexibacteria AM13,1 strain	Tryptanthrin	Antibiotic	Wagner-Dobler et al., 2002



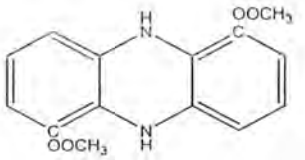
Pyocyanin



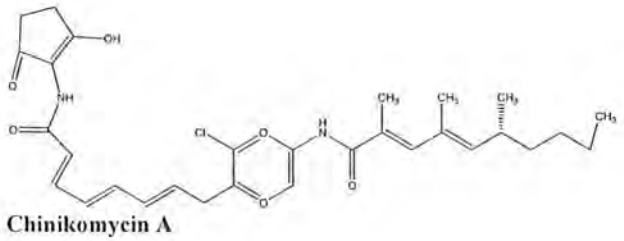
1 Hydroxyl phenazine



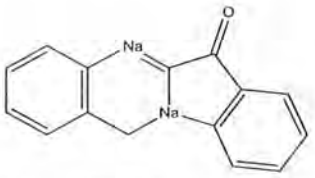
Prodigiosin



5,10-Dihydrophencomycin methyl ester phenazine derivatives



Chinikomycin A



Tryptanthrin

Fig. 2.1: Structures of Bacterial pigments/ colored metabolites

2.3.3.2 Fungal pigments/colored metabolites

The diversity among fungal pigments has been considered as the second most after plant flavonoids. Fungal pigments are secondary metabolites and can be broadly categorized as polyketides and carotenoids on the basis of their chemical nature. Fungal polyketide pigments are very important group of pigments ranging in structure from tetra to octaketides (4-8 Carbon units) contributing to the polyketide chain (Mapari et al., 2010). Some of these polyketides are produced through mixed biosynthesis by involvement of other pathways (terpenoid or amino acid synthesis), in addition to the polyketide pathway. Representative classes are anthraquinones, azophilones, hydroxyanthraquinones and naphthoquinones with diverse color arrays. Ascomycetous, basidiomycetous fungi and lichens produce a variety of colors belonging to several chemical classes of pigments. Only 355 species of fungal kingdom have been investigated for the production of natural pigments and colorants. Among them, 37 species belong to lichens while 318 belonged to mushrooms and other fungi (Maldonado et al., 2005). Mushrooms and lichens possess a rich history for being used as a promising source of pigments for dye stuff industry. Mycelial extracts of some mushrooms *Bankera violascens* gives greens, *Chroogomplus vinicolor* gives red tints and *Collybia iocephala* gives blues (Maldonado et al., 2005). All of them possess a high potential for dyeing silk fabrics and wool.

But such fungi are not feasible to grow under lab conditions hence, not appropriate for scale up production. Whereas, ascomycetous fungi are relatively more suitable for biotechnological production because they can be easily grown under controlled conditions and higher yields can be obtained by the existing culturing methods (Mapari et al., 2009). Fungi have been source of more than 1000 identified pigments. Fungi are non-photosynthetic as they do not contain chlorophyll. Carotenoid distribution in fungi is limited to specific orders (e.g., Pharagmobasidiomycetidae, Discomycetes) while, flavonoids are also rare in fungi e.g., riboflavin imparts the yellow color in the genera *Lyophyllum* and *Russul*. Other pigments are frequently found in fungi and plants i.e., melanins, a small number of carotenoids, betalains and certain anthraquinones.

Recently industrial approach for using fungi as pigment producer is revived as, *Blakeslea trispora* has been the source of β-carotene in the Netherlands and has also been approved for use in food industry (Mapari et al., 2010). Carotenoids are basically orange to red natural colorants with about 750 identified structures (Britton et al., 1996). Ascomycetous polyketide class of pigments is being over looked as potential future food colorants.

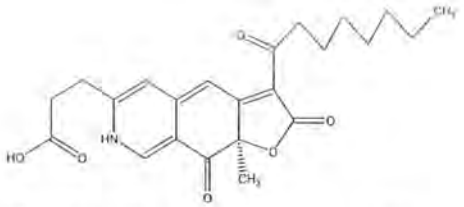
Table 2.2 Examples of different fungi with their specific pigments

Fungal species	Pigment composition (Color)	Biosafety level classification	Reference
P. atramentosum	Uncharacterized dark brown not characterized yet	Not known	Fisvad et al., 2004
P. atrosanguineum	Phoenicin (red) yellow and red not characterized	Not known	Christensen et al., 1999
P. atrovenetum	Norherqueinone (red), Atrovenetin (yellow)	Not known	Raistrick and Stossl, 1958
P. aurantiogriseum	Not characterized	1	Fisvad et al., 2004
P. brevicompactum	Xanthoepocin (yellow)	1	Fisvad et al., 2004
P. chrysogenum	Sorbicillins (yellow), Xanthocillins (yellow)	1	Fisvad et al., 2004
P. citrinum	Anthraquinones (yellow), Citrinin (yellow)	1	Duran et al., 2002
P. cyclopium	Viomellein (reddish-brown), Xanthomegnin (orange)	Not known	Fisvad et al., 2004
P. discolor	Uncharacterized	Not known	Fisvad et al., 2004
P. echinulatum	Uncharacterized (yellow)	Not known	Fisvad et al., 2004
P. flavigenum	Xanthocillins	Not known	Fisvad et al., 2004
P. freii	Vioxanthin, Xanthomegnin (orange), Viomellein (reddish-brown)	Not known	Fisvad et al., 2004
P. herquei	Herqueinones (red and yellow), Atrovenetin (yellow)	Not known	Robinson et al., 1992
P.oxalicum	Arpink redTM- anthraquinone derivative (red) Secalonic acid D (yellow)	Not known	Mapari et al., 2005, Sardaryan, 2002
P. persicinum	Uncharacterized (Cherry red)	Not known	Fisvad et al., 2004

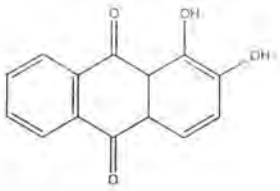
Fungal species	Pigment composition (Color)	Biosafety level classification	Reference
P. viridicatum	Vioxanthin, Viomellein (reddish-brown), Xanthomegnin (orange)	Not known	Fisvad et al., 2004
P. paneum	Uncharacterized (red)	Not known	Fisvad et al., 2004
T. macrosporus	Mitorubrin (yellow)	Not known	Frisvad et al., 1990
P. islandicum	Erythroskyrin (orange-red), Luteoskyrin (yellow), Emodin (yellow), Skyrin (orange)	Not known	Frisvad, 1989
P. funiculosum	Uncharacterized	Not known	van Reenen-Hoekstra et al., 1999
P. marneffei	Mitorubrinol, Monascorubramine (purplered), Purpactin, Rubropunctatin (orange), Secalonic acid D (yellow)	3	[unpublished]
P. pinophilum	uncharacterized	Not known	van Reenen-Hoekstra et al., 1999
P. purpurogenum	Mitorubrin (yellow), PP-R (purple red), Mitorubrinol (orange-red), Purpurogenone (yelloworange)	1	Buchi et al., 1965, Frisvad, 1989, Mapari et al., 2006
P. rugulosum	Rugulosin (yellow)	1	Frisvad, 1989
P. variabile	Rugulosin (yellow)	Not known	Frisvad, 1989
E. amstelodami	Auroglaucin (orange) Erythroglaucin (red) Physcion (yellow) Flavoglaucin (yellow)	1	Frisvad and Thrane, 2004
E. chevalieri	Auroglaucin, Erythroglaucin, Flavoglaucin Physcion (yellow)	1	Frisvad and Thrane, 2004
E. herbariorum	Flavoglaucin (yellow), Aspergin (yellow), Physcion (yellow)	1	Frisvad and Thrane, 2004
A. ochraceus	Vioxanthin , Xanthomegnin (orange), Viomellein (reddish-brown)	1	Frisvad et al., 2005
A. melleus	Viomellein (reddish-brown) Xanthomegnin (orange), Rubrosulphin (red), Viopurpurin (purple)	Not known	Frisvad et al., 2005
A. sulphureus	Viomellein (reddish-brown) Xanthomegnin (orange), Rubrosulphin (red), Viopurpurin (purple)	Not known	Frisvad et al., 2005
A. westerdijkiae	Rubrosulphin (red), Viomellein (reddish-Nobrown) , Xanthomegnin (orange), Viopurpurin (purple)	Not known	Frisvad et al., 2004

Fungal species	Pigment composition (Color)	Biosafety level classification	Reference
A. niger	Flavioline (orange-red), Nnaptho-R-pyrones (yellow)	1	Samson et al., 2004
A. sclerotioniger	Uncharacterized yellow	Not known	Samson et al., 2004
Em. falconensis	Falconensins C-H (yellow), Zeorin (yellow), Falconensones (Yellow)	Not known	Ogasawara et al., 1997
Em. purpurea	Epurpurins A-C (yellow)	Not known	Hideyuki et al., 1996
A. versicolor	Sterigmatocystin (yellow)	1	Davies et al., 1960
Fusarium acuminatum	Aurofusarin (red), Antibiotic Y (yellow)	Not known	Thrane, 2001
F. avenaceum Antibiotic Y (yellow) Aurofusarin (red)	Antibiotic Y (yellow), Aurofusarin (red)	Not known	Thrane, 2001
F. culmorum	Aurofusarin (red), Fuscofusarin (yellow), Rubrofusarin (red)	Not known	Thrane, 2001
F. fujikuroi	Bikaverin (red), Norbikaverin (red), O-demethylanhydrofusarubin (red)	1	Medentsev and Akimenko, 1998
F. graminearum	Rubrofusarin (red), Aurofusarin (red),	Not known	Thrane, 2001
F. oxysporum	O-methyljavanicin, 2,7-dimehoxy-6-(acetoxyethyl)juglone (yellow), Bikaverin (red), Bostrycoidin (red) , O-methylanhydrofusarubin (orange-red), Nectriafurone (yellow), Norjavanicin (red) ,O-methyl-6- hydroxynorjavanicin (yellow), O-methylfusarubin (red)	2	Medentsev and Akimenko, 1998
F. poae	Aurofusarin (red)	Not known	Thrane, 2001
F. sambucinum	Aurofusarin (red)	Not known	Thrane, 2001
F. solani	O-methyldihydrofusarubin, Fusarubin (red), somarticins (red) O-ethylfusarubin (red)	2	Medentsev and Akimenko, 1998
F.sporotrichioides	, Lycopene, Aurofusarin (red)	Not known	Thrane, 2001
F. stilboides	Antibiotic Y (yellow), Aurofusarin (red), Nectriafurone (yellow)	Not known	Unpublished
F. tricinctum	Antibiotic Y (yellow)	Not known	Thrane, 2001
F. venenatum	Aurofusarin (red) Rubrofusarin (red)	Not known	Thrane, 2001
F. verticillioides	O-demethylfusarubin, O-methyljavanicin, Fusarubin, O-methylsolaniol (orange-red)	Not known	Medentsev and Akimenko, 1998

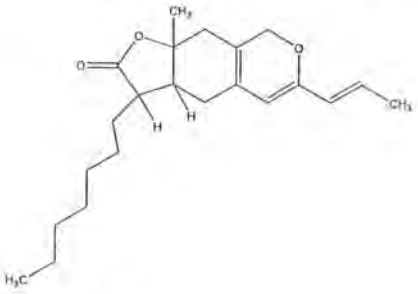
Fungal species	Pigment composition (Color)	Biosafety level classification	Reference
Alternaria dauci	Uncharacterized (red)	1	Andersen et al., 2008
Alt. porri	Altersolanol A (yelloworange)	1	Andersen et al., 2008
Alt. solani	Altersolanol A (yelloworange)	1	Andersen et al., 2008
Alt. tomatophila	Altersolanol A (yelloworange)	Not known	Andersen et al., 2008
Cladosporium cladosporioides	Calphostins (A, B, C, D) (red)	1	Andersen et al., 2008
Cordyceps unilateralis	Erythrostominone (red), Deoxyerythrostominol (red), Deoxyerythrostominone (red) Epierythrostominol (red), 4-O-methyl erythrostominone (red)	Not known	Unagul et al., 2005
Curvularia lunata	Chrysophanol (red), Cynodontin (bronze), Helminthosporin (maroon)	1	Duran et al., 2002
Drechslera spp.	Cynodontin (bronze), Tritisporin (redish-brown), Catenarin (red), Helminthosporin (maroon)	Not known	Duran et al., 2002
*Epicoccum nigrum**	Epicoccarines A & B, Isobenzofuran derivatives (yellow to brown Chromanone (yellow), Flavipin (brown), Orevactaene (yellow), Epicocconone (fluorescent yellow), Epipyridone (red)	Not known	Mapari et al., 2006, Kemami Wangun and Hertweck, 2007, Lee et al., 2007
Paecilomyces sinclairii	Uncharacterized (red)	Not known	Cho et al., 2002
Monascus pilosus	Citrinin (yellow)	1	Wang et al., 2005
M. purpureus	Monascorubramine, Ankaflavin (yellow), Citrinin (yellow), Monascin, Monascorubrin, Rubropunctamine (purple-red), Rubropunctatin (orange)	1	Wang et al., 2005
M. ruber	Monascorubramine, Monascorubrin, Rubropunctatinm (orange), Rubropunctamine (purple-red), Ankaflavin (yellow), Citrinin (yellow), Monascin (red)	1	Wang et al., 2005



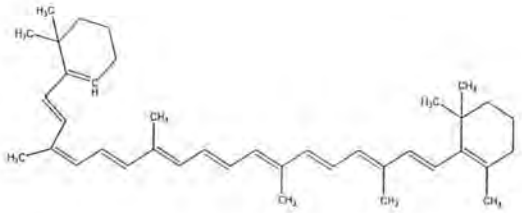
12 carboxyl Monascorubramine or PPV



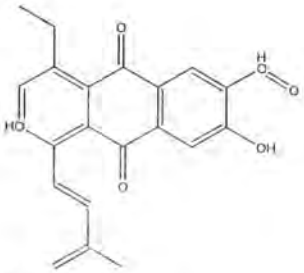
Alizarin



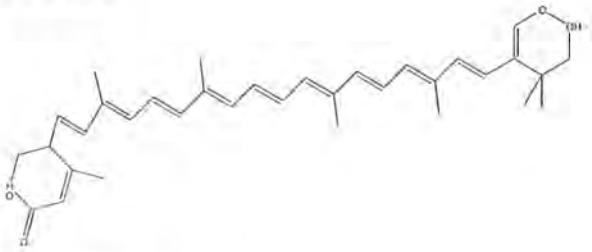
Ankaflavin



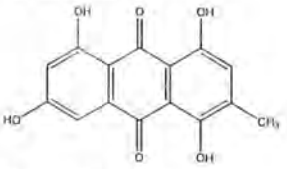
β Carotene



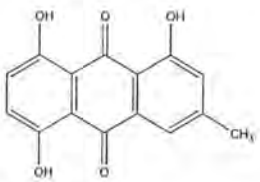
Arpink red



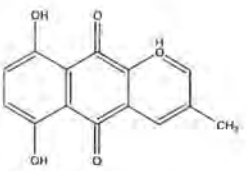
Astaxanthin



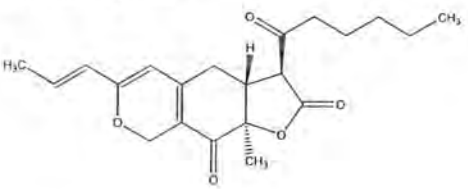
Catenarin red



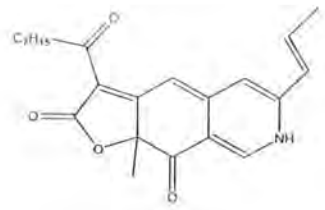
Helminthosporin



Helminthosporin



Monascin



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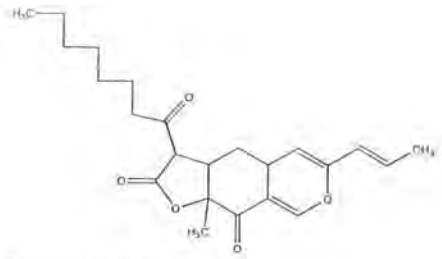

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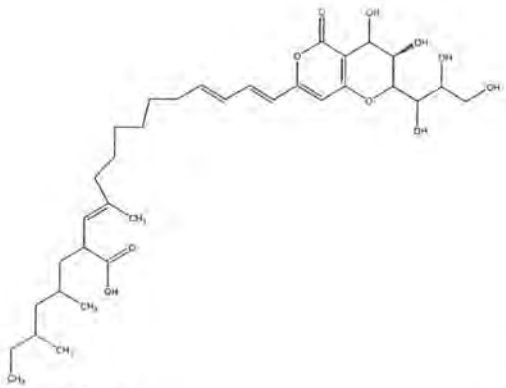

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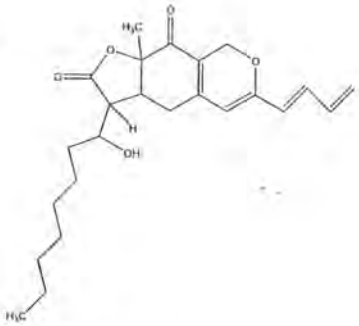

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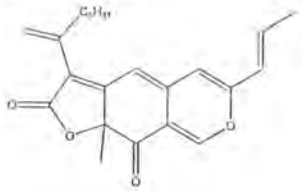
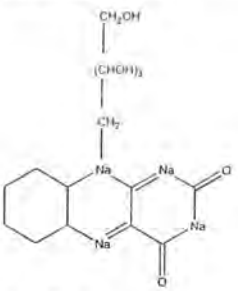
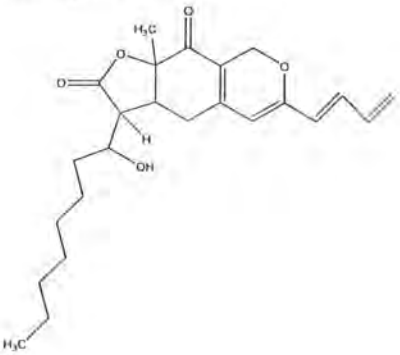


Rubropuntamine



Riboflavin



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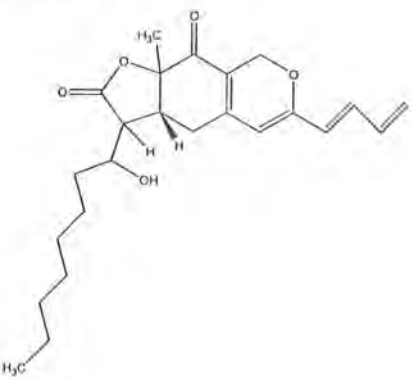
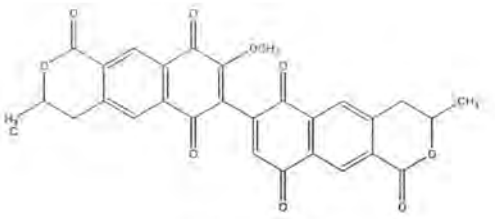


Viomellin



Xanthomagnin

Fig. 2.2: Structures of some fungal pigments

2.4. Characteristics of pigments/colored metabolites

A number of species produce an extensive array of pigments that are essential to cellular physiology and existence. Numerous of these natural metabolites have antibiotic, anticancer and immunosuppressive activities. These secondary metabolites produced by microorganisms have the capability to inhibit the growth of or even kill bacteria and other microorganisms even at very low concentrations. Due to such diverse and promising activities against different disease causing agents, these compounds can play a significant role in both pharmaceutical and agricultural sectors.

Fungal pigments like plant pigments also play an ecological role for the producing organism. For example, melanins also known to be produced by bacteria, (polymerized phenolic and/or indolic compounds) provide resistance to a variety of adverse environmental factors such as desiccation and irradiation (Nosanchuk and Casadevall, 2003). If the producer is in soil, then melanins accumulate in the soil humus, where they may play an important role in manipulating soil chemistry (Valmaseda and Martinez, 1989). Carotenoids are known to have a protective action against photooxidation. Flavins act as cofactors in enzyme catalysis. In the past, polyketide pigments of ascomycetous fungi have been utilized mostly for species identification and differentiation (Frisvad and Samson, 2004) in addition to their applications with respect to food.

However, the specific role of all kinds of pigments, like many other secondary metabolites is scarcely found in the literature. The reason that why these compounds are pigmented is also unexplored. Some studies showed that loss of pigmentation by mutation resulted in loss of antibiotic activity (Holmstrom et al., 1996; Bruhn et al., 2007). One of the auspicious biological activities of the microbial colors

is their cytotoxic effect against cancer cells. Regardless of many investigations, the exact molecular mechanism of the cytotoxicity of pigmented compounds remains unexplored and needs further investigations. For example, violacein can cause apoptosis in tumorous cells (Duran et al., 2007). Nevertheless, the pathways leading to cell death have not yet been related to the possible effects of the pigment, which also displayed its effect on signal transduction agents such as, enzymes of family protein kinase and protein phosphatase playing important part in cell differentiation and proliferation.

2.5 Toxicity studies of bio-colorants

Information on the toxicological aspects of natural pigments is rare compared to synthetic color additives. In general, the only color exempted from certification is known to be caramel. Presently, both certified and exempted color additives are subject to the same toxicological requirements. To obtain the Acceptable Daily Intake (ADI), it is necessary to establish a dose at which no detectable effect can be witnessed. This dose is termed as "no observed adverse effect level" (NOAEL). An additional value generally found in the literature is LOAEL, a low level at which health damage is not observed. NOAEL and LOAEL are obtained as an outcome of long-term animal *in vivo* studies. In these experiments, it is presumed that responses witnessed in humans are similar to most sensitive animal species.

It is evident that metabolism is different among animal species whereas, studies with humans are not conducted most of the time. So, these concerns are taken into account to determine the ADI value. During acute toxicity studies, LD_{50} (The dosage that results in the death of 50% of the experimental animals) is used as an important parameter.

TABLE 2.3: Examples of toxicity studies of natural pigments/colored metabolites

COLORANT	MODEL	EXPERIMENTAL CONDITIONS	RESULTS
Monascus pigments	Mutagenicity in *Salmonella*, hepatocyte and microsome assays	0, 50, 100, 500, 1000, and 5000 µg/plate, comparison with citrinin	In all *Monascus* extracts, citrinin is identified at up to 1.8 µg/g extract and citrinin is a mycotoxin; *Salmonella* microsome assay did not exhibit mutagenicity with or without activation; in the *Salmonella* hepatocyte assay, mutagenicity is dose dependent and this is related with the effect of citrinin; thus, it has been suggested that mutagenicity of *Monascus* is mediated by a complex activation processing in hepatocytes.
Riboflavin	Mutagenicity Umu test, SOS Chormotest, and Ames *Salmonella* assay	0–100 mg/mL, with or without activation (S9 or cecal extract)	Lumiflavin and its activated metabolite have been analyzed; riboflavin did not show mutagenicity but activated lumiflavin showed a significant effect in all the tests; it is suggested that the activated product acts as an DNA intercalating agent.
Carmine	Rats	50, 150 or 500 mg/kg bw/day/109 wk following *in utero* exposure	No clastogenic effect. No carcinogenic activity.
		50, 150, or 500 mg/kgbw/day/three generation	No adverse effect.
		0, 250, 500, and 1000 mg/kg bw/day/90 days	No toxicity observed but only reduced growth rates at the two highest doses.
Caramel	Mutagenicity *S. typhimurium assay* and chromosomal damage in Chinese hamster ovary cells	2.5, 5.0, 10.0, and 20.0 mg/plate, caramel I, II, III, and IV; with or without S9 activation	Neither mutagenic nor chromosomal damage observed; hence, it is concluded that caramelization do not produce detectable levels of mutagens.
	Mice	0, 1.25, or 5% in drinking water/96 weeks followed by 8 weeks of recovery, caramel III	Cumulative mortality has been found at 5% level. Clear pathological differences are not observed; only significant effect is an increase of the total leukocyte counts.
		2.5, 5.0, or 10.0 g/kg bw/day/104 weeks,	No effects.

		caramel IV	
		0, 2, or 10% w/v in water up to 9 weeks	No difference is observed in the number of leukocytes or in the relative number of lymphocytes and neutrophils; however, significant reductions of the T-helper and T-cytotoxic/suppressor cells are observed at 10% dose; lymphopenia is not observed.
Betalains	Rats	Two-generation reproduction study in rats provided with 17g in the parental and 24 in the first generation	Mammary fibroadenomas are observed.
		Two-generation reproduction study in rats provided with 17 g/animal	No effects.
Anthocyanins	Rats Dogs	7.5 or 15% dietary concentration/multi-generational	No adverse effects on reproductive system.
		15% of grape color powder in diet/13 wk or grape color extract/ 90 day	No toxicity.
L-Carotene	Human Rat	60 mg/day/3 months	No adverse effect.
		1000 ppm/2 years	No adverse effect.
1-Apo-8´-carotenoic esters	Rats	1% in the diet/2 years Up to 500 mg/kg bw/day/34 days	No adverse effects.
Canthaxanthin	Rats	250 mg or more/kg bw/day/life-time	Increased enzymatic activity and weight of liver in the females.
		5% by weight in feed/98 days	No toxic effects.
		250–1000 mg/kg bw/day/three generations	No reproductive teratogenic effects.

Several natural colorants	Tissue culture Rat spleen lymphocytes	0, 10, 100, or 1000 μM	Production of immunoglobulins has been evaluated (IgE, IgG or IgM); higher concentrations of *Monascus* pigments than 1 μM induce IgE production but inhibition is exhibited at lower concentrations; at 1 μM, carthamus yellow, betanin, and *Monascus* pigment shows a strong inhibition of IgM and IgG; water-insoluble pigments (bixin, gardenia yellow, and laccaic acid) inhibit the IgE production at all concentrations; thus, it is suggested that lipophylic coloring can be stimulant of the humoral system and by inhibition of IgE production, an antiallergenic agent.

JECFA (1975), Khera and Munro (1979), Parkinson and Brown (1981), Hallagan et al., (1995), Kuramoto et al., (1996), Linnainmaa et al., (1997), Tanaka et al., (1997), Lu et al., (1998), Sabater-Vilar et al., (1999) and Imazawa et al., (2000)

2.6 Strategies for enhanced pigment/ colored metabolites production

Enhanced pigments production and their application are the hallmark of various investigations. Researchers have proposed some techniques which can help in development of sustainable procedures for production of these compounds. It is well renowned that colored metabolite production by microorganisms might be controlled both qualitatively and quantitatively through specific nutritional inputs into the artificial growth media (Adinarayana et al., 2003; Moita et al., 2005; Vahidi et al., 2005; Gunasekaran and Poorniammal, 2008). Moreover, integration of genetic modulation and molecular engineering techniques can really improve gene expression and related yield of such compounds far beyond expectations (Mapari et al., 2005)

2.6.1 Process development with fungal cultures

Nowadays, food grade pigments which are available in the market are produced by conventional fermentation techniques, for example; pigments from *Monascus* sp. and other fungi i.e., *Xanthophyllomyces dendrorhous* (astaxanthin), *Penicillium oxalicum* (Arpink red color), *Ashbya gossypii* (Riboflavin) and *Blakeslea trispora* (b-carotene) (Johnson and An, 1991; Armstrong and Hearst, 1996; Kim *et al.*, 1997; Santos et al., 2005). Pigment production can be achieved either through submerged fermentation or solid state fermentation.

There are many specific factors that influence growth and pigment production from fungi including; culture conditions, pH, temperature, Carbon, Nitrogen sources and C:N ratio. Different factors and fermenters are discussed below:

2.6.1.1 Carbon (C) source

The type and concentration of carbon source is primarily vital in growth and secondary metabolism of any microbe (Lee et al., 2007). A number of C sources including monomeric sugars i.e., glucose and fructose and various complex C sources i.e. lactose, starch and ethanol have been trialed under artificial laboratory conditions in order to support and harness pigments production (Matin and Edward, 1990). Among these, the most suitable C source has been monomeric sugars like glucose but some studies suggested sucrose and ethanol as superior C source in order to increase biomass and then enhanced pigment production (Yoshimura et al., 1975; Broder and Koehler, 1980; Matin and Edward, 1990; Lin and Demain, 1991). Whereas lactose, xylose and fructose as a C source resulted in reduced growth and thus decline in pigment production (Lin, 1973; Lin and Demain, 1991).

Besides, the concentration of glucose also has an effect on biomass yield and pigment production. For example, it has been suggested that glucose concentration should be less than 20g/L to avoid crab tree effect i.e., shift from aerobic to anaerobic condition in metabolism resulting in reduced growth rate, pigment yield and ethanol production (Chen and Johns, 1994; Carvahlo et al., 2003). When complex C sources like starch are used for pigment production in submerged culture by *Monascus*, it resulted in low pigment yield and biomass because of the low oxygen transfer to media as viscosity of media is high. However, rice has been successfully used as a suitable complex C source in solid state cultures for enhanced pigment production (Lin, 1973; Teng and Feldheim, 1998; Tseng et al., 2000). Fungus *Monascus ruber* grown over synthetic media composed of glucose and glutamate in submerged cultures was evaluated for the production of red pigment and a mycotoxin citrinin (Hajjaj et al., 2000). Fungus (*Penicillium purpurogenum* GH2) in submerged culture showed highest production of pigment with xylose as a Carbon source (Mendez, 2011).

2.6.1.2 Nitrogen source

Different organic (Yeast extract, Peptone) and in-organic nitrogen sources (ammonium chloride, nitrates) have been exploited for supporting pigment productions by fungi (Juzlova et al., 1996; Matinkova and Patakova, 1999). In general, these nitrogen sources and their relative concentrations have been known effecting fungal growth, sporulation and pigments yield. Generally, utilization of different nitrogen sources by fungi resulted in a change in pH of the media towards more acidic (Wong and Bau, 1977; Pirt, 1985; Matinkova and Patakova, 1999). Organic nitrogen sources such as monosodium glutamate and some amino acids have been reported to cause increase in pigment production in *Monascus* species. While, the use of yeast extract enhanced growth but not pigment production in some other reports (Lin, 1991; Yongsmith et al., 1993; Chen and Johns, 1994; Juzlova et al., 1996; Cho et al., 2002; Gunasekaran and Poorniammal, 2008).

The addition of ammonium chloride (Inorganic nitrogen source) acidifies the culture media and promotes production of orange pigments. The reason is, red pigment is produced by the reaction of orange pigments with amino acids but by acidification, this reaction is halted. The addition of sodium nitrate to media declines both pigment yield and growth rate (Juzlova et al., 1996; Matinkova and Patakova, 1999). A specific C:N ratio in the culture media has therefore been shown to favor red and yellow pigments in the case of chemotaxonomically selected, potentially harmless *P. purpurogenum* IBT11181 (Mapari et al., 2009). The optimum nitrogen source for pigment production by *Paecilomyces sinclairii* was 1.5% (w/v) meat peptone (Cho et al., 2002). *Monascus* genus when screened for co-production of pigment and citrinin by using ethanol as an inducer of citrinin showed that the biosynthesis of citrinin seemed to be strain-specific and does not correlate with the pigments biosynthesis of genus *Monascus* (Pisarevaa et al., 2005). *Fusarium verticillioide* produced naphthoquinone pigment which is supported by the addition of peptone and yeast extract but malt extract exhibited the opposite results (Boonyapranai et al., 2008). The pigment production by *Monascus purpureus* was optimum at around 20–22.5 g/l of peptone (Silveira et al., 2008).

2.6.1.3 Trace metals

Trace metals like Zinc, magnesium and Potassium proved to have varying degrees of effects on pigment yield by the fungi. Metals are known to effect growth, spore formation and results in antibiotic production in microbes (Basak and Majumdar, 1975). In some cases they might be involved in enzyme system of metabolic pathways of the specific product of fungi (Ketaki and Majumdar, 1975). Generally, too high concentration of a trace metals proved to have negative effects on pigment production. The optimum concentration of zinc has found to be varying between 1 to 500ug/L (Cochrane, 1958). Zinc might be involved at some place in enzyme system of secondary metabolite formation (Ketaki and Majumdar, 1975), it can be toxic in higher concentrations (Johnson and Mchan, 1975). The ideal zinc concentration for *Monascus* has been found 800ug/L for both growth and pigment production. Phosphorus is another important source and it is known to effect phospholipid production. Increasing potassium phosphate in the media has been known to have negative effects on the pigment yield in *Monascus* (Lin, 1991; Lin et al., 2007). Potassium chloride is known to decrease pigment yield when its amount is greater than 3mM. Similarly, magnesium is known to affect pigment production and fungal growth. The concentration of magnesium sulphate greater then 2mM is known to reduce both pigment production and biomass yield in many fungi (Lin, 1991; Lin and Demain, 1991).

2.6.1.4 pH

In uncontrolled fermentation conditions, the pH of the media depends on N source (Wong and Bau, 1977; Pirt, 1985; Matinkova and Patakova, 1999). The ideal pH range for pigment production in some fungi range from 4 to 7 and for growth it ranges from 2.5 to 8 (Yongsmith et al., 1993). The color produced by *Monascus* varies with pH and ranged from yellow orange to red. Yellow pigments are produced at lower pH values and with increasing pH up to 5; the production of red pigment is obtained in case of *Monascus* species. In submerged conditions, the production of red pigments has been enhanced even under alkaline conditions (Yoshimura et al., 1975). Fungus *Paecilomyces sinclairii* produces maximum pigment at initial pH 6 (Cho et al., 2002). Fungus (*Penicillium purpurogenum* GH2) in submerged culture showed highest

production of pigment (2.46g/L) at the pH value of 5 (Mendez et al., 2011) and pH value of 8 was optimum in *Fusarium* (Boonyapranai et al., 2008). The endophytic fungus *M. castaneae* had optimum pH 5 for pigment production (Visalakchi and Muthumary, 2009).

2.6.1.5 Temperature

Temperature affects pigment production and biomass by influencing different biochemical reactions and their rates. The optimum temperature for biomass and pigment production in most fungi range from 25 to 30 °C, however, the conditions vary with different species (Shepherd, 1977; Lin, 1991; Carvahlo et al., 2005). Sometimes, greater yields in pigment production have also been endorsed at higher temperatures (Carvalho et al., 2005). Temperature can easily be controlled in submerged cultures but not in solid cultures where substrates are not mixed. Pigment producing fungus *Paecilomyces sinclairii* produced pigment at optimum temperature i.e., 25 °C (Cho et al., 2002). *Monascus* genus showed pigments production on the standard conditions of 28 °C (Pisarevaa et al., 2005).

Fungus (*Penicillium purpurogenum* GH2) in submerged culture showed highest production of pigments at a temperature of 24 °C and at 30 °C in *Fusarium* sp. (Boonyapranai, 2008; Mendez et al., 2011; Velmurugan et al., 2011), *Trichoderma virens, Alternaria alternanta* and *Curvularia lunata* produced pigments under optimal temperature 28 °C (Sharmala et al., 2012). The endophytic fungus *M. castaneae* at a specified temperature (24 °C) produced antibacterial pigment (Visalakchi and Muthumary, 2009).

2.6.1.6 Culture media; a combination of nutrients sources

Different fungi are able to produce *Monascus*-like azaphilone pigments and their extracellular derivates on solid state media (Sardaryan, 1999; Sardaryan, 2004; Mapari et al., 2009) and liquid media (Mapari et al., 2009). Although submerged fermentation has ease of operation and its conditions can be controlled, the solid state fermentation has advantage of increased production of pigment as compared to submerged fermentation.

Trichoderma virens, alternaria alternanta and *curvularia lunata* produced pigments under optimal culture conditions in potato dextrose broth under static conditions (Sharma et al., 2012). The endophytic fungus *M. castaneae* in optimized CYA/CYB produced pigment showing antimicrobial activity significantly inhibiting the growth of human pathogenic bacteria viz., *Klebsiella pneumonia, Staphylococcus aureus, Vibrio cholerae* and *Salmonella typhi* (Visalakchi and Muthumary, 2009). Pigment production was seen in submerged fermentation by *Monascus purpureus* using grape waste as a growth substrate (Silveira et al., 2008).

About seven *Penicillium* strains belonging to *Penicillium purpurogenum, P. aculeatum* and *Penicillium funiculosum* are reported to be the producers of *Monascus*-like azaphilone pigments and their derivates on solid state media (Mapari et al., 2009) in addition to the *Penicillium aculeatum* and *P. pinophilum* (Sardaryan, 1999; Sardaryan, 2004) strains belonging to *Penicillium subgenus Biverticillium*. Among them four strains are capable of producing extracellular pigments in liquid media (Mapari et al., 2009). *Monascus purpureus* yielded 25.42 OD Units/gram of dry weight pigments in solid state fermentation (SSF) utilizing substrate i.e., corn cob powder under optimized culture conditions (Velmurugan et al., 2011). Solid state fermentation (SSF) of *Monascus purpureus*, while utilizing jack fruit seed powder as a substrate under optimal process parameters resulted in pigment yeild upto 25 OD Units/g dry fermented substrate (Babitha et al., 2007). Magenta and red pigment producing ability was seen in *Phoma herbarum* (Chiba et al., 2006; Quereshi et al., 2010; Singh et al., 2010). Four different strains of *Monascus* (NRRL 1991, NRRL 2897, CCT 3802 and LPB 31) when cultivated over cooked rice, produced minimum citrinin and maximum pigment which could be used in food industry (Carvalho et al., 2005).

2.6.2 Fermenters

Process development in terms of bioreactor design is a crucial step for obtaining high yield of bio-colorants from a fungal source. The most common type of fermenters used in submerged cultivation are conventional stirred tank in which various sugars, starches and agricultural waste were used as carbon sources.

The traditional bamboo tray method was used as a technique of solid state fermentation for production of *Monascus* pigments but it cannot be scaled up as it is laborious and require a lot of space. Moreover, contamination and quality of product are two other draw backs in tray fermentation. The low productivity in submerged cultures is due to product inhibition as the hydrophobic pigments remain in fungal mycelia and thus product formation is reduced, a type of feedback inhibition. While, in solid state fermentation, the pigments are diffused out from mycelia onto solid substrate (rice or grains) thereby, product inhibition is reduced. Static flasks, packed bed columns, rotating drum fermenters and open trays have been used in solid state fermentation of pigments like *Monasucs* (Han and Mudget, 1992; Rosenblitt et al., 2000; Lee et al., 2002; Carvahlo et al., 2006).

The fungus *Blakeslea trispora* is known for b-carotene production through fermenters i.e., stirred tank and fed batch resulting in enhanced pigment yeild (Cerdá-Olmedo, 2001; Yan et al., 2013).This fungus yielded maximum amount of the bio-colorant when the liquid culture media was provided with bio-sufractants in industrially important agitated and aerated fermenter (Kim et al., 1997) and by co-fermentation (Membrane filter) of the two sexual types of the fungus in specific proportion as sexual stimulation i.e., release of hormones (Trisporic acid) results in maximum production of carotene upto 35mg/g (Van den Ende, 1968; Murillo et al., 1978). Another fungi *Phycomyces blakesleeanus* (Ootaki et al., 1996) is also known for b-carotene production. The mutation of wild type to mutant resulted in an increase of carotene yield from 0.05mg/g to 10mg/g of dry mass (Murillo et al., 1978). However, this production rate can further be enhanced in engineered bioreactors (mechanical engineered, material science and by sexual stimulation) (Cerdá-Olmedo, 2001).

Several species of *Monascus* are exploited for commercial production of yellow and/or red pigments using solid substrate like rice as a media. Such techniques are

frequently practiced in China and Japan (Fabre et al., 1993). Whereas using stirred tank bioreactor, pigment production from *Monascus* was also enhanced (Mohamed et al., 2009). Pigment producing fungus *Paecilomyces sinclairii* under optimized conditions can give nine fold enhanced pigment production in batch fermenter (Cho et al., 2002).

Commercial production of Astaxanthin, a carotenoid has been attained through liquid state stainless steel fermentor using red yeast, *Xanthophyllomyces dendrorhous* (formally *Phaffia rhodozyma*) (Andrews et al., 1976; Vazquez et al., 1988; Flores Cotera and Sanchez, 2001; Roy et al., 2008). Another bacterium *Paracoccus carotinifaciens* is also a good source of Astaxanthin (Tsubokura et al., 1999). Yeasts of the genus *Rhodotorula* and the most species *R. glutinis* has been used to synthesize carotenoids while other main species are *R. rubra, R.gracilis* and *R. graminis* (Sakaki et al., 2001; Simova et al., 2004; Tinoi et al., 2005).

Algae are used as the most potent commercial source of Astaxanthin. Astaxanthin is produced by three ways by *Haematococcus* species. The different processes used for the production of this compound includes; One step process i.e., jacketed column type) (Del Rio et al., 2007), Phtoinduction bioreactors with attached cultivation (Wan et al., 2014) and Outdoor batch column photobioreactors (Wang et al., 2013).

Wood after treatment with hydrolyzates like Pine treated with cellulases from *T. reesei* and cellobiase from *A. niger* are most likely substrates for carotenoid production (Yamane et al., 1997). Similarly, the broth hydrolyzed with enzymes can be utilized for growth of *X dendrorhous*, resultant in high growth rate and cell density with pigment (Carotenoid) yield up to 1.8 mg/l (Parajo et al., 1997). *E. nigrum* has been explored for the industrialized production of natural colorants i.e., polyketides in liquid media as well as solid rice based media (Mapari et al., 2009). Extracellular pigment produced in repeated batch processes by immobilized *Monascus purpureus* C32 resulted in negligible Ca-alginate cell leakage and maximum pigment production (Fenice et al., 2000).

2.6.3 Production management by genetically modified (GM) microbes

With the isolation of plant color compounds specific biosynthetic genes, their regulation in response to environmental and developmental factors has been likely to be elucidated. With the aid of recombinant DNA technology, plants having potential of overproducing carotenoid have been generated due to possibility of isolation of biosynthetic gene. By biotechnological methodology, engineering of yeast strains for over production of carotenoids is made possible. Mutants of *Phycomyces blakesleeanus* carS have the capability to accumulate up to 100 times β-carotene as compared to the wild type (2 to 5 mg/g dry weight) (Hempel and Bohm, 1997). *X. dendrorhous* JB2 which is a bulk producer of Astaxanthin shows production enhancement of 2.3 times relative to the wild type and this fungus has been employed commercially for Astaxanthin production utilizing cheap substrates i.e., corn by-products and by metabolic engineering of the astaxanthin biosynthetic pathway (Bon et al., 1997; Visser et al., 2003; 2005). Three different strains of *L. sulphureus* produced variable yield of orange pigment ranging from 0.1 to 6.7mg/g dry weight. Manikprabhu and Lingappa, (2012) isolated one red pigment producing fungi illustrated as *Lasiodiplodia* spp. *Fusarium sporotrichioides* was genetically modified by Jones et al., (2004) for the improved production of lycopene by using cheap corn fibers as substrate. This pigment had dual functions i.e., a colorant and an antioxidant. Using an innovative, common technique for the sequential cloning of multiple DNA sequences, the isoprenoid pathway operating in fungus was redirected toward the carotenoids synthesis. Strong promoter and terminator sequences of bacterium *Erwinia uredovora* were added to carotenoid biosynthetic genes. The chimeric genes were introduced and expressed in the fungus at levels equivalent to those detected for endogenous biosynthetic genes.

The heterogeneity with respect to morphological, developmental and yield in natural saffron population is due to genetic and environmental factors (Raina et al., 1996). Considerable improvement in productivity has been achieved by development of productive genotypes using existing Saffron pigment genes (Xuabin, 1992). Golden rice is one of the major innovations in this regard (Zimmermann and Qaim, 2004; Paine et al., 2005). The β-Carotene biosynthesis pathway is introduced into rice endosperm by genetic engineering to overcome vitamin A deficiency (Beyer et al., 2002).

2.7 Characterization of fungal pigments

Various purification i.e., Thin layer chromatography, Column chromatography (C8, C18 and Sephadex) and analytic tools i.e., Nuclear magnetic resonace, Liquid chromatography mass spectrometry, 3D X ray crystallography have been used for characterization of natural pigments.

2.7.1 Chromatography

The word 'chromatography arose from the Greek word 'Khroma' meaning color and 'graphein' meaning to draw, and it is usually employed to describe the procedure of splitting mixtures of pigments. The general principle of chromatography is movement of a mixture of compounds over a mobile phase, which passes over stationary phase, a selectively adsorbing surface.

In order to purify the pigments or any natural compound, column chromatography is done with suitable solvent system and TLC helps in combining of similar fractions obtained after column chromatography. Whereas, with the help of preparative TLC, the colored band appearing on the TLC plate can be scratched and processed for further analysis.

2.7.1.1 Column chromatography

Column chromatography is developed on the basis of differential adsorbing of compounds on solid adsorbent (silica or alumina) depending on their polarity in addition to other chemical and structural properties. Some compounds adsorb to the stationary phase more strongly as compared to others, due to their slow rate of elution through column. Different compounds are separated from mixture based on their elution rate.

2.7.1.2 Thin layer chromatography

Thin layer chromatography (TLC) is a chromatography method utilized for separation of non-volatile mixtures. Thin layer chromatography is carried out on a sheet of glass, plastic or aluminium foil, coated with a thin layer of stationary phase i.e., usually silica gel, aluminium oxide or cellulose. After applying the sample on the plate, a mobile phase (a solvent or solvent mixture) is drawn up the plate by capillary action. Due to differential ascending rates of the different analytes on TLC plate, separation is achieved. Thin layer chromatography can be useful in monitoring the reaction progress, compound identification present in a given mixture in addition to determining purity of a compound. Difference in polarity and nature of the compounds results in their differential movement rates on TLC plate based upon the variations in their attraction to the stationary phase and differences in solubility in the solvent.

2.7.1.3 Liquid chromatography mass spectrometry (LCMS)

The LC-DADMS was effectively used as a prevailing tool to check the presence or the absence of different organic compounds using accurate standards and to identify pigments based on UV-vis and mass spectra. Since, UV-vis spectroscopy in the identification and de-replication has been known to be quite handy though LC-DAD-MS proved to be comparatively more efficient chemotaxonomic tool. Liquid chromatography-mass spectrometry (LC-MS) permits acquisition of structural data during a single chromatographic run. Molecular mass information, used in conjunction with PDA UV/vis spectra is often sufficient for the assignment of components. HPLC coupled to tandem MS (LC-tandem MS) adds a further dimension and can be used to identify structural differences that do not affect the UV/Vis absorption properties or to distinguish ions with the same mass-to-charge ratio (isobaric ions). Furthermore, LC-tandem MS is particularly powerful if MS/MS spectra of an unknown compound is compared with a structurally related and identified compound.

2.7.2 Nuclear magnetic resonance spectroscopy

NMR spectroscopy is a research method that works on basis of the magnetic properties of certain atomic nuclei. NMR spectroscopy is frequently used by chemists and biochemists for investigations of the properties of organic molecules.Whereas, it is appropriate for any type of sample containing nuclei which possess spin. Appropriate samples range from small compounds which are analyzed with 1-dimensional proton or carbon-13 NMR spectroscopy to large compounds i.e., proteins or nucleic acids using 3 or 4-dimensional proton and carbon NMR. NMR spectroscopy has a major impact on the chemical and natural sciences because it provides variety of information about diverse samples including solutions and solids. It distinguishes different functional groups and even similar functional groups with differing neighboring substituents give distinguishable signals. It requires complete analysis and interpretation of the entire spectrum, although larger amounts of sample are needed than for mass spectrometry but NMR is non-destructive and sample can be retrieved easily. http://www2.chemistry.msu.edu/faculty/reusch/VirtTxtJml/Spectrpy/nmr/nmr1.htm;http://en.wikipedia.org/wiki/Nuclear_magnetic_resonance spectroscopy).

2.7.3 Raman spectroscopy

It identifies the compound by providing finger print of the molecule. The fingerprint region of organic molecules is in the (wave number) range 500–2000cm^{-1}. Similarly, changes in chemical bonding can also be studied by this technique e.g., as when a substrate is added to an enzyme (http://en.wikipedia.org/wiki/Raman_spectroscopy).

2.7.4 UV/Vis spectrophotometer

A spectrophotometer is an instrument which measures the reflection or absorbance characteristics of a sample such as pigments. The predetermined electromagnetic radiation wavelengths for ultra-violet (UV) (300-400nm), visible (vis) (400-700nm) and near infra-red (nir) (765-3200) radiation are defined (http://www.vam.ac.uk /content/journals/conservation-journal/issue-01/uv-vis-nir-spectrocopy-what-is-it-and-what-does-it-do/). Chromophore is substance or its part that can be excited by absorption of wavelength of light. Organic chromophores that absorbs strongly in the

UV or visible parts of the spectrum approximately always employs multiple bonds such as C=C, C=O or C=N (http://www.andor.com/learning-academy/absorption-transmission-reflection-spectroscopy-an-introduction-to-absorption-transmission-reflection-spectroscopy).

2.8 Applications

Natural dyes and pigments are arising as a significant substitute to potentially harmful synthetic dyes (Sivakumar et al., 2009). Leather hood food industry (LFI) declares that the market share for natural colorants is flourishing, indicative of natural colorants to exceed synthetic colorants in market value in the near future. Thus current market price for natural colorants is about USD 1/g, thus restricting their application to very expensive natural colored garments only. To defeat this constraint, it is suggested to exploit the potentiality of other biological sources such as fungi, bacteria and cell cultures (Mapari et al., 2005).

2.8.1 Food industry

Natural colorants now comprise 31% of the colorant market, compared to 40% for synthetics (data from Leatherhead Food International LFI) (www.leatherheadfood.com). Recent investigations have indicated some other ascomycete sources of pigments including polyketide class (Mapari et al., 2009) which were used for species differentiation and identification (Frisvad and Samson, 2004), and can be used safely as food colorants. Polyketide pigments of ascomycetous fungi including *Monascus* pigments have the capability to produce a variety of yellow, red, orange, green and blue hues (Bachmann et al., 1986; Robinson et al., 1992; Jung et al., 2003).

Commercially available polyketide pigments of *Monascus* have been used for hundreds of years as food colorants and as medicine (Dufosse, 2006; Lin et al., 2008). Some *Monascus* species also co-produce the mycotoxin citrinin (Blanc et al., 1994), but *Monascus* pigments are advantageous for food industry when produced by

citrinin free strains of *Monascus* and some other potentially safe fungi (Jung et al., 2003; Dufosse, 2006; Lin et al., 2008). A decade ago, the production of pigments from fungi was limited to the production of Riboflavin (yellow colorant, vitamin B2) using the fungi *Eremothecium ashbyii* and *Ashbya gossypi* (Stahmann et al., 2000; Santos et al., 2005). However, Riboflavin is light sensitive pigment (Downham and Collins, 2000). Stricker and colleagues nearly about 30 years ago reported the potential of water soluble yellow pigment of *Epicoccum nigrum* for being used as food colorant because it possessed antioxidant property (Stricker et al., 1981). In both Europe and the US, most of the listed color additives are derived from natural sources (Mapari et al., 2005).

Recent studies showed that *Penicillium* and *Epicoccum* species produce hues of the pigments similar to the colorants of present day natural red and yellow food colorants (Carroll, 1999; Mapari et al., 2006; Mapari et al., 2009). The yeast *Phaffia rhodozyma* as a source of Astaxanthin (Andrewes et al., 1976; Johnson et al., 1979; Johnson and An, 1991; Flores Cotera and Sanchez, 2001), *Blakeslea trispora* used for the industrial production of b-Carotene (Ende, 1968) and anthraquinone pigment isolated from *Penicillium oxalicum* currently being established for use as a 'natural' food additive (Sardaryan et al., 2004; Mapari et al., 2005; Dufossé et al., 2006) are some of the examples of pigments utilized in food industry due to low toxicity.

2.8.2 Textile and Leather industry

The application of fungal pigments like anthraquinones and pre anthraquinones in cotton, silk and wool dyeing has been reported in several studies (De Santis et al., 2005; Nagia & EL-Mohamedy, 2007). But still laboratory scale trials are being carried out to assess their successful utilization in leather and textile industry in a cost effective way. Fungal pigments were used since centuries to add color to ornamental wood products (Blanchette et al., 1992). *Monascus purpureus* C322 yielded red to orange pigments and were used in wool dying (De Santis et al., 2005). Assessment of the dyeing properties of pigments in leather and textile from five fungi i.e., *Monascus purpureus, Isaria farinosa, Emericella nidulans, Fusarium verticillioides* and *Penicillium purpurogenum* showed them as a promising source of dyes in future (Velmurugan et al., 2009). Pigment producing fungus i.e. *Trichoderma virens,*

Alternaria alternanta and *Curvularia lunata* produced pigments which dyed Wool and silk with an additional advantage of good wash & rub fastness (Sharma et al., 2012).

2.8.3 Medical importance

Fungi are rich source of bioactive colored compounds with huge implications in biomedical sciences (Raisanen, 2001). A variety of antibacterial anthraquinone derivatives have been recognized from many species of fungi and lichens (Yagi et al., 1993). Besides, anthraquinones derivatives of fungal origin have also been reported for their antiprotozoal and cytotoxic activities (Nelson and Marasas, 1983; Okamura et al., 1993). Similarly, an anthraquinone pigment isolated from *Penicillium oxalicum* has anticancer effects (Mapari et al., 2005; Dufossé et al., 2006). Cotton fabrics and leather dyed from pigments of *M. purpureus* and *P. purpurogenum* exhibited excellent activities against diverse group of pathogenic microbes. Other fungi (*I. farinosa*, *E. nidulans*, *F. verticillioides*) pigments also showed inadequate activity to all the bacterial pathogens with the exception of *S. aureus*, whereas, dyed fibres also showed considerably activity thus increasing the scope of antibacterial clothings (Velmurugan et al., 2009).

Monascus species pigments are commonly utilized as food additives and antimicrobial agents. Some *Monascus* pigments have lipase inhibitory, anti-atherogenic and antioxidant activities acting as nutraceuticals when added to the specific food commodity (Kim et al., 2006; Yang et al., 2006). The components isolated from this fungus exert several biological actions and produce hypocholesterolemic (Endo, 1979; 1980; Tobert et al., 1982), liver-protective and antitumor effects (Yasukawa et al., 1996; Aniya et al., 1998). g-Aminobutyric acid (GABA) in the aqueous extract of *Monascus*-fermented rice could decrease blood pressure in vivo (Kohama et al., 1987; Kushiro et al., 1996). Dimerumic acid and polyketide pigments isolated from red koji showed several physiological functions (Izawa et al., 1997; Aniya et al., 2000). Antioxidant properties of methanolic extracts of *Monascus* and the rice colored with Monascal pigments were also reported (Yang et al., 2006). Poultry meat product in which seitan colored by red Yeast rice (RYR) of

Monascus purpureous was added showed positive effect in terms of product look, taste and the consistency of the product (Baranovi and Malh, 2008).

Extracts of *Monascus pilosus*-fermented rice (red-mold rice) contained six azaphilones, Monascin, Ankaflavin, Rubropunctatin, Monascorburin, Rubropunctamine and Monascorburamine, two Furanoisophthalides, Xanthomonasin A and B and two amino acids, (+)-monascumic acid and (-)-monascumic acid showing moderate inhibitory effects on NOR 1, a nitric oxide (NO donor) activator (Akihisa et al., 2004). Brevianamides which was antitubercular agent is obtained from a Marine-Derived isolate of *Aspergillus versicolor* (Song et al., 2012).

Monascus pigments enhanced IgE production at 1 m*M* in rat spleen lymphocytes, but constrained it at lower concentrations. Thus, *Monascus* pigment can be used as an anti-allergenic substance (Kuramoto et al., 1996). Red and yellow pigments of *Monascus anka* and *M. purpureus* did not induce mutagenesis. Thus, these natural colorants could inhibit the mutagenicity of activated forms of food pyrolysate mutagens. Further screening of colors in plants and other natural sources for their antimutagenic activities is important (Izawa et al., 1997).

Oral administration of *Monascus* pigment suppressed tumor promotion by tetradecanoyl phorbol acetate (TPA) in mice following initiation by 7, 12-dimethylbenz[*a*] anthracene. Treatment with *Monascus* pigment caused a 66 and 58% reduction in the average number of tumors per mouse at week 20. There was no difference regarding body weight between the control group and two treated groups during the experiment. Therefore, foods additives may prove to be important for the chemoprevention of cancer (Yasukawa et al., 1996).

Another motivating biological activity of *Monascus* was linked with its antibiotic activities against *Bacillus subtilis* and *Candida pseudotropicalis*. The active compounds are pigments i.e., Rubropunctatin and Monascorubrin. Immunosuppressive activity on mouse T splenocytes has been most pronounced with compounds Monascin and Ankaflavin. The immunosuppressive effect of Monascin is more prominent than that of Rubropunctatin and Monascorubrin. *Monascus*

preparations have also shown favorable dietetic effects, involving reduction of serum cholesterol and triglycerides in mice (Martinkova, 1999).

2.9 Biosynthesis of aromatic polyketides

The biosynthesis of aromatic polyketides shares remarkable similarities with the fatty acid biosynthesis (World of polyketides, 2007). Polyketides are synthesized by a collection of enzymes called polyketide synthases (PKSs). Component domains of PKSs consist of acyl transferases, acyl carrier domain (with an SH group), a serine-attached 4'-phosphopantetheine (ACP, which hold the growing macrolide as a thiol ester), 3-ketoacyl synthase (catalyzing chain extension), 3-keto reductases (responsible for the first reduction), dehydratase (elimination of water to give an unsaturated thiolester), enoyl reductase (the final reduction to full saturation), and finally a thiolesterase (product release and cyclization). The biosynthesis occurs in a stepwise manner from simple 2-, 3-, or 4-carbon building blocks acting as starters. The common starters are acetyl- CoA (S-acetyl transferase, EC 2.3.1.38) and its activated derivatives malonyl-CoA and methylmalonyl- CoA. The major extender units are acetyl-CoA, malonyl-CoA, methylmalonyl-CoA, propionyl-CoA, butyryl-CoA, isobutyryl-CoA, pentanoyl-CoA, hexanoyl-CoA, octanoyl-CoA, benzoyl-CoA, cinnamoyl-CoA, p-coumaroyl-CoA, caffeoyl-CoA, feruoyl-CoA etc. S-malonyltransferase (EC 2.3.1.39) provides the malonyl groups for polyketide biosynthesis and the action of β-oxoacyl- ACP synthase (EC 2.3.1.41) yields the poly-β-keto chain, -[CH2-C(=O)]n.

The key chain-building step is decarboxylative condensation. Unlike in fatty acid biosynthesis, however, in which each successive chain elongation step is followed by a fixed sequence of the oxo group reduction (3-oxoacyl-ACP reductase, EC 1.1.1.100), dehydration (3-hydroxyacyl-ACP dehydratase, EC 4.2.1.61), and enoyl reduction (enoyl-ACP reductase, EC 1.3.1.10), the individual chain elongation intermediates of polyketide biosynthesis undergo all, some, or none of these functional group modifications, resulting in chemical complexity in the products.

Dieckmann condensation

It is the base-catalysed intramolecular condensation of dieste to give cyclic β-keto esters. Hence, the enolate component and the carbonyl component become parts of a single, larger molecule. The enzyme catalysis prevents the need for a base and esterification of the second carboxyl to produce β-keto ester.

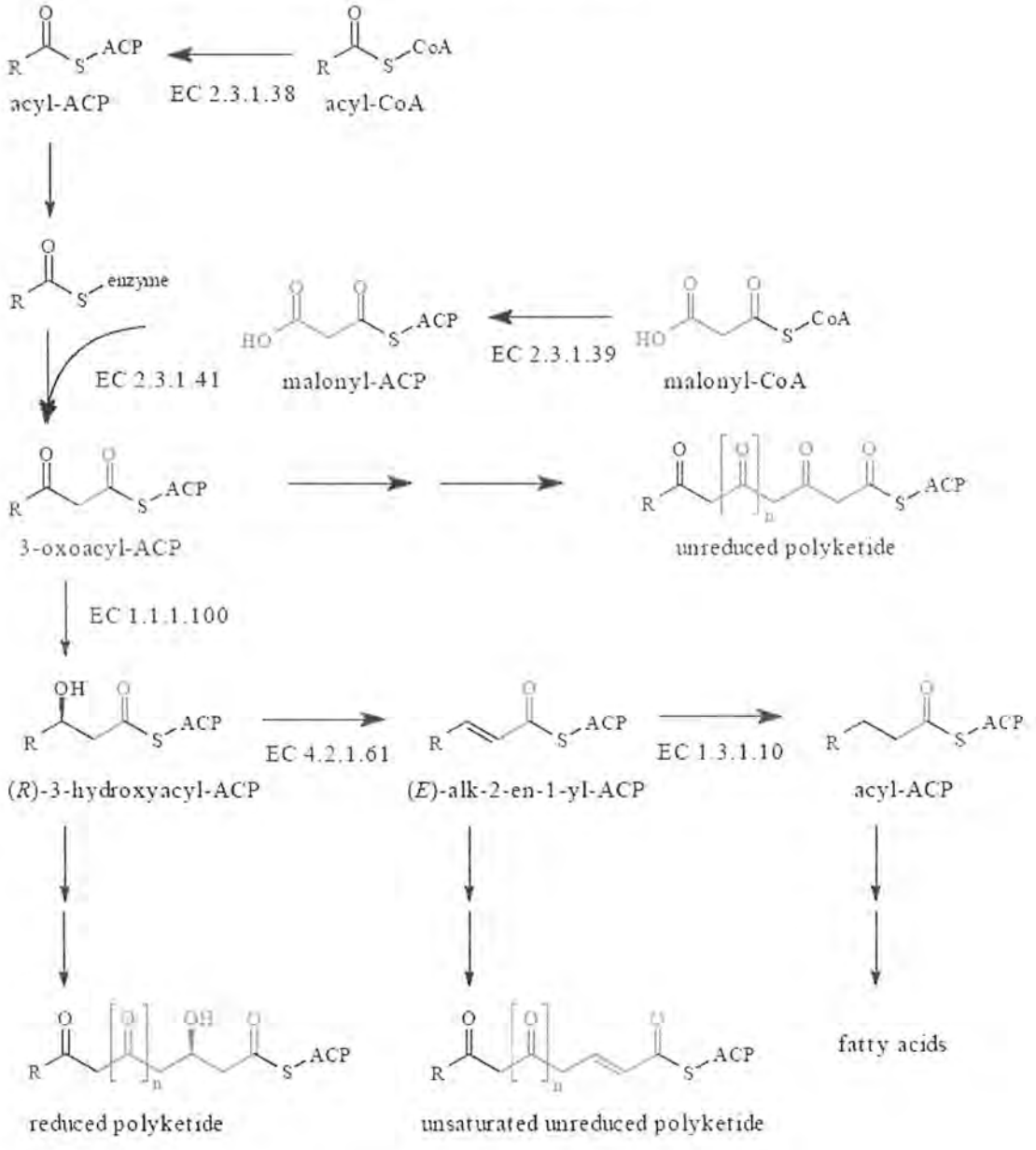


Fig 2.3: Biosynthesis of aromatic polyketides

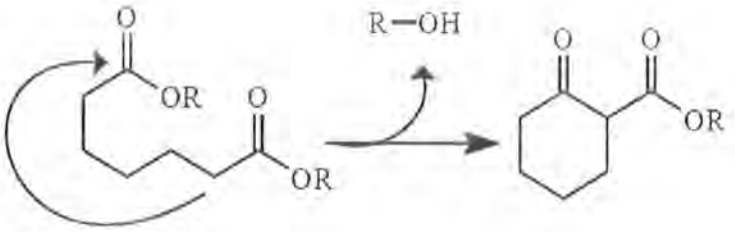


Fig 2.4: Dieckmann condensation

Considering in view the literature cited, there is a need of further exploration with respect to new sources of pigments i.e., fungi from various competitive environments not studied previously in search of potential and better candidates. The colorants obtained should be evaluated with respect to enhanced pigment production at varying operational parameters. Moreover, their stability should be tested in order to see their future compatibility with industrial level applications. The toxicity of these colorants is a missing gap in most of the literature cited. It should be evaluated for development of non-toxic, environment friendly colorants.

MATERIALS AND METHODS

3 Material and Methods

3.1 Isolation of pigment producing fungi

Pigment producing fungi were isolated by serial dilution and pour plate method from soil samples of Kala Pani Forest (34°12'30N 73°19'20E and at an altitude of 1762 meters) located in Khyber Pakhtunkhuwa Pakistan. Soil suspension of each sample was prepared by adding 1.0 gm per 100 mL distilled water of soil and agitated on orbital shaker for 15 minutes (Nazir et al., 2007). Soil suspensions were serially diluted to 10^{-6} gm/L pipetted and equally spread onto potato dextrose agar (PDA) petri plates, and incubated at 30°C for 7 days. Similarly, endophytes were isolated from woody parts of *Taxus* plant by the following procedure (Petrini, 1986). First, the plant material was washed with distilled water to remove any dust. Afterwards, the woody parts were carefully cut in to small pieces and their surface was sterilized by sodium hypochlorite (1-14%) for 5-10 minutes followed by 75% ethanol for 1 minute. The plant material was then immersed in sodium hypocholoride followed by 75% ethanol again for 30 seconds. Later, the plant material was rinsed with distilled water thrice followed by blotting on sterilized filter paper. 4-6 segments of sterilized plant material were placed on agar water plates at 27 °C and growth was seen after 2 weeks of inoculation.

Chloramphenicol antibiotic (0.03 mg/L) was added into the media to avoid bacterial contamination. Each colony (colony formation units = cfu) that appeared on the plates was then purified by sub-culturing it. These fungi were then transferred to PDA plates for screening of pigment producing fungi. The fungi producing extracellular pigments/colored metabolites on culture media were then identified on morphological bases; vegetative and reproductive structures using taxonomic guides and standard procedure (Gilman, 1944; Domsch et al., 1980).

3.2 Molecular identification of fungal isolates

Fungal pellets were filtered and dried after being grown in sabouraud dextrose broth culture media for 7 days at 25°C. Genomic (g) DNA was isolated from the fungal pellets with the Master Pure kit Gene Elute™. The respective D2 (0.6 Kb) and ITS regions of the 28S and 18S ribosomal genes were amplified through PCR using

purified gDNA samples (QIAquick PCR clean up kit, Qiagen) and specific universal primers 18S (ITS1 5'TCC GTA GGT GAA CCT GCG G 3' and ITS4 5'TCC TCC GCT TAT TGA TAT GC 3'), ITS5 (GGAAGTAAAAGTCGTAACAAGG) (White et al.1990) and 28S (D2 region, ITS4 and ITS5 GGAAGTAAAAGTCGTAACAAGG) (Ferrer, 2001).

Programmable thermocycler (MJ Mini Biorad) was used for PCR. The reaction mixture (25 µL) contained 5 µL of DNA template, 3 µL of 25 mMol $MgCl_2$, 100 µM of each dNTP, 25 pM of each primer and 1 U of Taq DNA polymerase (Fermentas). The amplification conditions involved 1 cycle at 95°C for 5 min followed by 35 cycles with a denaturation step at 95°C for 30 seconds, annealing step at 55°C for 1 min and an extension step at 72°C for 1 min followed by 1 cycle at 72°C for 6 min. The PCR reaction products were analyzed by agarose gel electrophoresis. The DNA was sequenced with the ABI Big Dye terminator chemistry. The consensus sequences were compared to reported DNA sequences in public databases to confirm the identity of fungi. After sequencing of purified DNA, comparison with the help of Basic Local Alignment Search Tool (BLAST) of the GeneBank by Bankit program was carried out to get the accession numbers for each sequence (http://www.ncbi.nlm.nih.gov) (ACGT, Inc. USA). By using Neighbor-Joining method, the evolutionary history of relevant fungi was inferred. Maximum Composite Likelihood method (Tamura et al., 2005) was used for computing evolutionary distances. The distances were in the units of number of base substitutions per site. Phylogenetic analysis was carried out in MEGA4 by Tamura et al., (2005).

3.3 Optimization of Natural Pigments production in liquid state fermentation

33.1. *Selection of Culture Media*

Five complex media including; Potato dextrose broth (PDB), Sabouraud dextrose broth (SDB: 10 g/l peptone; 40 g/l glucose), Yeast extract malt extract broth [YMPGB: 10 g/l glucose; 5 g/l peptone; 3 g/l yeast extract (Scharlau, Germany); 3 g/l malt extract (Scharlau)], Minimal salt media (MSM) with different carbon sources (Fructose 3g/L, Sucrose 6g, Glucose 1g, NH_4NO_3 4g, $MgSO4.7H_2O$ 0.5g, KH_2P0_4, 0.5g, NaCl 0.6g, Sodium Acetate 1g, Sodium Benzoate 0.1g), and Czapek dox broth were used to evaluate optimum pigment production in each fungus. The shake

flask experiments were performed by first growing the inoculum in SDB in 250 ml Erlenmeyer flask containing 100 ml of culture media at room temperature (25°C) on a rotary shaker at 120 rpm for 7 day. The inoculum obtained was further introduced into the flasks containing above mentioned five media for pigment production under static conditions in flask culture experiments at room temperature (25°C). Pigments estimation were done as described by Tseng et al., (2000) in which the optical density of the pigmented filtrate was expressed as a function of the pigment concentration at its specific λ maximum i.e., 510nm for red pigment (*Penicillium*), 380 for yellow pigment (*Aspergillus*) and 430 (*Chaetomium*) for pink pigment. The optical density obtained at a specific λmax was multiplied but the dilution factor to get the final pigment concentration. Experiments were carried out in triplicate under standard sterilized conditions. The best complex media for production of extracellular pigment was used to screen for the best carbon source, nitrogen source, initial medium pH and temperature for flask culture experiment. LSD and SEM were applied on the triplicate results.

3.3.2 *Effect of initial pH and temperature*

The most optimum culture media was used to get the most ideal temperature for growth and pigment production by each fungus. Initially, the innoculated culture media of each fungus was placed at different temperatures (15, 25, 30 and 37°C) by taking 100 ml medium in 250 ml Erlenmeyer flask and operating it under static condition. After temperature optimization, different pH viz. 3.0, 5.5, 7 and 9 conditions were evaluated for their effects with respect to pigment production in each fungus using aforesaid conditions. LSD and SEM were applied on the triplicate results.

3.3.3 *Effect of Carbon Sources/Concentrations*

Carbon (sugars) sources including glucose, fructose, sucrose, lactose and glycerol (20 g/l) were used in PDB medium. Experiments were run as previously prescribed at optimized conditions of temperature and pH for each fungus. No additional nitrogen source was added in PDB medium. After selection of best carbon source, its

concentration (40, 20, 10, 5, 2, 1 g/l) was then optimized for pigment production in each fungus. LSD and SEM were applied on the triplicate results.

3.3.4 *Effect of Nitrogen Sources*

Two groups of nitrogen sources, organic and inorganic were used in culture media for pigment production. Peptone and yeast extract (3g/l) as organic while, sodium nitrate and ammonium nitrate (3g/l) as inorganic nitrogen sources were utilized for each fungus. Experiments were run on specified optimized conditions as previously prescribed for each fungus. Un-innoculated PDB media was used as basal control. Experiments were carried out in triplicate in 250 ml flask containing 100 ml media and SEM and LSD were applied on the triplicate results.

3.4 Extraction of Secondary Colored Metabolites in different media

Solid state fermentation conditions were used to produce pigment production in each fungi on five different media i.e., PDA (Potato dextrose agar), YMPG (yeast extract, malt extract, peptone, glucose) Agar, Czapek Agar, Malt extract Agar (MEA) and SDA (sabouraud dextrose agar). Experiments were performed by inoculating each fungus on five different media in petri-plates and incubating them at 25°C. The soluble extracellular pigments were extracted by adding 3 times (3x) of 70% methanol followed by overnight shaking at 150 rpm at 40 °C. The soluble fraction was then purified by filtering it through Whatmann No.1. The organic layer was further dried on rotary evaporator at 40°C. This filtrate was then used for further processing.

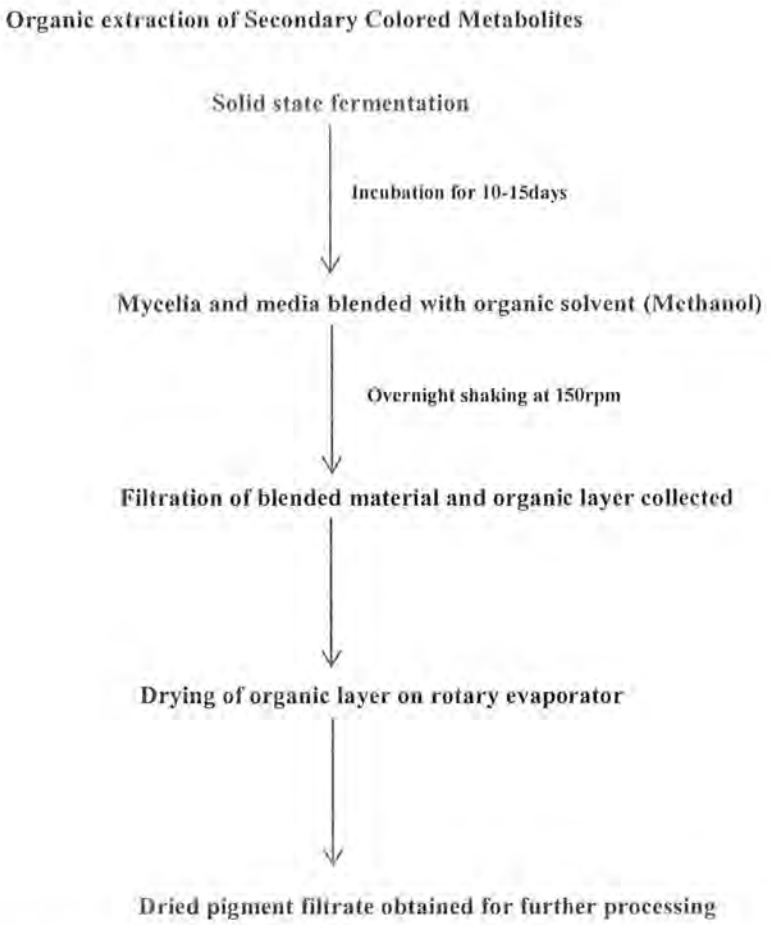


Fig. 3.1: Schematic representation of the pigment extraction from fungi

3.5 Biological screening of pigmented culture filtrate

3.5.1 *Antibacterial Assay*

Pigmented filtrate obtained by culturing fungi in five different media i.e., PDA, SDA, Czapek dox, Minimal salt media and MEA were subjected to antibacterial assay using agar well diffusion method reported by (Bagamboula et al., 2003). Gram positive and Gram negative bacterial strains of *Staphylococcus aureus* (ATCC 6538), *Micrococcus luteus* (ATCC 10240), *Escherichia coli (ATCC* 25922), *Pseudomonas aeruginosa* (ATCC 9721) and *Klebsiella species* (Clinical isolate) to be tested were cultured on nutrient agar plates. 100 µL of each sample (20 mg/ml of DMSO) was added to the respective well. The plates were incubated at 37°C for 24 hours. SEM and LSD were calculated statistically.

3.5.2 *Antifungal Assay*

Pigmented filtrate obtained by culturing fungi in five different media i.e., PDA, SDA, Czapek dox, Minimal salt media and MEA were tested for their antifungal activity by well diffusion method as reported by Duraipandiyan and Ignacimuthu, (2009). Fungal strains used as a test organisms were; *A. niger* and *A. terreus*. Fungal spore suspension was made in normal saline and 2 ml spore suspension of the test fungal strains was spread evenly on PDA plates and 100 µL of each pigmented extract of fungus (20 mg/ml of DMSO) was poured in respective wells. Incubation at 27°C was carried out and results were noted after 48 hours. SEM and LSD were applied on triplicate results.

3.5.3 *Phytotoxicity Assay*

Phytotoxicity bioassay was performed with PDA extract of three fungi by radish seeds (*Raphanus sativa*) according to the method recommended by Turker and Camper, (2002). An aliquot of 5 ml of samples (mg/ml) at various concentrations (2, 5 and 10mg/ml) were added to sterilized petri plates containing sterilized 9 cm filter paper (Whatman No. 1). The filter paper in each plate containing sample was filled with Sterilized radish seeds (n=25). Petri plate containing distilled water was used as control. Plates were incubated at room temperature i.e., 25 °C in presence of light. The Phytotoxicity was determined by number of seeds germinated and inhibited on 5^{th} day.

3.5.4 *Cytotoxicity assay*

The brine shrimp cytotoxic assay was performed with fungal PDA extracts by using the method of Meyer-Alber et al., (1992). Various concentrations (1000, 100 and 10 ppm) of the pigmented filtrate obtained by culturing fungi in PDA were used in the experiment. Controls having no samples (pigmented filtrate) were also used. The samples were mixed with sea water in vials containing viable shrimp larvae. These vials were then incubated at 25 °C in presence of lamp as a light source. The cytotoxicity was determined by counting viable shrimp larvae after 24 and 48 hrs intervals. IC_{50} was calculated by table curve software using non linear regression.

3.6 Biochemical screening of Crude extract

3.6.1 *DPPH Free Radical-Scavenging Assay*

The ability of the PDA pigmented filtrates of the three fungi to scavenge DPPH free radicals was analyzed by the method of Brand-Williams et al., (1995). The experiments were carried out in triplicate in 96-wells micro titter plate. Test samples (dissolved in DMSO) at a concentration of 4mg/ml (10µl) and DPPH (190µl) solution were mixed in each well of 96 well plates. The plates containing the solution were incubated at 37°C for 30 minutes in dark. After incubation, absorbance at 517 nm was taken on plate reader. The variations in wavelength absorbance were related to change in color of DPPH solution from deep-violet to yellow. Ascorbic acid was used as positive control whereas pure DMSO as negative control. The following formula was used for calculation of scavenging activity.

$$\textbf{\textit{Scavenging effect}}\ (\%) = [1 - \frac{As}{Ac}] \times 100$$

Where "Ac" is absorbance of control and "As" is absorbance of the test sample.

IC_{50} value was determined with the help of table curve software using non linear regression.

3.6.2 *Determination of total flavonoid content*

This assay was performed by aluminium chloride colorimetric method as described by Kim et al., (2003). Test samples (Pigmented filtrates of fungi in five different media PDA, SDA, MEA, Czapek dox, Minimal salt media) were dissolved in DMSO. The concentration of sample used was 4mg/ml. Microtitre plates were used in the experiment. Solution was prepared by mixing 10µl of sample (extract) with 10µl of 1.0 M potassium acetate. Further, 10µl of 10 % aluminum chloride was added to the solution followed by 160µl of distilled water in wells of microtitre plate. The microtitre plate was incubated at room temperature for 30 minutes. Absorbance was taken in plate reader at 405 nm. Quercitin was used as standard. Flavonoid contents in extracts were expressed in terms of Quercitin equivalent (mg of QU/g of extract).

3.6.3 *Determination of total phenolic content*

The total phenolic content was determined according to the method proposed by Park et al., (2008) using Folin-ciocalteu reagent. Sample solution was prepared by dissolving pigmented culture filtrate of five different media (PDA, SDA, MEA, Czapek dox, Minimal salt media) in DMSO at a concentration of 4mg/ml. 10µl of sample was mixed with 90µl of Folin-ciocalteu reagent in 96-well microtiter plate. This solution was placed for 5 minutes at room temperature followed by addition and gentle mixing of 90µl of 6% sodium carbonate. This final solution was incubated at 25°C for 90 minutes. Afterwards, absorbance was measured at 630nm on plate reader. The calibration curve of gallic acid corresponds to the total phenolic content. The results were expressed as mg of gallic acid equivalents (GAEs) per g of extract.

3.6.4 *Determination of total reducing power*

The method described by Oyaizu et al., (1986) was used for determining total reducing power of the pigmented filtrates of the three fungi. Sample solutions were prepared by dissolving pigmented culture filtrate of five different media (PDA, SDA, MEA, Czapek dox, Minimal salt media) in DMSO at a concentration of 4mg/ml. Pigmented samples were first dissolved in 0.2 M of phosphate buffer followed by addition and subsequent mixing with 1% potassium ferricyanide. This solution was kept at 50°C for 20 minutes. Further, 10% trichloroacetic acid was added to the mixture and then centrifugation at 3000 rpm for 10 minutes was done. Finally, 0. % ferric chloride was added to the upper layer of centrifuged solution. Absorbance was taken at 630nm on micro plate reader using ascorbic acid as standard. IC_{50} value was determined with the help of table curve software using non linear regression.

3.6.5 *Determination of total antioxidant activity*

Total antioxidant capacity was carried out by following method of Umamaheswari and Chatterjee, (2008). Samples (PDA extracts) were prepared by dissolving pigmented culture filtrate in DMSO (4mg/ml of DMSO). 0.1ml of sample was mixed with 1 ml of the reagent solution (0.6 sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). This solution was placed in 95°C water bath for 90 minutes. After cooling of the reaction mixture, absorbance was taken at 630nm on

microtiter plate reader. Ascorbic acid was used as standard. The antioxidant capacity of each sample was expressed as ascorbic acid equivalent. The readings were taken by running the experiment in triplicate for more accurate and authentic results. IC_{50} value was determined with the help of table curve software using non linear regression.

ABTS free radical scavenging activity

This activity was carried out by following the method of Re et al,. (1999). ABTS (7mM) was dissolved in deonized water followed by addition of potassium persulphate (2.45mM). This reaction was left in dark overnight at room temperature. Positively charged ABTS resulted in dark color of the mixture which was diluted by addition of phosphate buffer saline (0.01M; pH 7.4). The absorbance was taken at 734nm. After wards the pigmented filtrates of the three fungi were diluted with ABTS solution. Absorbance was taken after one minute. Experiments were carried out in triplicates. IC_{50} value was determined with the help of table curve software using non linear regression.

3.7 Qualitative evaluation of pigmented filtrate of fungi

Phytochemical screening was carried out in order to check the presence of major constituents and bioactive classes of compounds in the PDA extracts of different fungi using standard qualitative procedures (Harborne, 1973; Trease and Evans, 1989; Sofowora, 1993).

3.7.1 *Test for tannins*

Presence of tannins in pigmented filtrate of the three fungi in PDA was determined by the method of Sofowara (1993). 50 mg of pigmented extract of three fungi was boiled in distilled water and filtered through filter paper. The solution was observed for change in color upon addition of few drops of 0.1% $FeCl_3$. If the color of the solution turned brownish green, it showed the presence of tannins in the extract.

3.7.2 *Determination of flavonoids*

Detection of flavonoids in pigmented filtrate of the three fungi in PDA was attained by the method proposed by Harbrone (1973) and Sofowara (1993). Mixing of the

sample (50 mg of each extract) with distilled water (100 ml) resulted in a suspension and consequently filtrate. Afterwards, dilute ammonia solution (5ml) was added to filtrate (10ml). Change of solution color to yellow by addition of few drops of concentrated H_2SO_4 confirmed the presence of flavanoids.

3.7.3 *Terpenoids test (Salkowski test)*

Presence of terpenoids in pigmented filtrate (PDA) of the three fungi was determined according to Harbrone (1973). 5ml (1mg/ml) of PDA pigmented filtrates of the three fungi were added to few drops of chloroform followed by concentrated H_2SO_4 (3ml). If the solution color turned brown, it indicated the presence of terpenoids in the pigmented extracts.

3.7.4 *Test for alkaloids*

Alkaloids in pigmented filtrates (PDA) of the three fungi were detected according to Harbrone (1973). Pigmented filtrate (0. g) of the three fungi obtained by culturing them in PDA was mixed with 1% HCl (8ml). This solution was then warmed. Further, this warmed solution was filtered through filter paper. Titration of each filtrate (2ml) separately by means of potassium mercuric iodide (Mayer's reagent) and potassium bismuth (Dragendroff's reagent) was done. Turbidity of precipitation confirmed the alkaloid's presence in pigmented filtrates.

3.7.5 *Cardiac glycosides determination (Keller-Killani test)*

Presence of cardiac glycosides in each PDA pigmented filtrates of the three fungi was performed according to method of Trease and Evans, (1989).

3.7.6 *Coumarins identification*

Presence of coumarins in pigmented filtrates (PDA) of the three fungi was analyzed following method of Trease and Evans, (1989). 0.3g of each pigmented filtrates (0.3 ml) was taken in test tubes and these test tubes were covered with 1N NaOH moistened filter paper. The filtrates in test tubes were boiled for few minutes. The filtrates were then examined under UV light after removal of filter paper from the test

tubes. Confirmation of coumarins presence in pigmented filtrates was shown by yellow florescence in UV light.

3.7.7 *Anthraquinone detection*

Method of Trease and Evans, (1989) was implemented for detection of anthraquinones presence in pigmented filtrates (PDA) of the three fungi. Each filtrate (200 mg) was mixed with 1% HCl (6 ml) and then boiled followed by filtration of the solution. Benzene (5ml) was added to filtrates and shaken followed by addition of 10% NH_4OH. The change in color of the filtrate to pinkish, violet or reddish color by addition of base confirmed the presence of anthraquinone.

3.8 Stability study of the pigmented culture filtrate by FTIR and spectrophotometer

Stability study of the pigmented culture filtrate of each fungus in PDA was done at various temperatures (-20_-100°C) and pH (3-9) by means of spectrophotometer and FTIR. In order to detect presence of various functional groups in the pigmented filtrates of each fungi, FTIR (Perkin Elmer spectrum 65) with ATR equipped spectrophotometer was used. The samples (300mg/ml) were placed on top of FTIR plate and fixed. The samples were scanned at range of 600 – 4000 wave-number/cm for their further analysis. To detect the changes in intensity of different peaks, overlays were recorded for every 2 hours interval of incubation at various temperatures and pH. UV/Vis spectrophotometer was also used in order to check the stability of pigmented filtrate of the three fungi obtained by culturing them in PDA at different pH (3-9) and temperatures (-20-100°C) by taking ODs at the respective λmax.

3.9 Column chromatography for fractionation and purification of culture filtrate of the three fungi

In order to do separation and purification of pigmented culture filtrate obtained by culturing fungi in PDA, column chromatography was done. Fungi were grown in bulk on most productive media PDA. The soluble extracellular pigments were extracted with Methanol by adding 3 times of 70% Methanol and shaking at 150 rpm at 40°C

for 12-16 hrs. The pigment dissolved in organic solvent were decanted and filtered through Whatmann No.1 filter paper in order to remove impurities. These dried pigmented filtrates (Rotary evaporator) obtained from three fungi were further subjected to column chromatography to obtain colored metabolite containing fractions to be analyzed by LCMS and two pure colored compound.

The dried extracts obtained were initially processed by using normal phase silica gel (200-400micro metre) column chromatography. The sample was loaded on blank silica with ratio of 1g sample/1.5 gram of silica. Loaded sample was then filled at the top of filled column. Then sample was eluted in first column (Silica) run with the SS1 while in second column run wherever it is used, SS2 was used as stated below.

1. Stationary phase silica gel (SP1)

Solvent system 1 (SS1) ——————→n- Hexane100%: Chloroform 0%____100% Chloroform:0 % MEOH_____50% Chloroform:50 % MEOH (Gradient)

Solvent system 2 (SS2) ——————→ n-Hexane 50%: Ethyl acetate50%_____10 % Ethyl acetate: 90% Methanol (Gradient)

2. Stationary phase LH20 (SP2)

Solvent system (SS3) ——————→Methanol: Water (Gradient)

TLC is a qualitative method to determine purity of an isolated compound and different fractions. The solvent system to be used during column chromatography can also be established and optimized by using TLC at every stage of column chromatography. Analytical TLC was performed on precoated TLC plates with Si gel 60 F_{254} (0.2 mm, Merck) and RP-2 (0.2 mm, Merck). Different solvent systems can be used for developing TLC especially for semi polar compounds. Preparative TLC was done in suitable solvents to obtain the colored compounds that are scratched for further processing. However, two solvent systems were used on preparatory TLC in the present study.

SST1——————→Chloroform 15: Methanol 85:1Aammonium hydroxide.

SST2 ——————→Ethyl acetate 15: Methanol 85:1 Ammonium hydroxide.

3.9.1 *Column chromatography of Penicillium verruculosum*

After 1st column run on SP1 with SS1 solvent system, total 76 fractions of 150 ml each were collected. These fractions were analyzed by TLC and then combined to get 7 fractions (A,B,C,D,E,F,G). Fractions B, E, F were found to have colored metabolites. Fraction E which was 5 grams was again subjected to SP1 with SS2 solvent system used and total 60 fractions 100 ml each were obtained and analyzed by TLC. TLC analysis led to combination of fractions again to get 6 sub fractions (a-f) which being less in amount were subjected to LCMS directly.

Fraction B contained red, purple and yellow colored spots apparently and was subjected to SP1 with SS2 solvent system and 50 fractions were obtained that were combined into a,b,c,d,e,f based on TLC analysis. Fraction B-e which was in good amount and contained maximum number of colored spots was subjected to SP2 with solvent system SS3. Then 76 fractions 1ml each were collected and combined into 18 fractions B-e1-B-e18. The colored fractions (B.e7- B.e12) were loaded on preparatory TLC plate (250-500µm) with SST1 solvent system and the colored bands were scratched for their LCMS analysis to analyze them further for possible elucidation of the compounds. These fractions because of fewer amounts were processed through LCMS. An important fraction B.d being uncolored and less in amount was also subjected to LCMS directly. Yellow spot was observed in fraction B.c which was 800 mg. This fraction c was subjected to gel filtration chromatography by using SP2 eluted with SS3. Total 150 fractions 1ml each was collected. Then, these fractions were combined into 40 fractions. Fraction 27 and 28 contained yellowish colored spot as analyzed by TLC. The yellow colored compound was precipitated in $CHCl_3$ on standing. These precipitates were dried and crystalized and 3D XRD of these crystals is done.

Another fraction F was also subjected to SP1 with SS2 solvent system to chase some colored/bioactive spot. Total 92 fractions were obtained each 50ml and combined after TLC analysis into a-g. Fraction F.c containing reddish spot was subjected to SP2 with SS3. 40 fractions were obtained 1ml each. They are then combined into 15 fractions F.c1-F.c15. LCMS of the fractions was done which were in fewer amounts.

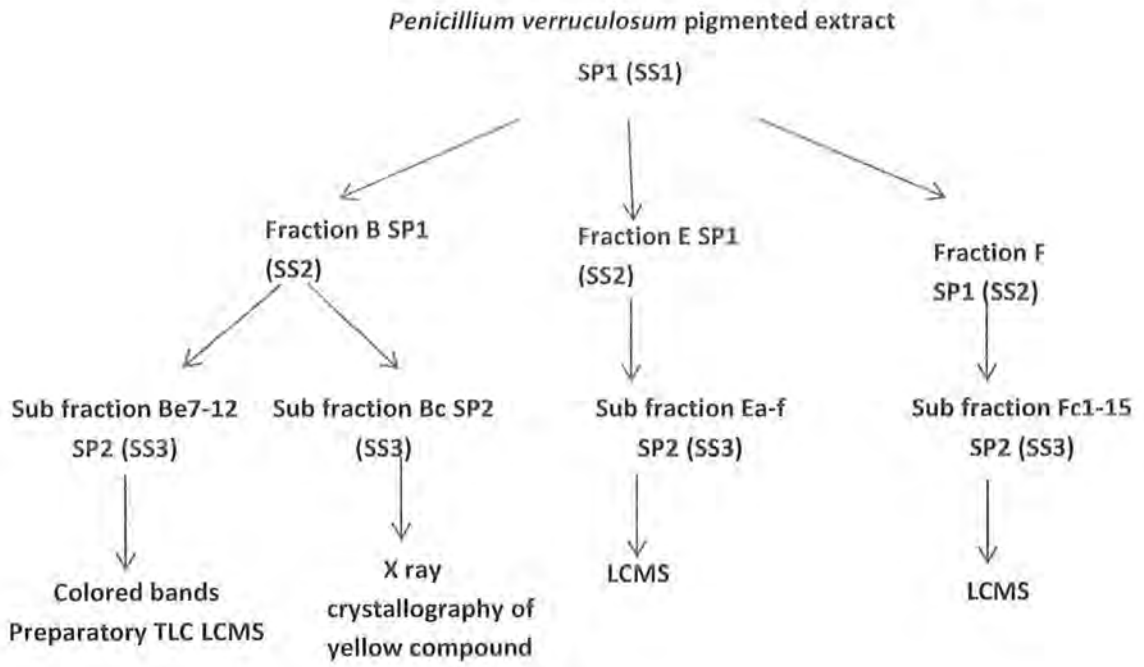


Fig 3.2: Schematic flow sheet representing Column chromatography of *Penicillium verruculosum* and ultimate fate of the different selected fractions

3.9.2 *Bulk production and Column Chromatography of Chaetomium strumarium*

After 1st column SP1 with SS1 solvent system, Total 85 fractions of 150 ml each were collected. These fractions were analysed by TLC and then combined to get 6 fractions (A, B, C, D, E, F). Fraction C, E, F was found to have colored metabolites.

Fraction E which was 6 grams was again subjected to normal phase silica gel column eluted with SS2 and total 60 fractions 100 ml each was obtained and analysed by TLC. TLC analysis led to combination of fractions again to get 7 fractions (a-g). Orange spot was observed in fraction d which was 700 mg. This fraction d was subjected to gel filtration chromatography by SP2 and eluted with SS3. Total 142 fractions 1ml each was collected. Then these fractions were combined into 35 fractions. Fraction 31 and 32 contained orange colored spot as analyzed by TLC. These fractions were combined and orange colored compound was precipitated in a mixture of ethyl acetate and methanol on standing. These precipitates were dried and dissolved in DMSO to check for LCMS and Proton NMR and labeled as compound S2.

Fraction C contained reddish colored spot apparently and was subjected to SP1 with SS2 solvent system. 50 fractions were obtained that were combined into a,b,c,d,e,f. Fraction C-b was subjected to SP2 with SS3. Then 76 fractions 1ml each were collected and combined into 18 fractions C.b1-C.b15. Another fraction F was also subjected to SP1 with SS2 used for elution of fractions to chase another purplish spot. Total 92 fractions were obtained each 50 ml and combined after TLC analysis into a-g. Fraction F.c containing purplish spot was subjected to SP2 with SS3 for elution of various fractions. 40 fractions were obtained 1ml each. They are then combined into 10 fractions F.c1-c10. The fraction F.c3-F.c5 was subjected to preparatory TLC with SST1 and purple band was scratched to be processed through LCMS.

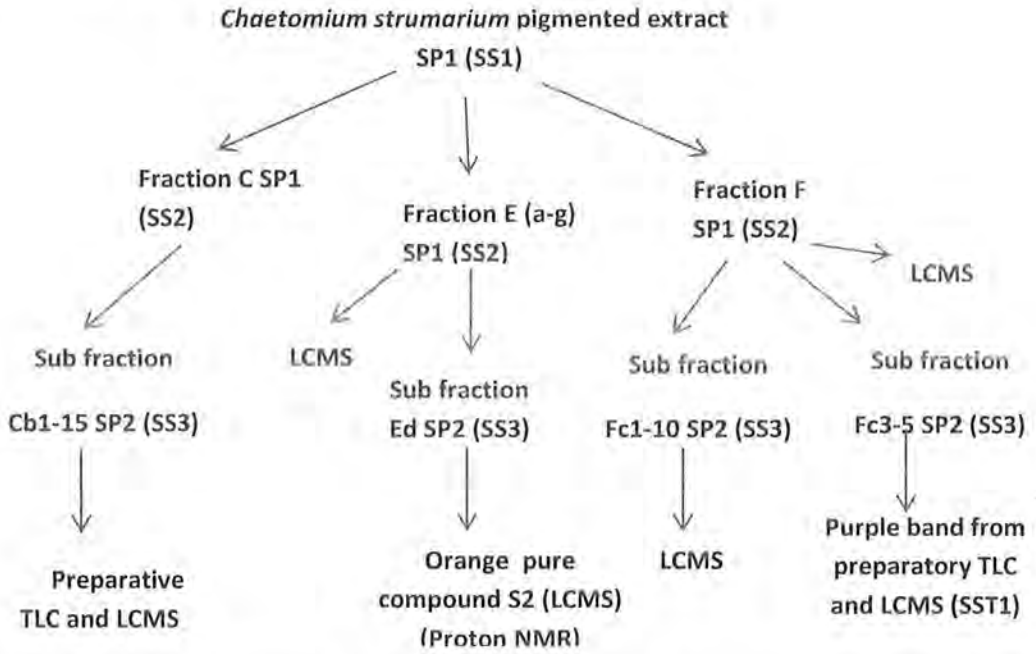


Fig 3.3: Schematic diagram representing Column chromatography of *Chaetmium strumarium* and ultimate fate of the different selected fractions

3.9.3 *Bulk production and column chromatography of Aspergillus fumigatus*

After 1st column run with SP1 and SS1 as solvent system used for elution of the sample, total 50 fractions of 100 ml each were collected. These fractions were analysed by TLC and then combined to get 5 fractions (A,B,C,D,E). Fraction B, C, D were found to have colored metabolites. Fraction B which was 4 grams, was again subjected to SP1 with SS2 and total 60 fractions 100 ml each was obtained and

analyzed by TLC. TLC analysis led to combination of fractions again to get 4 fractions (a-d).

Fraction C contained red and orange spots apparently was subjected to SP1 with SS2 solvent system used for elution of the fraction. 40 fractions were obtained that were combined into a,b,c. Fraction C-a which was in good amount and contained red colored spot was subjected to SP2 with SS3 solvent system for elution. Then 50 fractions 1ml each were collected and combined into 14 fractions C.a1-B.a14. The colored fractions (C.a5-C.a10) were loaded on preparatory TLC plate (250-500µm) with SST1 solvent system to develop TLC and the red colored bands were scratched for their LCMS analysis. The remaining fractions because of fewer amounts were processed through LCMS.

Sub fractionation of main fraction D after SP1 with SS2 as solvent system lead to 70 fractions which were further combined after TLC analysis in to 5 fractions (Da-e). These fractions were subjected to LCMS directly.

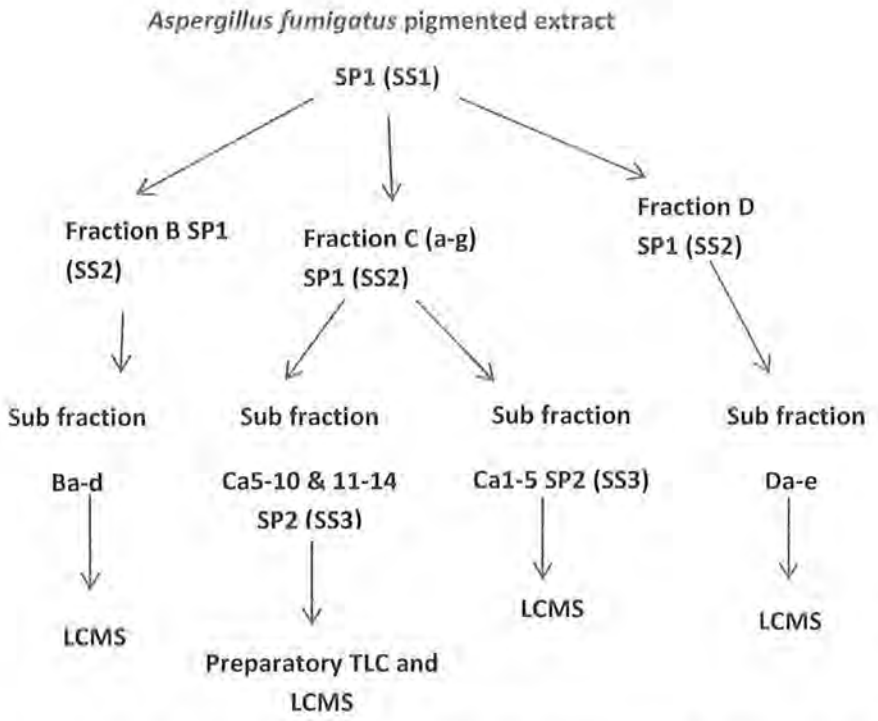


Fig 3.4: Schematic diagram representing Column chromatography of *Aspergillus fumigatus* and ultimate fate of the different selected fractions

3.10 Liquid chromatography Mass spectrometry (LCMS)

Liquid chromatography-diode array detection-mass spectrometry (LC-DAD-MS) was performed in order to detect presence of colored and bioactive compounds in different fractions (C8, Sephadex, Preparatory TLC) of colored filtrate of fungus. Liquid chromatography-diode array detection-mass spectrometry (LC-DAD-MS) was performed on an Agilent eclipse HP 1100 LC system with a DAD and a 5 μ XDB C18 column (Phenomenex, Torrance, CA). The LC system was coupled to a LCT orthogonal time-of-flight mass spectrometer (Waters-Micromass, Manchester, United Kingdom) with a Z-spray electrospray ionization (ESI) source and a LockSpray probe and controlled by the MassLynx 4.0 software. The positive ESI mode was used for operating MS system with solvent system of water-acetonitrile gradient. This method was previously described by Nielsen et al., (2005). The compounds were identified by comparing LCMS and LCMS/MS spectra with library and literature by their m/z value, wavelength absorbance pattern in addition to their logical fragmentation pattern through chemdraw software.

3.11 Cytotoxicity Assays (Mammalian cell lines) of various fractions obtained after column chromatography

The cytotoxicity assays were carried out following the method of Shier (1983) and Abbas et al., (1992). Different fractions being subjected to LCMS and LCMS/MS were tested for their cytotoxicity against mammalian cell line and cytotoxicity analysis was done on 96 well plate method using 5 different cell lines. Different fractions and sub-fractions of all the three fungi containing biologically active and colored compounds were subjected to these assays.

Stock Solution:

The sample is dissolved in 62.5µl of 100% ethanol then 62.5 µl of water (Conc = 40mg/ml) was added. Dilute the stock into sterile DME in a sterile vial by diluting 20 µl of stock into 2 ml DME (Conc. = 400 µg/ml).

Dilution:

The dilution of samples were made in DME ranging from 5 to 200 µg/ml and 96 well plates method was used. The dilutions were prepared according to the following table adding DME to the wells first.

Row	Final Conc. (µg/ml)	DME (µl)	Sample
A	200	--	100 µl of substock
B	100	50	50 µl of substock
C	50	75	25 µl of substock
D	20	90	10 µl of substock
E	10	171	9 µl of substock
F	5	50	50 µl of E
G	2	80	20 µl of E
H	1	90	10 µl of E

The trays were checked to make sure the same amount of medium is in each well.

Suspend the five mammalian cell lines i.e., KA31T (virally transformed cancerous cell line) or NIH3T3 cells (mouse embryonic fibroblasts), HSCT6 cells (rat hepatic stellate cell line), HEK293 (cell line derived from human embryonic kidney cells grown in tissue culture), MDCK Line (Madin-Darby Canine Kidney Epithelial Cells) with trypsin and add 100 µl of 2 x 10^4 Cells/ml in 10% Calf serum to each well. Cultures until control wells are confluent. Wash with medium, fix 30 min with formal saline, stain with 0.5% crystal violet, rinse away excess stain and estimate IC_{50}. Experiment was performed in triplicate and IC_{50} was calculated by means of table curve software.

3.12 3 D Xray crystallographic study of a yellow crystal obtained from *Penicillium verruculosum*

Yellow needle like crystal obtained in fraction B.c of *Penicillium verruculosum* was cut in order to fit in to the size of homogeneous part of the X-ray beam, which is mounted on topmost part of a glass fiber and aligned on a Bruker SMART APEX

CCD diffractometer (Platform with full three-circle goniometer). The crystal was cooled by using low temperature device (Bruker KRYOFLEX) to 100(1) K. Graphite monochromatized Mo-K _a radiation from a sealed ceramic diffraction tube (SIEMENS) performed the function of measuring intensity. A program SAINT was used for data integration and global cell refinement. Space group (XPREP) (Bruker et al., 2006) was determined through program suite SAINTPLUS. Patterson method resolved the structure of the compound; model's extension was accomplished by direct method and applied to difference structure factors using the program DIRDIF (Beurskens et al., 1999). Program PLUTO and PLATON package were used for final refinements, graphics and calculations.

3.13 Proton NMR (nuclear magnetic resonance) of orange compound obtained from *Chaetomium strumarium*

Fraction E.d containing orange fraction was crystallized in Ethyl acetate and Methanol to get the crystalline orange compound. These crystals were subjected to 1D proton NMR experiments which were acquired using 30° pulse width, 128 scans, 1 s recycle delay and 2.3 s acquisition time on a Bruker Avance 700-MHz NMR spectrometer with a TXI cryoprobe.

RESULTS

CHAPTER 4

4. Result

4.1 Isolation and Screening of pigment producing fungi

In order to evaluate the pigment producing ability of fungi, five different fungi were isolated i.e., two fungi from Kala pani forest soil whereas the remaining three fungi were endophytes of *Taxus baccata* plant i.e. SG1, SG2 and SG3 were isolated from woody parts of the *Taxus* plant. All these five fungi were initially screened on potato dextrose agar (PDA) for their extracellular pigment producing ability. Initially, the two fungi from Kala Pani Forest were identified morphologically and were identified as *Penicillium* sp SG, and *Aspergillus* sp SG4. *Penicillium* sp. SG produced cherry red extracellular pigment whereas *Aspergillus* sp produced purple extracellular pigment. The three endophytic fungi were identified morphologically as *Chaetomium* sp SG1, *Epicoccum* sp SG3 and *Penicillium* sp SG2 producing extracellular magenta, dark brown and yellowish orange color/pigments respectively.

These fungi were screened visually on five different media for their pigment producing ability including Malt extract agar (MEA), Sebouraud dextrose agar (SDA), Czapek dox agar (Czapek dox), Yeast extract malt peptone agar (YMPG) and Potato dextrose agar (PDA). *Penicillium* sp. SG producing extracellular red pigment on PDA also produced red pigment in other media but the intensity of the red color was less in remaining four media with least color found in Czapek dox agar (Fig. 4.1). SDA and MEA helped good pigment production followed by YMPG in which yellow pigment production was also apparent. Pigment production started on third day and covered the whole PDA plate on 10^{th} day. The optimum time for pigment production was 10^{th} day in *Aspergillus* sp SG4 and it produced purple pigment on PDA whereas bright yellow pigment on other media i.e., SDA, MEA and YMPG (Fig. 4.2). In case of *Chaetomium* sp. SG1 pigment production started on 7^{th} day and covered the plate on 14^{th} day. Magenta extracellular pigment was produced in SDA and PDA whereas in MEA and YMPG, yellow pigment was produced (Fig. 4.3).

In *Penicillium* sp. SG2 (Fig. 4.4), pigment production started on 7^{th} day and covered the plate on 15^{th} day. Orange pigment was produced in PDA while, MEA and YMPG

showed yellow pigment production of almost equal intensity. Moreover in SDA, comparatively less pigment production was observed. In *Epicoccum* sp SG3, pigment production started at day 6 and reached maximum at day 13th. Pigment production was maximum in PDA and SDA followed by YMPG>MEA>Czapek dox (Fig. 4.5). Overall, pigments production was least to none on Czapek dox medium in all the fungal isolates.

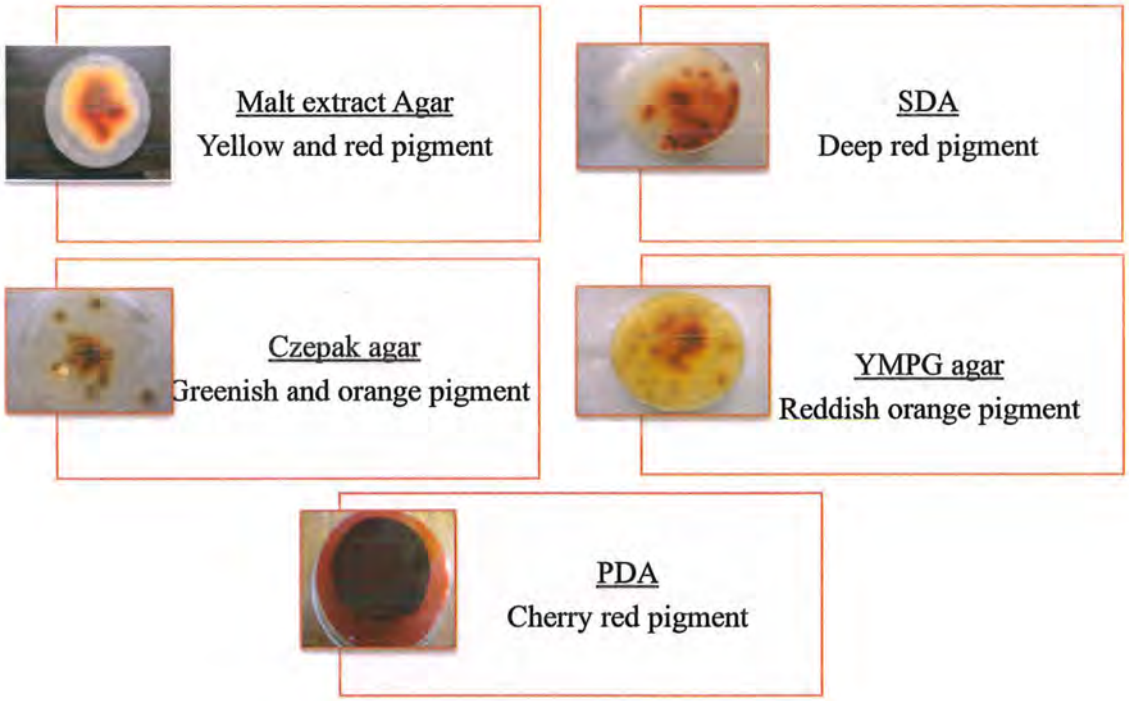


Fig 4.1: Extracellular pigment production in five different media by *Penicillium sp* SG.

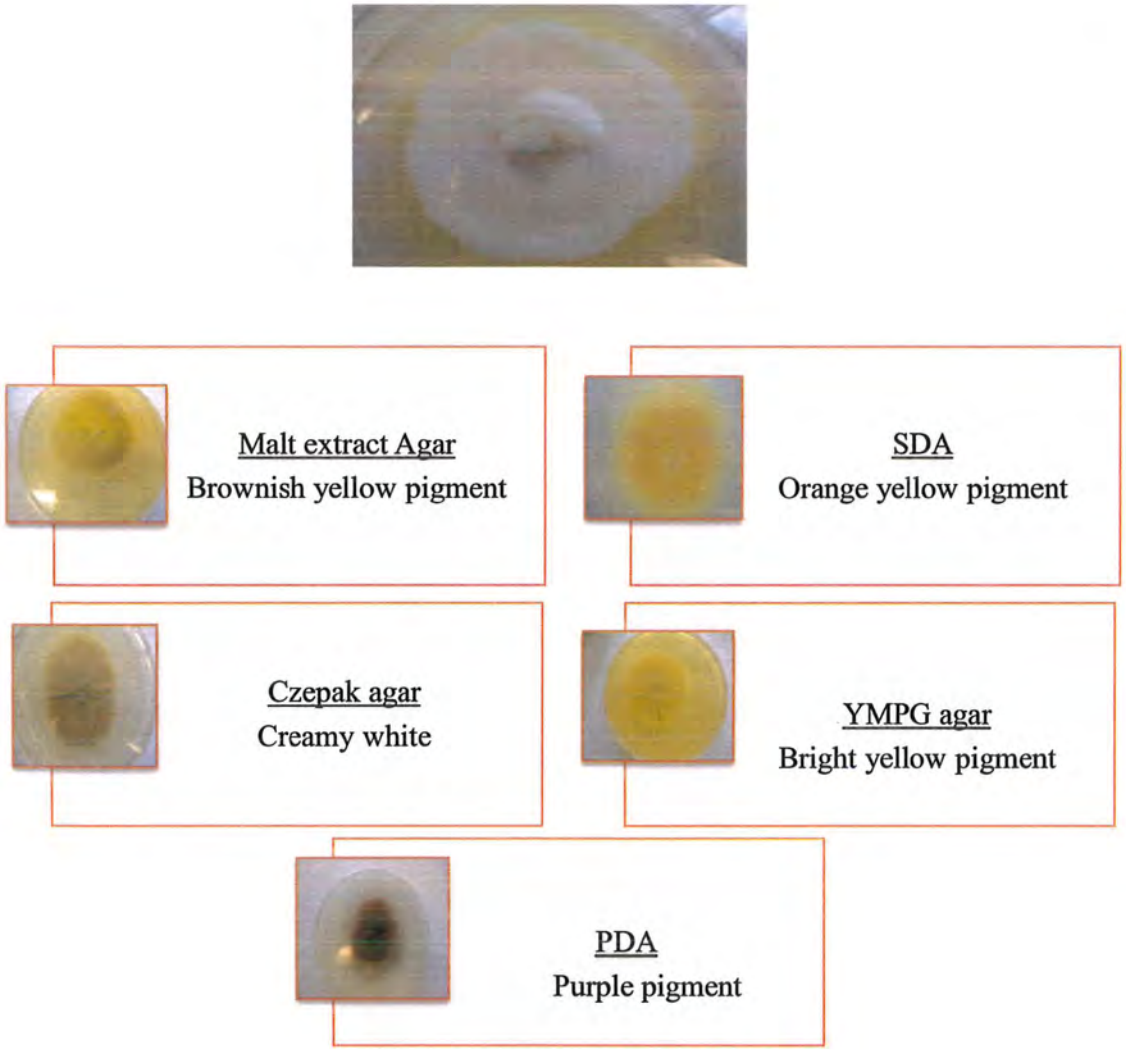


Fig 4.2: Extracellular pigment production in five different media by *Aspergillus* sp SG4.

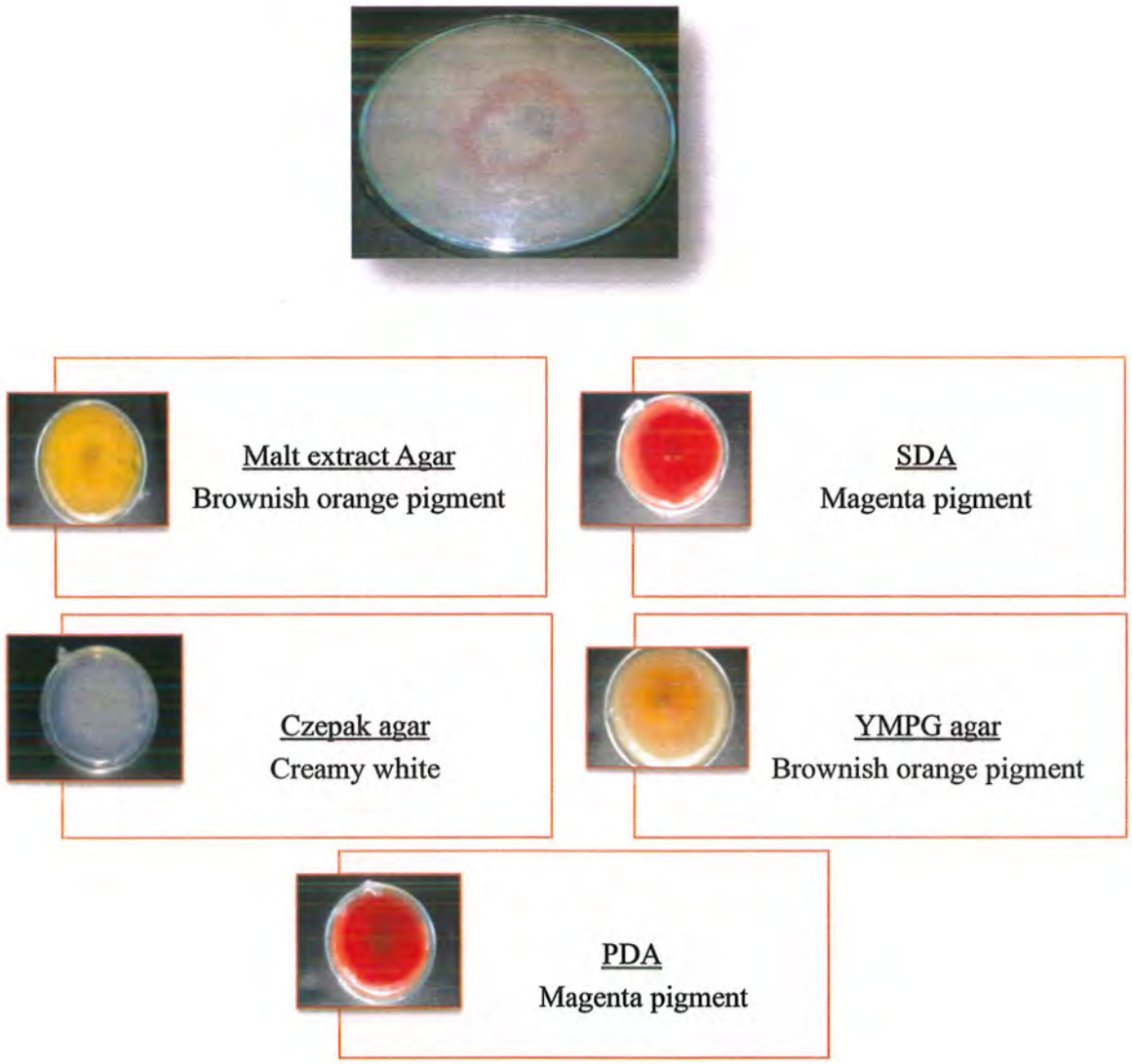


Fig 4.3: Extracellular pigment production in five different media by *Chaetomium* sp SG1.

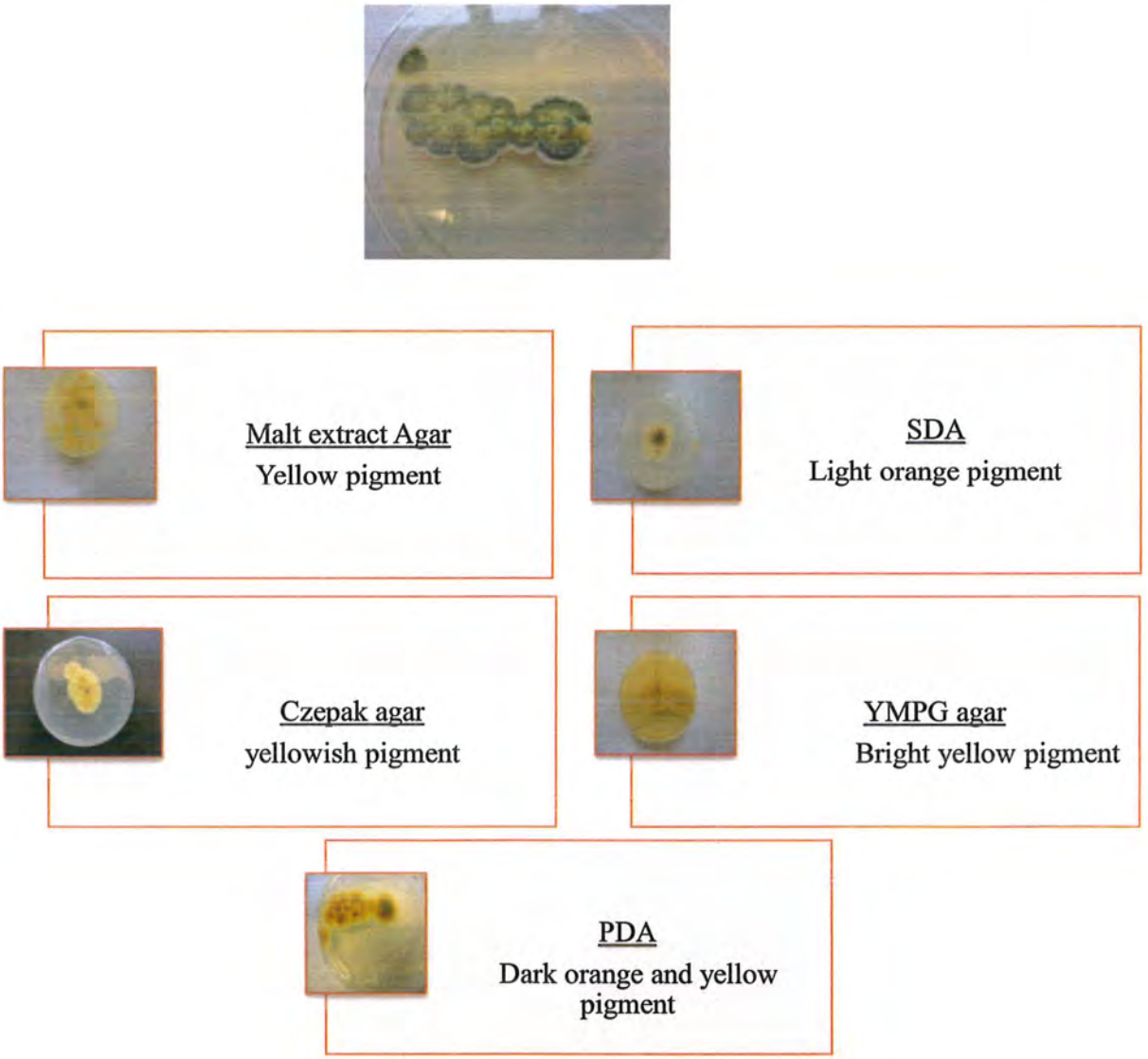


Fig 4.4: Extracellular pigment production in five different media by *Penicillium* sp SG2

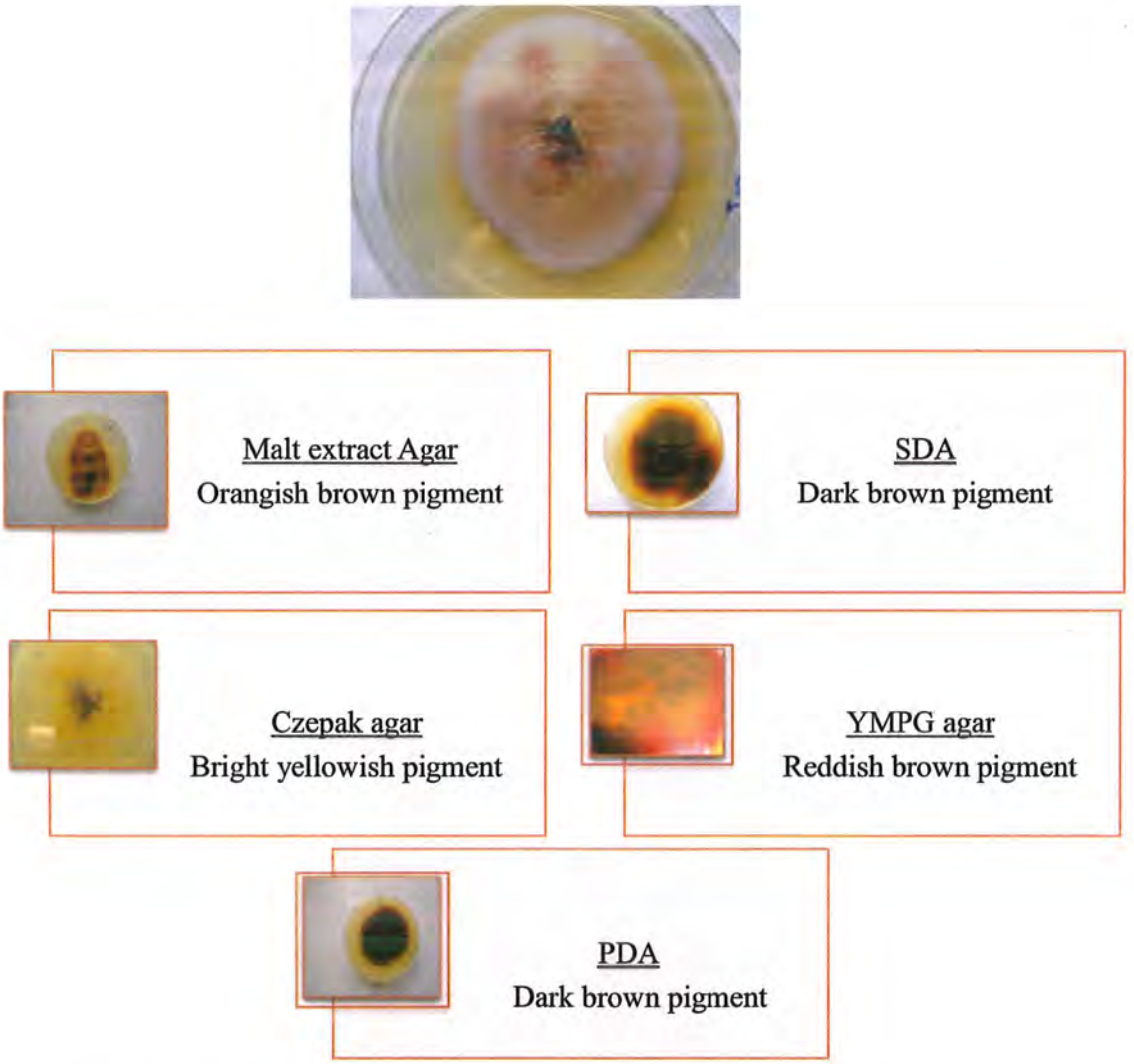


Fig 4.5: Extracellular pigment production in five different media by *Epicoccum* sp SG3.

4.2 Molecular identification of fungi

Out of the five fungi, three best pigment producing fungi were selected and identified to the species level. The detailed are as follows:

4.2.1 *Penicillium verruculosum (SG)*

The sequencing of ITS region of 28s (Fig. 4.7), 18s rRNA (Fig. 4.6) and D2 region of 28s rRNA (Fig. 4.8) proved this fungus to be *Penicillium verruculosum.* The sequences were submitted to the Gene Bank and accession numbers were obtained for each sequence. The detailed sequences of each amplified region are as follow:

Results

4.2.1.1 *Accession number KC698959 (28s ITS region)*

ATATGCTTAAGTTCAGCGGGTAACTCCTACCTGATCCGAGGTCAACCGTGGTA
AAATATGGTGGTGACCAACCCCCGCAGGTCCTTCCCGAGCGAGTGACAGAGC
CCCATACGCTCGAGGACCAGACGGACGTCGCCGCTGCCTTTCGGGCAGGTCC
CCGGGGGGACCACACCCAACACACAAGCCGTGCTTGAGGGCAGAAATGACG
CTCGGACAGGCATGCCCCCCGGAATGCCAGGGGGCGCAATGTGCGTTCAAAG
ATTCGATGATTCACGGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGC
GTTCTTCATCGATGCCGGAACCAAGAGATCCATTGTTGAAAGTTTTGACAATT
TTCATAGTACTCAGACAGCCCATCTTCATCAGGGTTCACAGAGCGCTTCGGCG
GGCGCGGGCCCGGGGACGAACGTCCCCCGGCGACCAGGTGGCCCCGGTGGG
CCCGCCAAAGCAACGGGTGTATAGAGACAAGGGTGGGAGGTTGGGCCACGA
GGGCCCGCACTCGGTAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTT
AC

4.2.1.2 *Accession number KC416630(18s rRNA)*

CTGCGGAAGGATCATTACCGAGTGCGGGCCCTCGTGGCCCAACCTCCCACCC
TTGTCTCTATACACCCGTTGCTTTGGCGGGCCCACCGGGGCCACCTGGTCGCC
GGGGGACGTTCGTCCCCGGGCCCGCGCCCGCCGAAGCGCTCTGTGAACCCTG
ATGAAGATGGGCTGTCTGAGTACTATGAAAATTGTCAAAACTTTCAACAATG
GATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAAT
GTGAATTGCAGAATTCCGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC
CTGGCATTCCGGGGGGCATGCCTGTCCGAGCGTCATTTCTGCCCTCAAGCACG
GCTTGTGTGTTGGGTGTGGTCCCCCCGGGGACCTGCCCGAAAGGCAGCGGCG
ACGTCCGTCTGGTCCTCGAGCGTATGGGGCTCTGTCACTCGCTCGGGAAGGA
CCTGCGGGGGTTGGTCACCACCATATTTTACCACGGTTGACCTCGGATCAGGT
AGGAGTTACCCGCTGAACTTAAGCATATCAAAAGG

4.2.1.3 *Accession number JX863916 (D2 region)*

GGAGGAAAAGAAACCAACCGGGATTGCCTCAGTAACGGCGAGTGAAGCGGC
AAGAGCTCAATTTGAAATCTGGCCCCTTTGGGGTCCGAGTTGTAATTTGCAGA
GGATGCTTCGGGTGCGGTCCCCGTCTAAGTGCCCGGAACGGGCGTCATAGAG
GGTGAGAATCCCGTCTGGGATGGGCGGCCGCGCCCGTGTGAAGCTCCTTCGA
CGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGCGGGTGGTAAATTTCATCTA
AAGCTAAATACTGGCCGGAGACCGATAGCGCACAAGTAGAGTGATCGAAAG
ATGAAAAGCACTTTGAAAAGAGAGTTAAACAGCACGTGAAATTGTTGAAAG
GGAAGCGTTGTCCACCAGACTCGCCCGGGGGGGTTCAGCCGGCACCTGTGCC
GGTGTACTCCTCTCCGGGCGGGCCAGCATCGGTTTGGGCGGCTGGTGAAAGG
CCCCGGGAATGTACCACCCTTCGGGGTGCCTTATAGCCCGGGGTGCCATACA
GCCAGCCTGGACCGAGGCCCGCGCTTCGGCGAGGATGCTGGCGTAATGGTGG
TCAACGGCCCGTCTTG

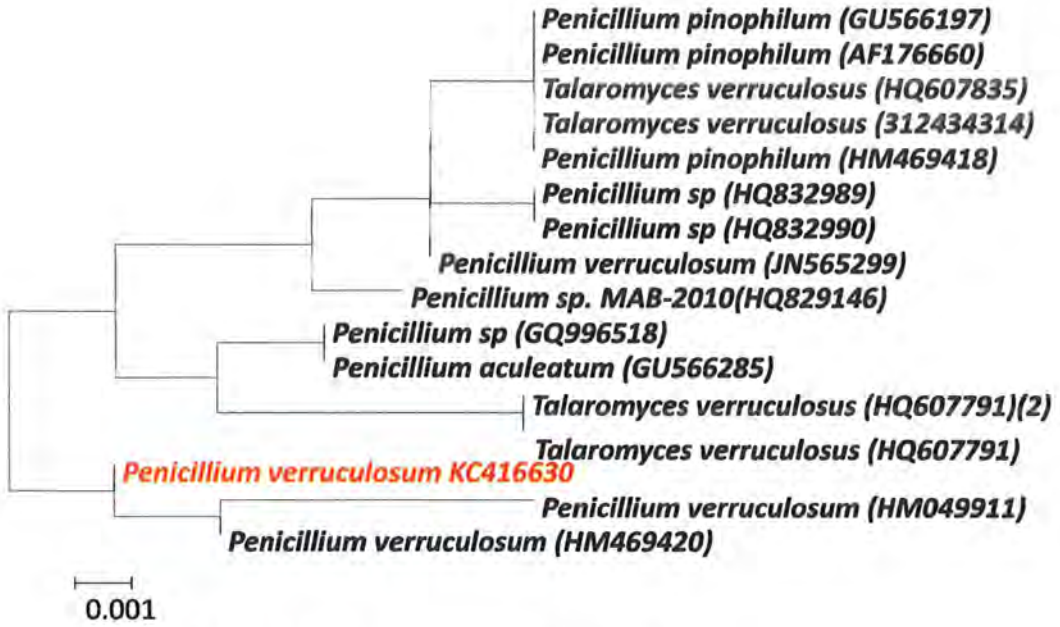


Fig 4.6: Phylogenetic tree of *Penicillium verruculosum* (18srRNA)

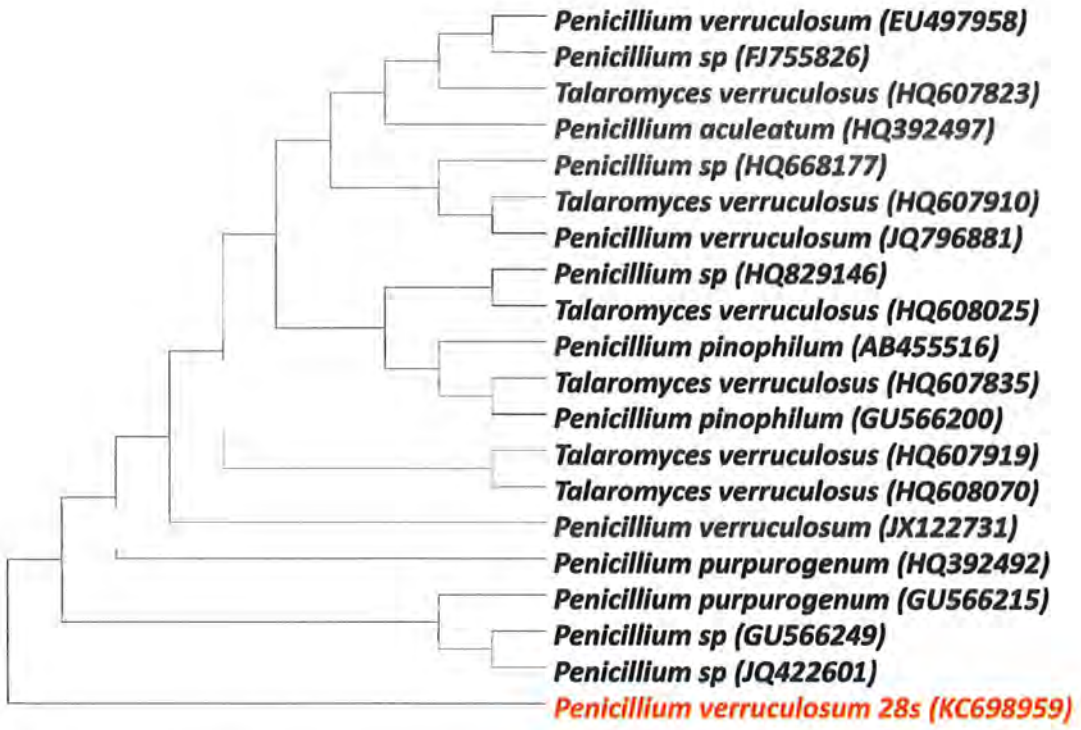


Fig 4.7: Phylogenetic tree of *Penicillium verruculosum* (28srRNA)

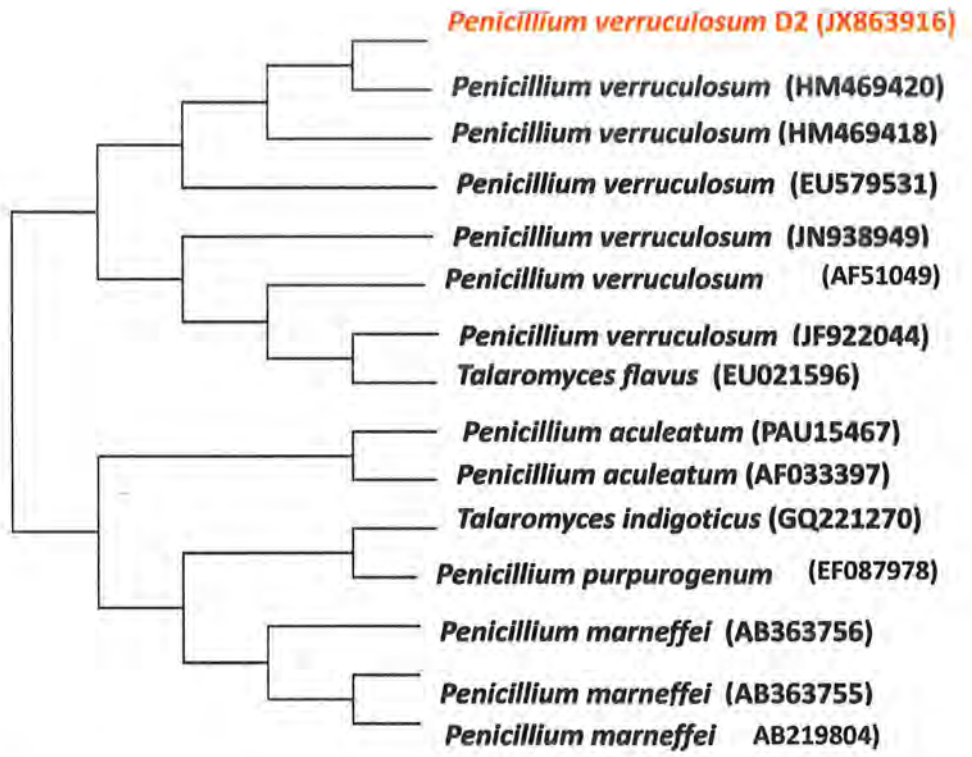


Fig 4.8: Phylogenetic tree of *Penicillium verruculosum* (28srRNA, D2 region)

4.2.2 *Aspergillus fumigatus* (SG4)

4.2.2.1 *Accesion number JX863917 (28s rRNA)*

The sequencing of D2 region of 28s rRNA proved this fungus to be *Aspergillus fumigatus*. The sequence was submitted to the Gene Bank and accession number was obtained for the sequence (Fig. 4.9). The detailed sequence of amplified region is as follow:

GAGGAAAAGAAACCAACAGGGATTGCCTCAGTAACGGCGAGTGAAGCGGCA
AGAGCTCAAATTTGAAAGCTGGCCCCTTCGGGGTCCGCGTTGTAATTTGCAG
AGGATGCTTCGGGTGCAGCCCCCGTCTAAGTGCCCTGGAACGGGCCGTCATA
GAGGGTGAGAATCCCGTCTGGGACGGGGTGTCTGCGTCCGTGTGAAGCTCCT
TCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAATGGGTGGTAAATTTCA
TCTAAAGCTAAATACTGGCCGGAGACCGATAGCGCACAAGTAGAGTGATCGA
AAGATGAAAAGCACTTTGAAAAGAGAGTTAAACAGCACGTGAAATTGTTGA
AAGGGAAGCGTTTGCGACCAGACTCGCTCGCGGGGTTCAGCCGGCATTCGTG
CCGGTGTACTTCCCCGTGGGCGGGCCAGCGTCGGTTTGGGCGGCCGGTCAAA
GGCCCTCGGAATGTATCACCTCTCGGGGTTGTCTTATAGCCGAGGGTGCAATG
CGGCCTGCCCGGACCGAGGAACGCGCTTCGGCTCGGACGCTGGCGTAATGGT
CGTAAATGACCCGTCTTG

Results

4.2.3 *Chaetomium strumarium* (SG1)

4.2.3.1 *Accesion number JX863914 (28s rRNA)*

The sequencing of D2 region of 28s rRNA proved this fungus to be *Chaetomium strumarium.* The sequence was submitted to the Gene Bank and accession number was obtained for the sequence (Fig.4.10). The detailed sequence of amplified region is as follow:

CGGAGGAAAAGAAACCAACAGGGATTGCCCTAGTAACGGCGAGTGAAGCGG
CAAGCTCAAATTTGAAATCTGGCTTCGGCCCGAGTTGTAATTTGTAGAGGAA
GCTTTAGGCGCGGCACCTTCTGAGTCCCCTGGAACGGGGCGCCACAGAGGGT
GAGAGCCCCGTATAGTTGGATGCCTAGCCTGTGTAAAGCTCCTTCGACGAGT
CGAGTAGTTTGGGAATGCTGCTCAAAATGGGAGGTAAATTTCTTCTAAAGCT
AAATACCGGCCAGAGACCGACACAAGTAGAGTGATCGAAAGATGAAAAGCA
CTTTGAAAAGAGGGTTAAATAGCACGTGAAATTGTTGAAAGGGAAGCGCTTG
TGACCAGACTTGCGCCGGGCGGATCATCCGGTGTTCTCACCGGTGCACTCCG
CCCGGCTCAGGCCAGCATCGGTTCTCGCGGGGGGATAAAGGCCCCGGGAACG
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TG

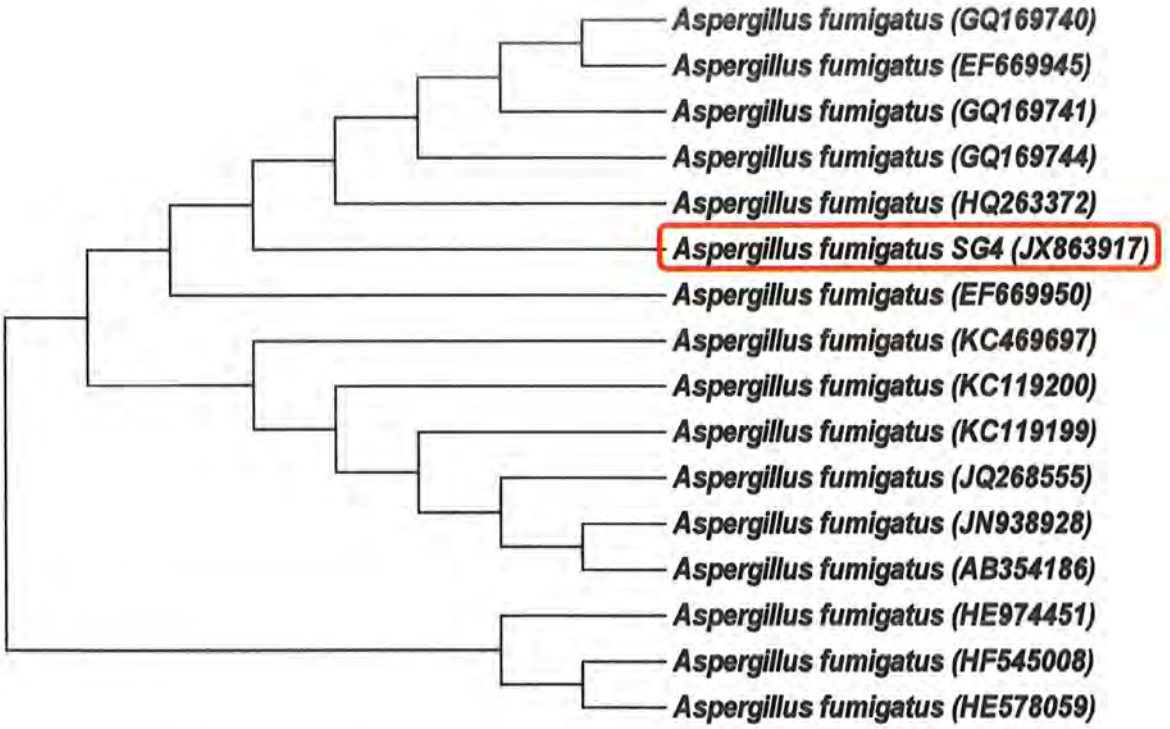


Fig 4.9: Phylogenetic tree of *Aspergillus fumigatus* SG4 (JX863917)

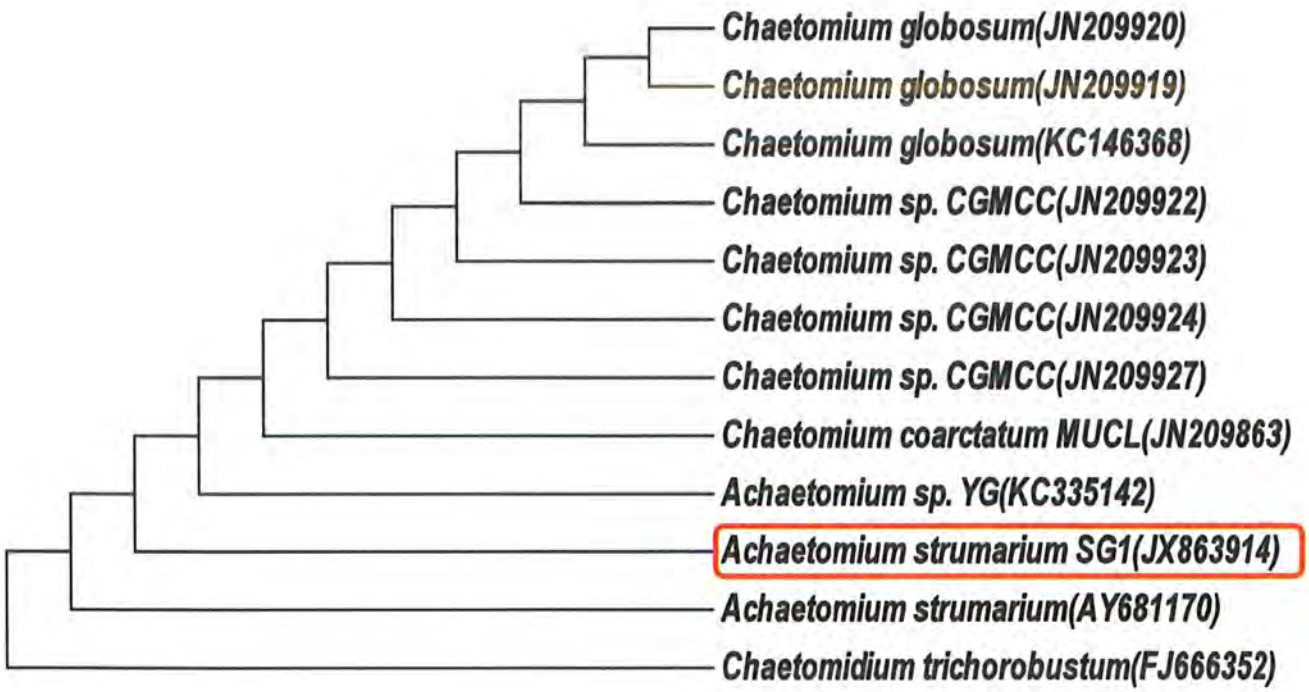


Fig 4.10: **Phylogenetic tree of *Chaetomium strumarium* SG1 (JX863914)**

4.3 Optimization of physiochemical parameters for pigments production

The three different fungi i.e., *Penicillium verruculosum* SG, *Aspergillus fumigatus* SG4 and *Chaetomium strumarium* SG1 which were identified to molecular level were selected for further evaluation with reference to pigments production under liquid state condition. Different parameters including pH, temperature, carbon source and concentration, nitrogen source and carbon nitrogen ratio (C: N) were optimized in order to get enhanced pigment production.

4.3.1 *Effect of Culture media and Time*

Five different media including PDB, SDB, YMPG, MSM and Czapek dox broth were used for liquid state fermentation of the fungi for pigment production. Out of these five different media, colored metabolites production [absorbance (AU) at λ maximum] was significantly ($p = \leq 0.05$) (LSD) higher in PDB with all the three different fungi (Table 4.1).

However, color metabolites production varied with time and initiated on 5th day with *Aspergillus fumigatus* SG4 and *Penicillium verruculosum* SG and on 8th day with *Chaetomium strumarium* SG1. Maximum pigments production was recorded in PDB on 10th day in case of *Aspergillus fumigatus* (9.5± 1.1) and *Penicillium verruculosum* (15±2) (Non-significantly differed from MSM), while on 14th day with *Chaetomium strumarium.* (8.5±0.5) (Non-significantly differed from MSM) at λmax 380, 510 and 480 nm for yellow, red and magenta pigments respectively (Fig. 4.11). Negligible pigments production was observed in Czapek dox media.

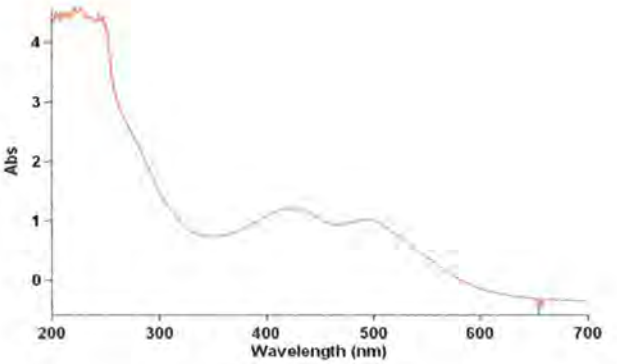


Fig 4.11(a): UV/Vis spectroscopy of methanolic extract of pigments (colored metabolites) by *Penicillium verruculosum*

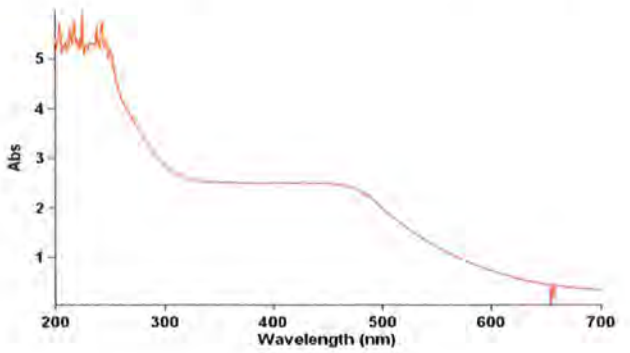


Fig 4.11(b): UV/Vis spectroscopy of methanolic extract of pigments (colored metabolites) by *Chaetomium strumarium*

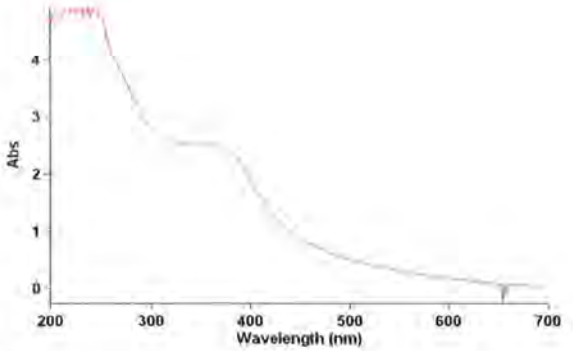


Fig 4.11(c): UV/Vis spectroscopy of methanolic extract of pigments (colored metabolites) by *Aspergillus fumigatus*

Table 4.1: Screening of five different media for pigments production by three different fungi [Least square difference (LSD), (Average values sharing a common letter do not differ significantly others differ significantly) ($p \leq 0.05$)]

	Optical density (AU) of culture filtrate in different media											
Fungi	**TM** Ave±SD (n=3)		**PDB** Ave±SD (n=3)		**CZAPEK** Ave±SD (n=3)		**SDB** Ave±SD (n=3)		**YMPG** Ave±SD (n=3)		**Combined** Ave±SD (n=5)
***Aspergillus* spp.**	7.5± 0.2	B	9.5± 1.1	A	1.0± 0.4	D	07± 0.83	B	6.0± 1.20	C	6.2±6.3
***Penicillium* spp.**	12± 0.9	B	15± 2	A	3.0± 0.6	D	11 ± 0.32	C	12.5± 1.10	B	10.5±9.0
***Chaetomium* spp.**	6.5±1.2	B	8.5± 0.5	A	0.75± 0.8	C	6 ± 0.77	B	7.0 ±0.12	B	5.7±5.8
Combined Ave±SD (n=3)	8.5±5.8	B	11±7.0	A	1.6±2.4	D	08±5.20	C	8.5±7.00	B	

Vertically ranking based upon absorbance units (AU) of the three fungi in their respective media. Horizontal ranking associated with AU units of each fungus in five different media. MSM-=Minimal salt media; PDA-=Potato dextrose broth; SDA= Sabouraud dextrose broth; YMPG= Yeast malt peptone glucose broth; LSD = Least Square Difference; R = Rank order; SD = Standard deviation; Ave = Average

4.3.2 *Effect of pH and temperature*

The general trend in pigmenst production at varying pH (3, 5.5, 7, and 9) was almost same. Overall, it was maximum at pH 5.5 followed by pH 7 >pH 3 and least pigments were produced at pH 9 (almost inhibited) in all the three fungi (Fig. 4.12; Table A2). Surprisingly, *Chaetomium strumarium* was even able to produce some pigments at pH 3. The trend of pigments production at various temperatures (15, 25, 30 & 37) was almost similar in *Chaetomium strumarium* and *Penicillium verruculosum* (Fig. 4.13; Table A1). It was significantly higher i.e., 17.5 and 15.3 AU in *Penicillium verruculosum* and *Chaetomium strumarium* respectively at 25°C (Fig. 4.13) and was considerably decreased at 15°C and 30°C in both the cases. In *Aspergillus fumigatus* maximum pigment production (15.5AU) was attained at 30°C (Fig. 4.13; Table A1).

Results

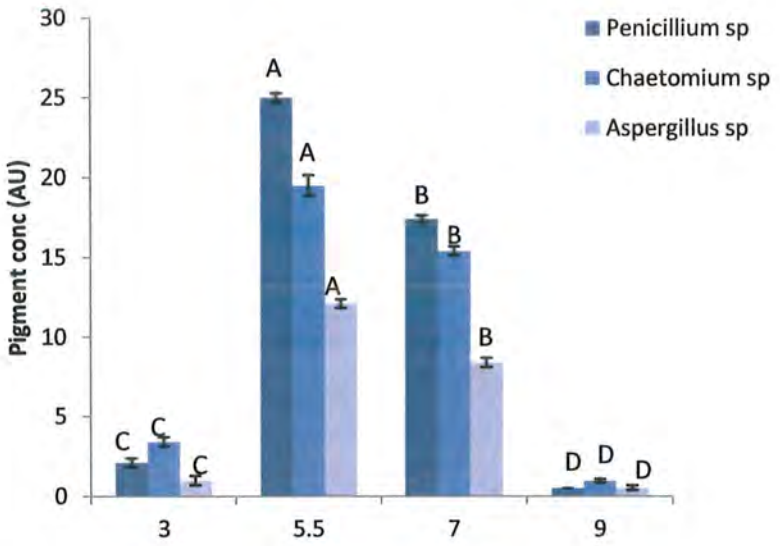


Fig 4.12: Pigments concentration (AU) at varying pH (3-9) by three pigment producing fungi (Table A2). [{Least square difference (LSD), (Average values sharing a common letter do not differ significantly others differ significantly) ($p \leq 0.05$)}]

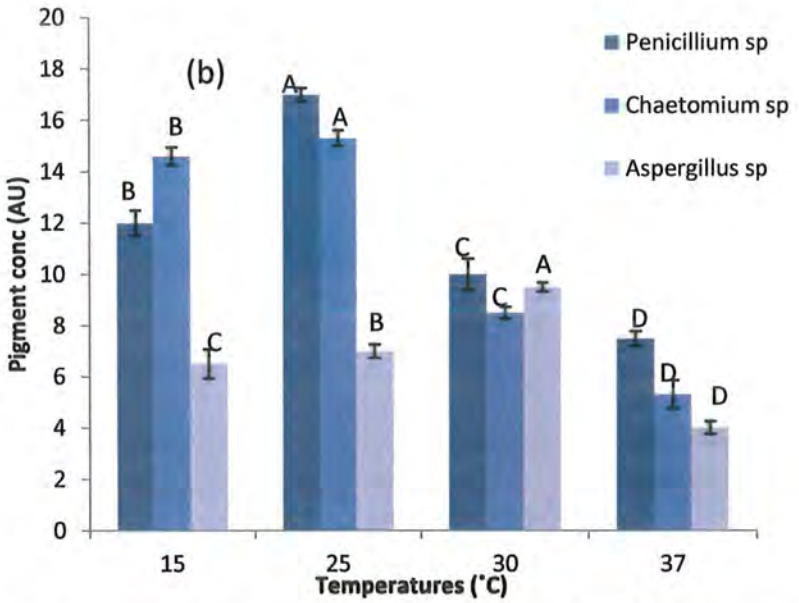


Fig 4.13: Pigments concentration at varying temperatures (15-37) by three pigment producing fungi (Table A2). [{Least square difference (LSD), (Average values sharing a common letter do not differ significantly others differ significantly) ($p \leq 0.05$)}]

4.3.3 *Effect of Carbon source*

Among different carbon sources, glucose which is monomeric sugar proved to be yielding significantly ($p \leq 0.05$) higher pigments followed by fructose (Monosccharide)> sucrose (Disaccharide) in all the three fungi (Fig. 4.14; Table A3). However, the pigments production was lowered significantly in case of lactose (Disaccharide) and glycerol (Polyol, sugar alcohol). The color metabolites productions were 25, 12, 19.5AU with glucose in *Penicillium verruculosum, Aspergillus fumigatus* and *Chaetomium strumarium* respectively (Fig. 4.14). Fructose was the second most important carbon source with reference to pigments production rates i.e., 21, 17, 10 AU in *P. verruculosum*, *Chaetomium strumarium* and *Aspergillus fumigatus* respectively. Lactose showed moderate pigment production with least pigment being found when glycerol was used as carbon source.

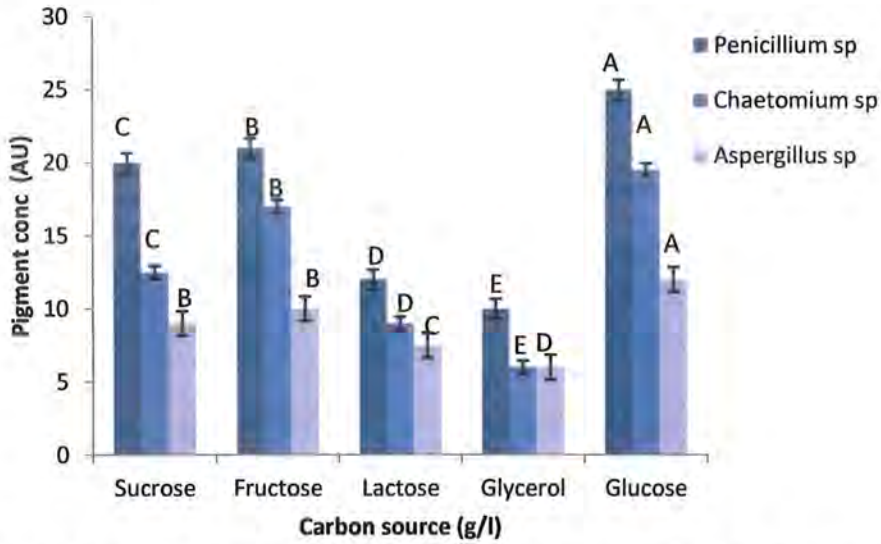


Fig 4.14: Pigments concentration at varying carbon sources by three pigment producing fungi (Table A3). [{Least square difference (LSD), (Average values sharing a common letter do not differ significantly others differ significantly) ($p \leq 0.05$)}]

4.3.4 *Effect of glucose concentration*

Pigments production and glucose concentration was directly related to each other in *Chaetomium strumarium* as pigments concentration (AU) reached maximum (20AU) at 40g/l which is the highest glucose concentration while minimum at (0.65 AU) 0.5g/l glucose (Fig. 4.15; Table A5).

Results

In case of *Penicillium verruculosum* and *Aspergillus fumigatus,* similar trend was seen and direct relation between pigments concentration and increase in glucose concentration was seen up to 20g/l of glucose after which the pigments production decreased. The trend for pigments concentration at various glucose level was as follows; in *Penicillium verruculosum* 20 (27AU)>10 (20.5AU) > 40 (19AU) >5 (17AU) >2 (20 AU) >1 (6AU) >0.5 (1.25AU) >0 while in *Aspergillus fumigatus* 20 (12AU)>10 (10.5AU) > 40 (9AU) >5 (7AU) >2 (5AU) >1 (2.5AU) >0.5 (0.75AU) >0.

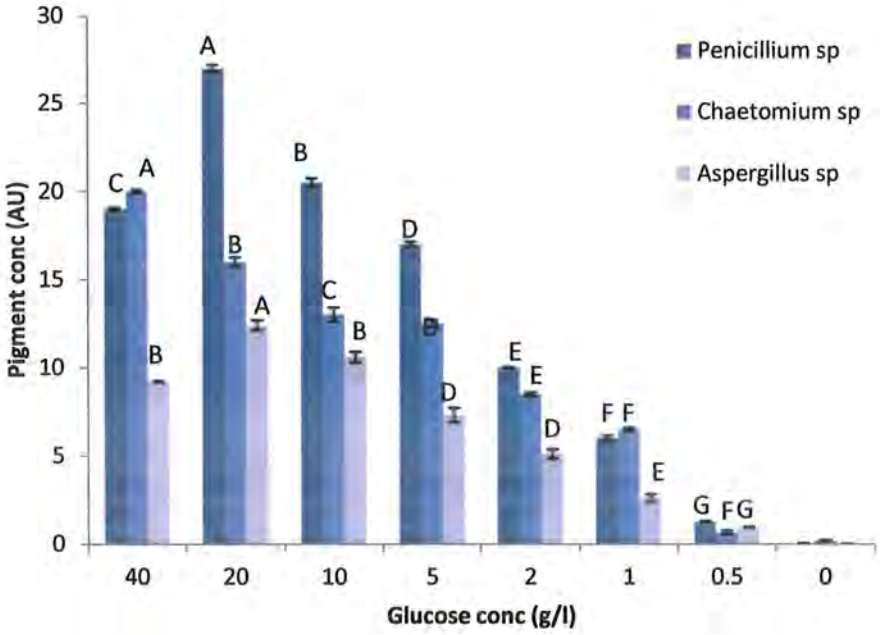


Fig 4.15: Pigments concentration at varying glucose concentration by three pigment producing fungi (Table A5). **[{Least square difference (LSD), (Average values sharing a common letter do not differ significantly others differ significantly) ($p \leq 0.05$)}]**

4.3.5 *Effect of nitrogen source*

Among different nitrogen sources used (3g/l), yeast extract and peptone helped greater production of colored metabolites as compared to inorganic N sources like ammonium nitrate and sodium nitrate in PDB (Fig. 4.16; Table A4). There was a significant ($p \leq 0.05$) decrease in pigments production with different nitrogen sources in following order, yeast extract> peptone>ammonium nitrae > sodium nitrate. Maximum pigments yield was 29, 25.5, 17.5 AU in yeast extract while minimum i.e. 9, 11 and 8.5 AU in sodium nitrate in *Penicillium verruculosum*, *Chaetomium strumarium* and *Aspergillus fumigatus* respectively (Fig. 4.16). Overall the pigments production was maximum in *Penicillium*

verruculosum followed by *Chaetomium strumarium* > *Aspergillus fumigatus*. Whereas, *Chaetomium strumarium* showed comparatively better pigment production in inorganic nitrogen sources i.e., $NANO_3$ (11AU) and NH_4NO_3 (19AU).

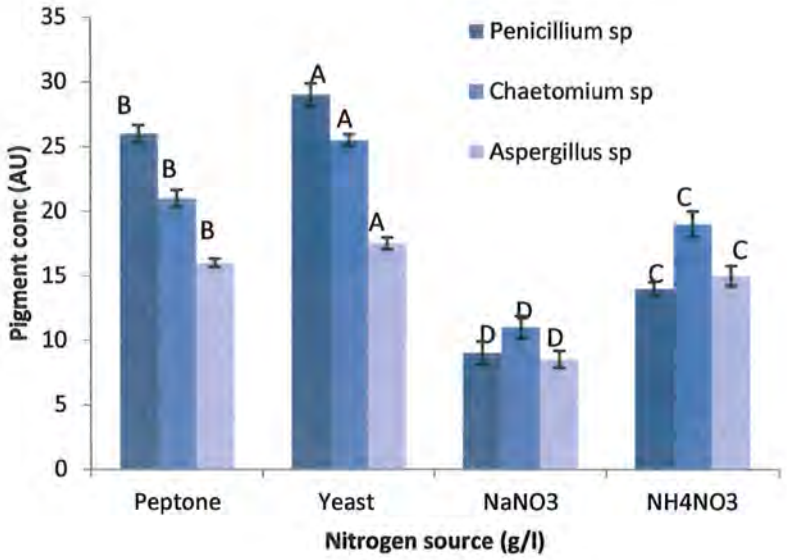


Fig 4.16: Pigments concentration at varying nitrogen sources by three pigment producing fungi (Table A4). **[{Least square difference (LSD), (Average values sharing a common letter do not differ significantly others differ significantly) ($p \leq 0.05$)}**

4.3.6 *Carbon nitrogen ratio (C: N)*

Pigments production was significantly ($p \leq 0.05$) higher at a carbon nitrogen ratio (C: N) 4:1 (35AU) followed by 5:1 (25.5AU) > 8:1 (24.5AU) >10:1 (22.5AU) > 20:1 (22AU) in *Penicillium verruculosum* (Fig. 4.17; Table A6). *Aspergillus fumigatus* yielded maximum pigments at C: N ratio of 8:1 (20AU) followed by 10:1 (17.5AU) (Non-significantly differed from 5:1) > 20:1 (15AU) > 4:1 (9.5AU). Whereas, *Chaetomium strumarium* has maximum pigments produced at 10:1 (30AU) followed by 8:1 (28.5AU) > 5:1 (24AU) >20:1 (17AU) (Non significantly differed from 4:1) (Fig. 4.17).

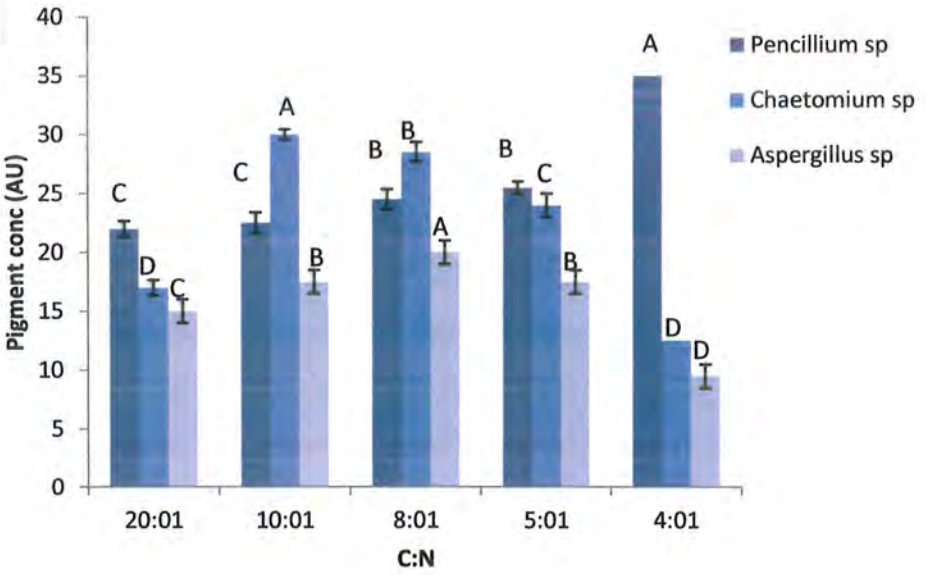


Fig 4.17: Pigments concentration at varying C: N ratio by three pigment producing fungi (Table A6). **[{Least square difference (LSD), (Average values sharing a common letter do not differ significantly others differ significantly) ($p \leq 0.05$)}**

Fig 4.18: Extracellular pigment production by three fungi in liquid state at static and optimizied conditions

4.4 FTIR spectroscopy of the pigmented culture filtrates of fungi

FTIR spectroscopy of the methanolic pigments filtrate was done in order to detect chemical classes of compounds based upon their functional groups present in the cultural filtrate (Fig. 4.19). In *Penicillium verruculosum,* a peak at 1750cm^{-1} showed C=O stretch indicating presence of pyrone of coumarin ring and peaks at 1500, 1600cm^{-1} corresponded to benzene ring's C=C stretch (Fig. 4.19a).

There was C-H bending peak at 1452cm^{-1} and 1449cm^{-1} in *Penicillium verruculosum* and *Chaetomium strumarium* respectively (Fig. 4.19 a,b). C=O stretching band at 1670cm^{-1}, 1648cm^{-1} and 635cm^{-1} in the culture filtrate indicated the presence 1.4-quinones of anthraquinones in *Penicillium verruculosum* (Fig. 4.19a), *Chaetomium strumarium* (Fig. 4.19b) and *Aspergillus fumigatus* (Fig. 4.19c) respectively. In *Penicillium verruculosum* and *Chaetomium strumarium*, the bands at 2800 cm^{-1} and 2900cm^{-1} corresponded to C-H stretch which has been reported in some alkaloids. Peaks at 3324cm^{-1}, 3282cm^{-1} and 3268cm^{-1} represented hydrogen bonded OH moiety in *Penicillium verruculosum, Chaetomium strumarium* and *Aspergillus fumigatus* respectively. C-OH vibrations between 1200 and 1300cm^{-1} was present in *Chaetomium strumarium* (Fig. 4.19b).

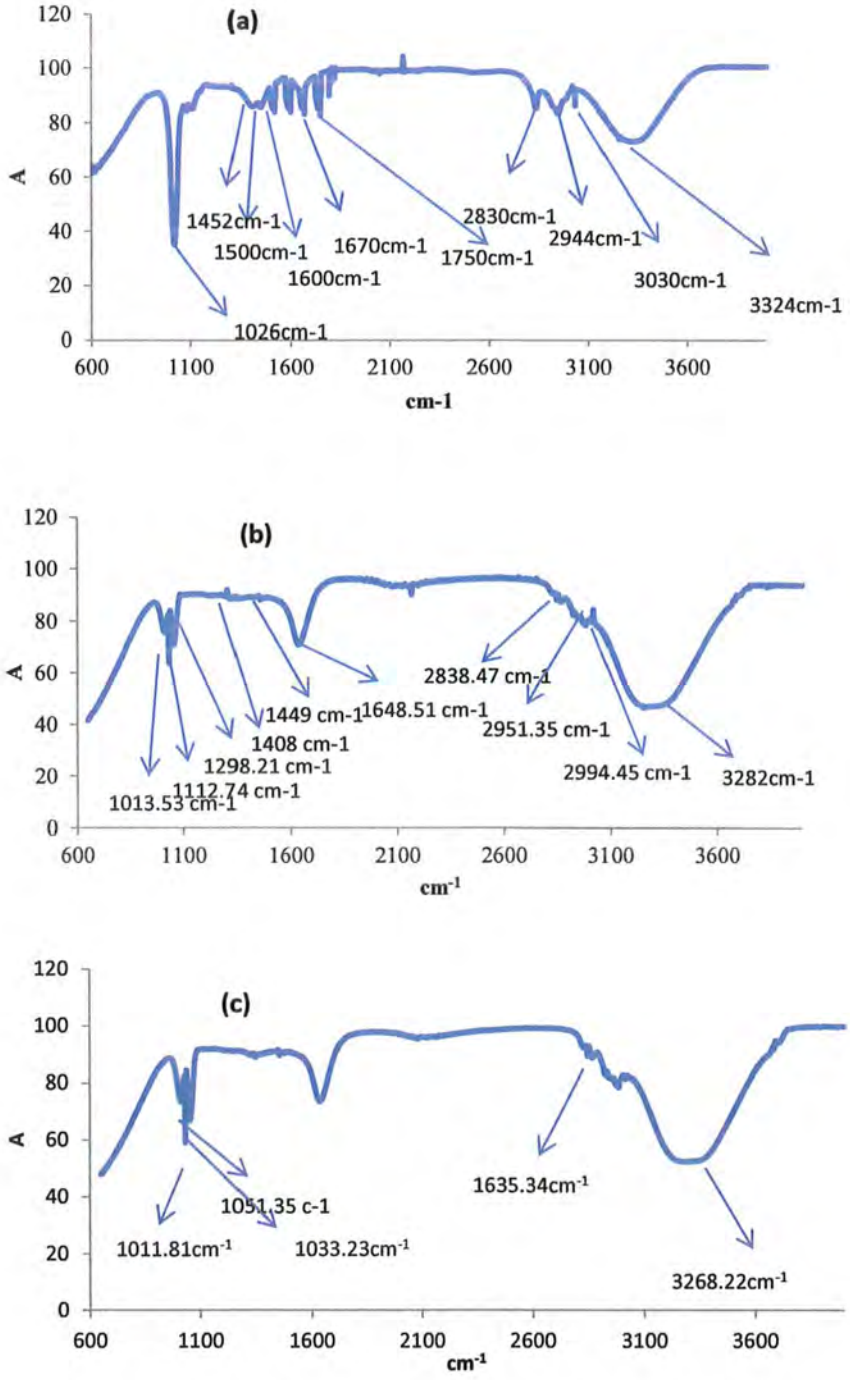


Fig 4.19: FTIR pattern of *Penicillium verruculosum* (a), *Chaetomium strumarium* (b) and *Aspergillus fumigatus* (c) showing presence of different functional groups in PDA media.

4.5 Stability studies on fungal pigments/colored metabolites at Different pH and temperatures

The stability of pigmented filtrates of fungi at different pH (Fig. 4.20 & 21) and temperatures (Fig. 22 & 23) was monitored in term of change in their absorbance (AU) and transformation patterns in UV/Vis spectroscopy and FTIR spectra. At pH 7, the colored filtrate of the fungi was most stable and only minor changes in absorbance (AU) were seen at pH 9. Whereas, the pigments in culture filtrates of *Chaetomium strumarium* and *Aspergillus fumigatus* were quite unstable at pH 3 as pigment concentration was reduced by 66 and 75% respectively whereas, the red pigment produced by *Penicillium verruculosum* was most stable at even acidic pH (3) with only 5% reduction in pigment concentration after 6 hours of incubation (Fig. 4.20; Table A7-A9). At pH 7, negligible changes in absorbance were observed and % reduction in pigment concentration was 4.2, 5 and 6.6 in *Penicillium verruculosum*, *Aspergillus fumigatus* and *Chaetomium strumarium* respectively and almost similar % reduction was seen at pH 9. The stability was also observed on FTIR spectrophotometer and no prominent change in peaks were observed showing no changes in chemical structure (Fig. 4.21).

The pigmented filtrates of all the fungi in PDA were almost stable (in terms of absorbance units at λ max.) at temperatures ≤ 100 °C after 6 hrs of incubation. .However, maximum decrease in concentrations/intensities of pigments was observed when incubated at 100°C. The % age reduction in pigments concentration at 100°C was 17% in *Penicillium verruculosum* (Fig. 4.22a; Table A10), 22.5% in *Aspergillus fumigatus* (Fig. 4.22c; Table A12) while, 16% in case of *Chaetomium strumarium* (Fig. 4.22b; Table A11). The decreasing order in pigments statbility at different temperatures in *Penicillium verruculosum* was like; 15°C > 37°C> 4°C> 50°C> 75°C> 100°C> -20°C. At -20°C the % reduction in pigment was 20. The pigmented culture filtrate of *Aspergillus fumigatus* was most stable at 15°C and 4°C followed by >20°C> 37°C> 50°C> 75°C> 100°C. In case of *Chaetomium strumarium,* the pigmented filtrate was most stable at 4°C followed by 15°C > -20°C > 37°C >50°C> 75°C> 100°C. Whereas, the FTIR pattern of the pigmented filtrates of all the three fungi did not show any significant difference in peaks neither any new peak appeared showing integrity of the chemical structure. Only the minor changes in intensity of peaks can be due to interaction with buffers used and evaporation and interaction with solvents used (Fig. 4.23).

Results

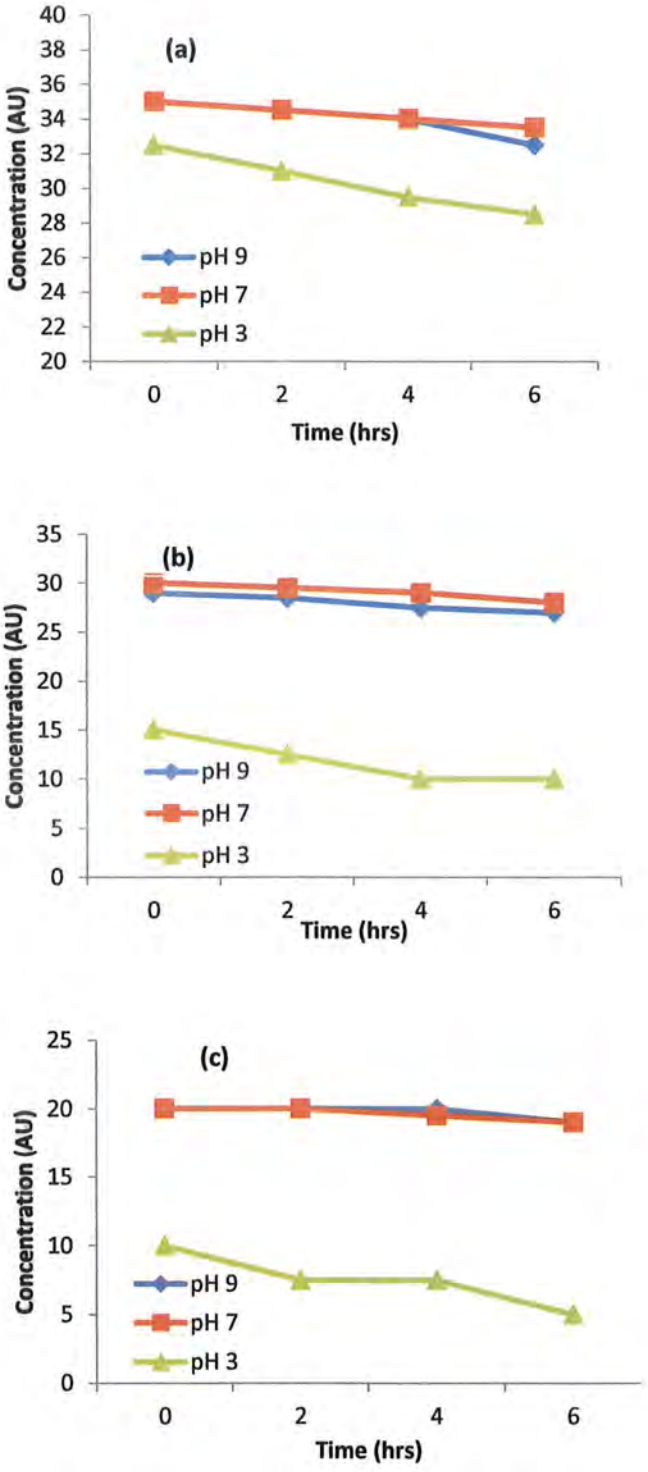


Fig 4.20: UV vis absorbance of the *Penicillium verruculosum* (a; Table A9), *Chaetomium strumarium* (b; Table A7) and *Aspergillus fumigatus* (c; Table A8) after treatment at different pH for 6 hrs.

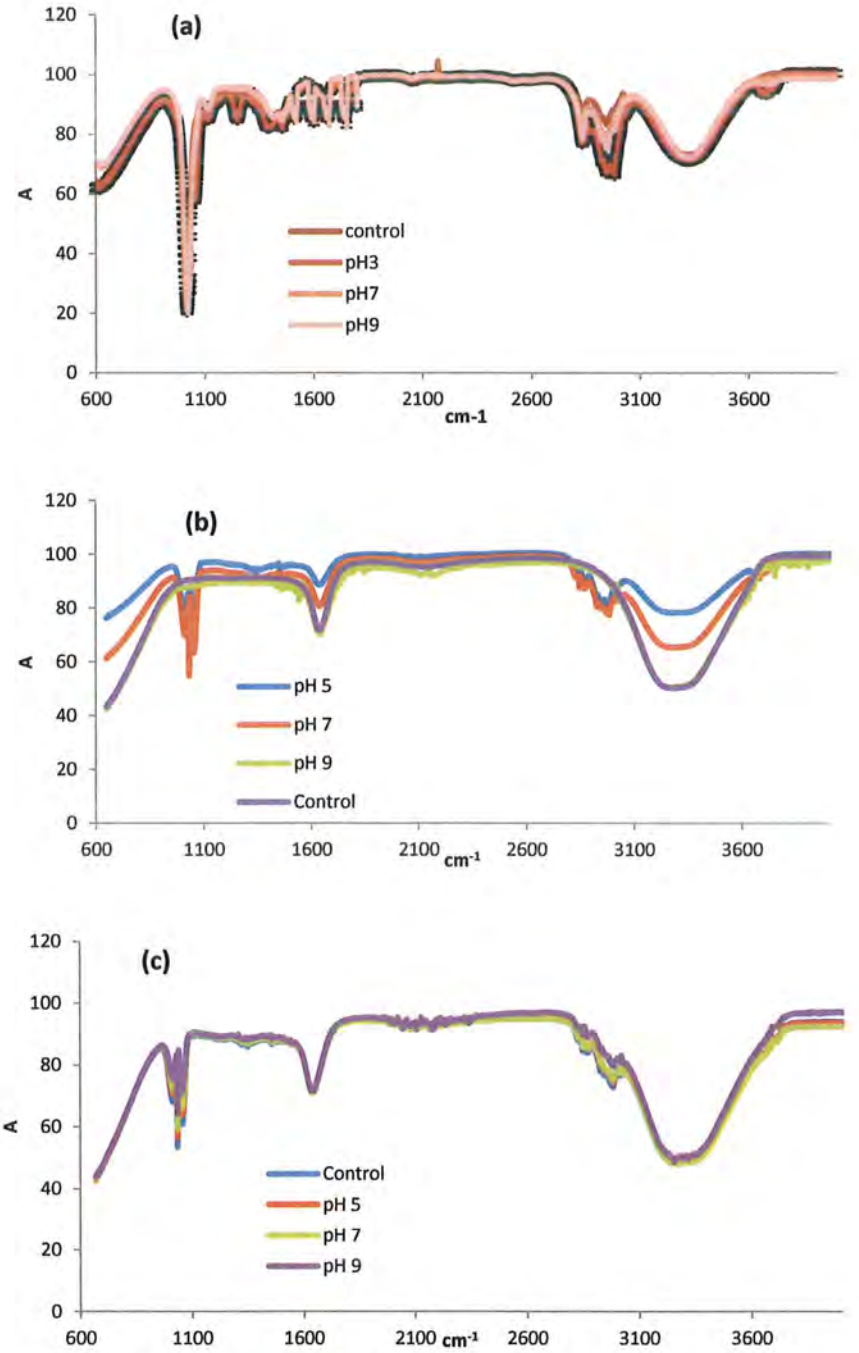


Fig 4.21: FTIR pattern of *Penicillium verruculosum* (a), *Chaetomium strumarium* (b) and *Aspergillus fumigatus* (c) showing presence of different functional groups in control in comparison to pH treated PDA media after 6 hrs.

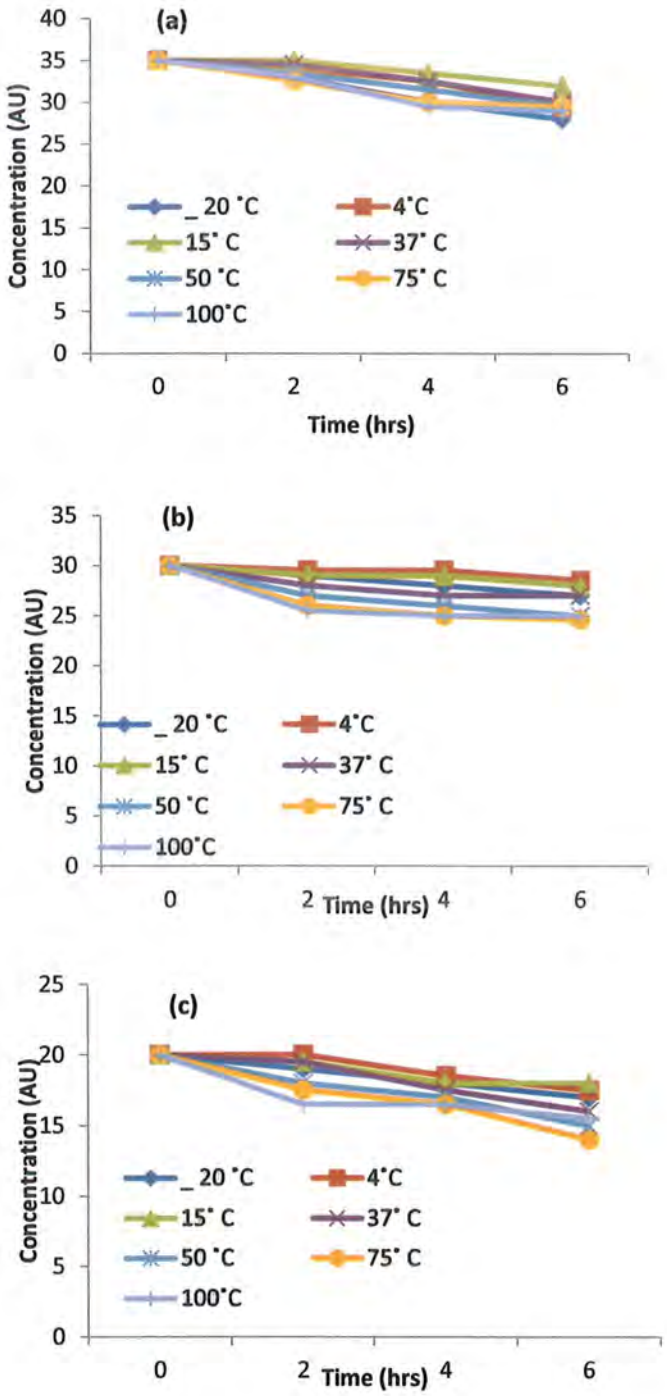


Fig 4.22: UV/Vis spectroscopy of the pigmented filtrates of *Penicillium verruculosum* (a; Table A1), *Chaetomium strumarium* (b; Table A11) and *Aspergillus fumigatus* (c; Table A12), after treatment at different temperatures for 6 hrs

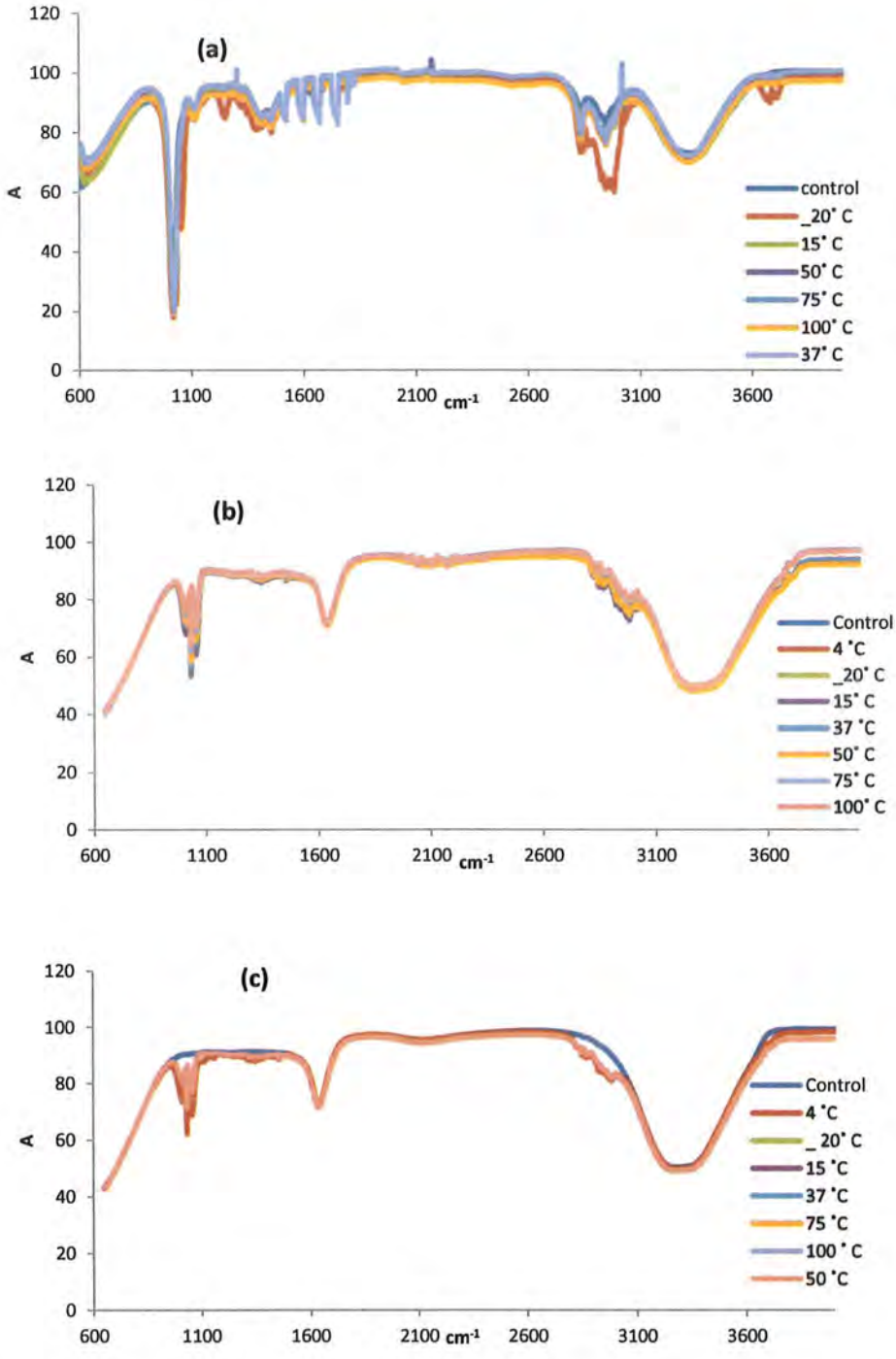


Fig 4.23: FTIR spectra of pigmented filtrates of *Penicillium verruculosum* (a), *Chaetomium strumarium* (b) and *Aspergillus fumigatus* (c) showing presence of different functional groups after treatment at different temperatures for 6 hrs

4.6 Phytochemical screening of pigmented culture filtrates of fungi

4.6.1 *Qualitative determination of different classes of bioactive compounds*

Qualitative analysis of pigmented filtrates in PDA showed presence of different important classes of compounds though it varied among different fungi. Presence of terpenes and anthraquinones were strongly (+++) indicated in colored filtrates of *Chaetomium strumarium* and *Penicillium verruculosum* whereas, moderately present in *Aspergillus fumigatus*. Alkaloids were strongly present in *Penicillium verruculosum* whereas, glycosides and flavonoids were moderate to slightly present in these fungi. Coumarins were strongly present in *Chaetomium strumarium* but moderately indicated in *Penicillium verruculosum* and *Aspergillus fumigatus*. Overall, tannins were completely absent in all the fungi (Table 4.2).

Table 4.2: Phytochemical screening of fungal cultural filtrate for the presence of important classes of bioactive compounds.

	Terpene	Alkaloid	Glycoside	Flavanoid	Anthraquinones	Tannins	Coumarins
Aspergillus sp.	+++	++	++	++	++	___	++
Penicillium sp.	+++	+++	++	++	+++	___	++
Chaetomium sp.	+	++	+	++	+++	___	+++

Foot notes: Strongly present=+++; Moderately present= ++; Weakly present= +

4.6.2 *Quantitative determination of total phenols and flavonoids in pigmented filtrates of fungi*

Total flavonoids and phenolic contents were determined by following methods developed by Kim et al., (2003) and Park et al., (2008) respectively. Total flavonoid content was maximum in *Penicillium verruculosum* (228 mg RTE/g extract) in PDA (Fig. 4.24c) followed by *Aspergillus fumigatus* (136.5 mg RTE/g extract) in YMPG (Fig. 4.24a) and *Chaetomium strumarium* (101 mg RTE/g extract) in SDA (Fig. 4.24b). Total phenolic contents were maximum in YMPG i.e., 29 GAE mg/g extract of *Penicillium verruculosum* (Fig. 4.25c) followed by PDA of *Chaetomium strumarium* (18 GAE mg/g of extract) (Fig. 4.25b) and then SDA for *Aspergillus fumigatus* (14 GAE mg/g of extract) (Fig. 4.25a).

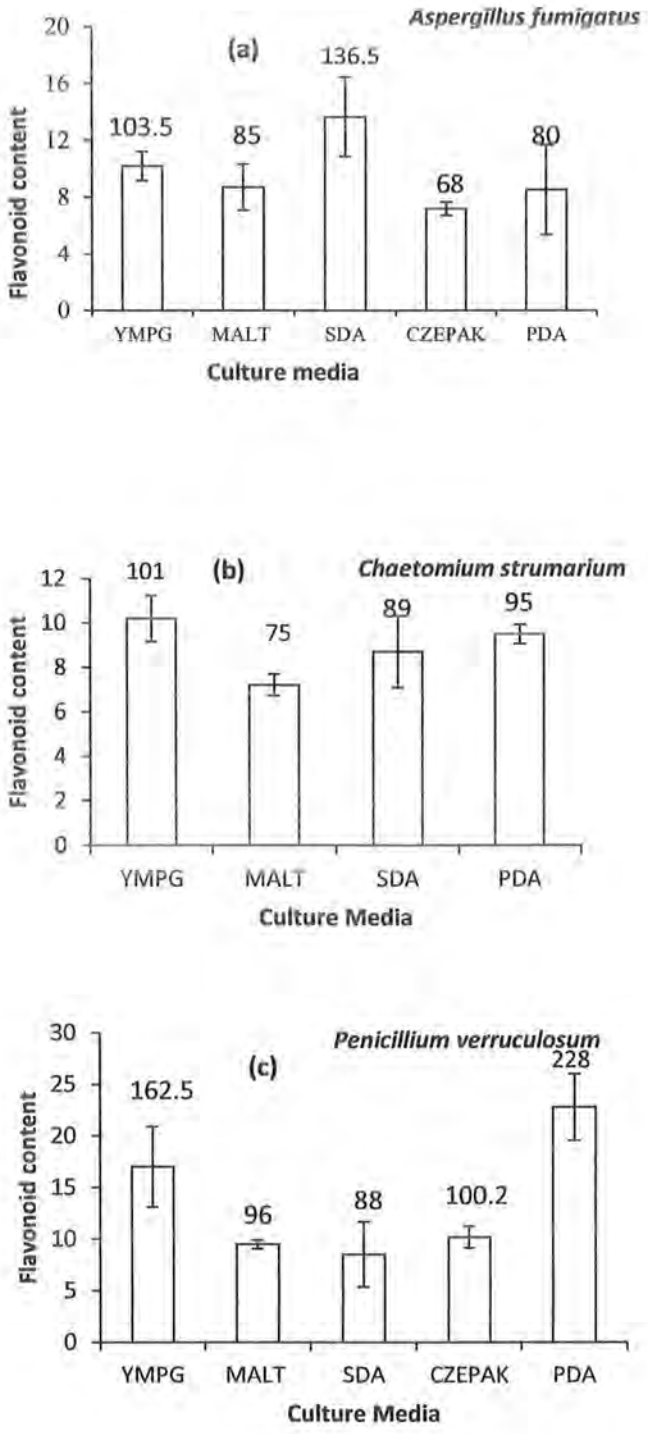


Fig 4.24: Total flavonoid content (mg RTE/g extracts) of *Penicillium verruculosum* (a), *Chaetomium strumarium* (b) and *Aspergillus fumigatus* (c)

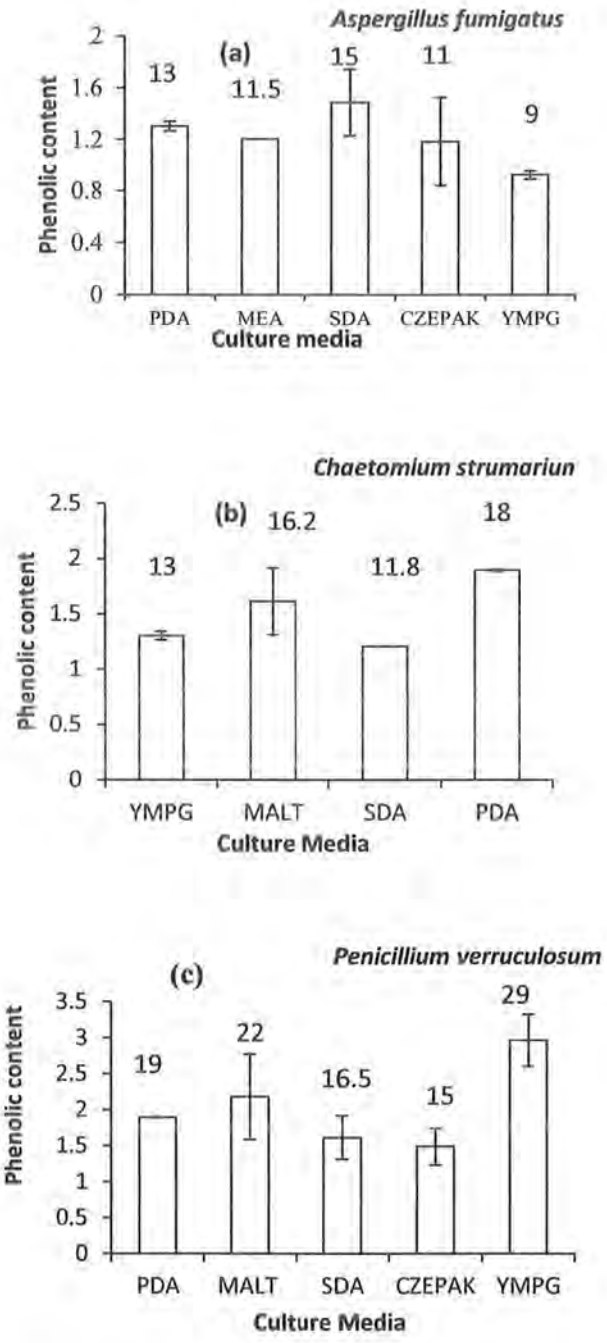


Fig 4.25: **Total Phenolic content (GAE mg/g) of *Penicillium verruculosum* (a), *Chaetomium strumarium* (b) and *Aspergillus fumigatus* (c)**

4.7 Biological activities of pigmented filtrates of fungi

4.7.1 *Antimicrobial activities.*

4.7.1.1 *Antibacterial activities*

The fungal extracts made in five different solid media were evaluated for their antimicrobial activities using agar well diffusion assay (Table 4.3). Overall, antibacterial activity (zone of inhibition = mm) of the culture filtrate of each fungus was maximum (10-24mm) in PDA against bacterial groups (non-significantly differed from SDA) followed by YMPG (0-16mm) (non- significantly differed from MEA in *Penicillium verruculosum* and *Chaetomium strumarium* but differed significantly from MEA in *Aspergillus strumarium*).

Whereas, fungal filtrates in czapek dox agar showed least antibacterial activity i.e., 0-12 mm against bacterial groups. Antibacterial activity of pigmented filtrate of *Chaetomium strumarium* was considerably higher against all tested bacteria (ave=12-15) except *S. typhimorium* (6.6±6.1), while, the antibacterial activities of culture filtrate of *Aspergillus fumigatus* against all the bacteria tested differed non-significantly from one another (ave=7.6-11). In case of *Penicillium verruculosum*, the antibacterial activity varied (ave=7-14) in five test bacterial strains and it was maximum for *E.coli* (Differed non-significantly from activity against all other bacteria except *B. spizizeni*) (Table 4.3).

4.7.1.2 *Antifungal activities*

Fungal species were also evaluated for their antifungal ability against two test pathogenic fungi *A.niger* and *A. terreus* (Table 4.4) and it was found that in *Chaetomium strumarium*, activities shown by PDA were significantly higher (ave=20±7) (differed non significantly from SDA). SDA was further non-significantly differed (ave=16.5±7.7) from YMPG which differed non-significantly (14.5±0.7) from MEA (14±1.4). Lowest antifungal activity was shown by Czapek dox media (4.5±6). Overall the *Chaetomium strumarium* culture filtrate was more active against *A. niger* (17± 6.5) (differed significantly from activity against *A. terreus*).

In case of *Aspergillus fumigatus*, the zone of inhibition shown by PDA was maximum (22±11) (Differed non significantly from SDA and YMPG). Overall, the culture media of *Aspergillus fumigatus* were more effective against *A. niger* (9±8.2) (Differed significantly from *A. terreus*). In *Penicillium verruculosum*, maximum zone of inhibition was shown by YMPG (13±2.8) (Differed non-significantly from PDA) whereas the PDA differed non-significantly (11.5±0.7) from SDA (11±2.8) and MEA (11±1.4). Overall, the activity of the *Penicillium verruculosum* pigmented filtrate was higher against *A. terreus* (10± 6) (Differed significantly from *A.niger*) (Table 4.4).

Table 4.3: Antibacterial activity of pigmented culture filtrate of different fungi in five different media against pathogenic bacteria [Least square difference (LSD), (Average values sharing a common letter do not differ significantly others differ significantly) ($p \leq 0.05$)]

Fungal Extracts		Bacteria											
Fungi	**Cultural media**	***E.Coli***		***B.spizizeni***		***S. Aureus***		***S.typhimorium***		***S. epidermidas***		***Combined effect***	
		Ave±SD (n=3)	R	Ave±SD (n=3)	R	Ave±SD (n=3)	R	Ave±SD (n=3)	R	Ave±SD (n=3)	R	Ave (n=5)	R
Chaetomium spp.	**Control (Strep.)**	14±0.10		32±0.99		26±0.59		30±1.4		31±1.21		27±7.4	
	YMPG	13±0.22	A	13±0.34	C	13±0.52	C	0.0±0.0	C	11±0.11	C	10±5.6	B
	MEA	12±0.34	A	12±0.21	C	16±0.62	B	09±0.65	B	10±0.65	C	12±2.7	B
	PDA	12±0.99	A	23±045	A	19±0.66	A	12±0.23	A	19±0.23	B	17±4.8	A
	SDA	13±1.10	A	21±0.33	B	15±0.77	B	12±0.87	A	20.0±65	A	16.2±4	A
	Czapek dox	09±0.67	B	00±0.00	D	12±0.71	C	0.0±0 .0	C	0.0±0.0	D	4.20±9	C
R	**Combined effect Ave (n=5)**	**12±1.64 A**		**14±9 A**		**15± 2.7 A**		**6.6±6.1 B**		**12±8 A**		**12±16**	
Aspergillus spp	**YMPG**	11±0.54	A	12±0.99	C	10±0.22	B	10±0.65	B	10±0.49	C	10.6±0.9	B
	MEA	08±0.43	C	0.0±0.43	D	14±1.0	A	9±0.32	B	0.0±0.21	D	6.2±6.00	C
	SDA	09±0.23	BC	20±0.54	A	13±0.82	A	10±0.87	B	19±0.66	A	14±5.00	A
	PDA	10±0.33	A B	24±0.66	B	12±0.81	A	12±0.54	A	22±0.54	B	16±6.00	A
	Czapek dox	0.0±0.45	D	00±0.77	D	10±0.87	B	0.0±0.32	C	0.0±0.21	D	5.0±4.50	D
R	**Combined effect Ave (n=5)**	**7.6±4.4 A**		**11±11 A**		**10±1.7 A**		**8±4.7 A**		**10±10.3 A**		**47±7.3**	
Penicillium spp.	**YMPG**	16±0.21	A	10±0.34	B	12±0.83	B	13±0.65	C	11±0.99	D	12.4±2.3	B
	MEA	13±0.67	B	0.0±0.29	C	13±0.82	B	11±0.43	D	14±0.43	C	10.0±6.0	B
	SDA	15±0.32	A	13±0.38	A	13±0.65	B	17±0.59	A	16±0.65	A	15.0±1.8	A
	PDA	16±0.11	A	14±0.25	A	15±0.99	A	22±0.83	B	18±0.21	B	17.0±3.0	A
	Czapek dox	10±0.33	C	0±0.49	C	12±0.12	B	**0.0**±0.0	E	10±0 .0	D	32.0±6.0	C
R	**Combined effect Ave (n=5)**	**14±2.6 A**		**7±6.9 B**		**13±1.2 A**		**12.6±8.2 A**		**14±3.3 A**		**61±13**	

Foot notes: Vertically ranking associated with zone of inhibition of different fungal culture filtrate against each bacteria. Horizontally ranking depict average of zone of inhibition value against all five bacteria by each culture filtrate, Strep=Streptomycin; PDA=Potato dextrose agar; SDA= Sabouraud dextrose agar; YMPG= Yeast malt peptone glucose agar; MEA=Malt extract agar

LSD = Least Square Difference; R = Rank order; SD = Standard deviation; Ave = Average

Results

Table 4.4: Antifungal acitivity of pigmented culture filtrate obtained by culturing three fungi in five different media against two pathogenic fungi [{Least square difference (LSD), (Average values sharing a common letter do not differ significantly others differ significantly) ($p \leq 0.05$)}]

Fungal Extracts		Fungi					
		A.terrus		*A.niger*		*Combined effect*	
Fungi	Culture media	Ave.±SD (n = 3)	*R*	Ave.±SD (n = 3)	*R*	Ave. (n = 2)	*R*
Chaetomium spp.	Control (N)	35±0.34		30±0.54		32.5±3.5	
	PDA	15±0.12	A	25±0.99	A	20.0±7.0	A
	SDA	15±1.20	A	22±2.30	B	18.5±5.0	AB
	MEA	15±1.20	A	13±0.99	D	14.0±1.4	C
	YMPG	14±0.22	A	15±0.21	C	14.5±0.7	BC
	Czapek dox	0.0±0.0	B	9.0±0.1	E	4.50±6.0	D
Cmbined effect Ave. (n = 5) R		12±6.6 B		17±6.5 A		17.0±5.5	
Aspergillus spp.	PDA	14±0.23	A	30±0.44	A	22.0±11	A
	SDA	11±0.88	B	22±0.77	B	16.5±7.7	A
	MEA	0.0±0.0	C	12±0.73	C	6.0±8.40	B
	YMPG	10±0.34	B	23±0.56	B	16.5±9.0	A
	Czapek dox	0.0±0.0	C	10±0.98	C	5.0±7.00	B
Combined effect Ave. (n = 5) R		7.0±6.5 B		9.0±8.2 A		8.0±1.4	
Penicillium spp.	MEA	12±0.56	BC	10±0.76	B	11±1.40	B
	SDA	13±1.1	B	9±0.69	B	11±2.80	B
	PDA	11±0.33	C	12±0.88	A	11.5±0.7	AB
	YMPG	15±0.44	A	11±0.21	A	13±2.80	A
	Czapek dox	0.0±0.0	D	0.0±0.00	C	0.0±0.0	C
Combined effect Ave. (n = 5) R		10±6 A		8.4±4.8 B		9.2±5.2	

Foot notes: Vertically ranking associated with zone of inhibition of different fungal culture filtrate against each pathogenic fungi. Horizontally ranking depict average of zone of inhibition values against pathogenic fungi by each culture filtrate, N=Nistatin; PDA=Potato dextrose agar; SDA= Sebaroud dextrose agar; YMPG= Yeast malt peptone glucose agar; MEA=Malt extract agar

LSD = Least Square Difference; R = Rank order; SD = Standard deviation; Ave = Average

Results

4.7.2 *Antioxidant and free radical scavenging activity*

With increase in concentration (0-300µg/ml) of the pigmented filtrate, there was an increase in antioxidant and % scavenging activity. In case of total antioxidant activity, EC_{50} (µg/ml) was lowest (7.36 µg/ml) for *Chaemtomium strumarium* followed by *Penicillium verruculosum* (7.89) which was even lower than standard (ASA 14.66) used in the process (Fig. 4.26a; Table A13). Lowest antioxidant activity was seen in *Aspergillus fumigatus* (21.09) (Fig. 4.25a). Comparatively, lowest EC_{50} (µg/ml) value i.e., 10.34 µg/ml in % DPPH scavenging activity was observed in case of *Penicillium verruculosum* which was comparable to standard Rutin (9.049 µg/ml) and Ascorbic acid (14.27 µg/ml) used as control (Fig. 4.26b; Table A14), followed by *Chaetomium strumarium* (37.56) and then *Aspergillus fumigatus* (38.37) (Fig. 4.26b; Table A14). Lowest EC_{50} (µg/ml) was found in case of *Penicillium* spp. i.e., 95.57 µg/ml followed by *Chaetomium strumarium* (116.9 µg/ml) >*Aspergillus fumigatus* (134.1 µg/ml) in ABTS scavenging activity when Ascorbic acid (54.12 µg/ml) was used as control (Fig. 4.27a; Table A15). Total reducing power (µg/ml) was highest in *Chaetomium strumarium* (558.9) followed by *Penicillium verruculosum* (574.8) >*Aspergillus fumigatus* (637.1) (Fig. 4.27b; Table A16) compared to Ascorbic acid (54.12) as standard.

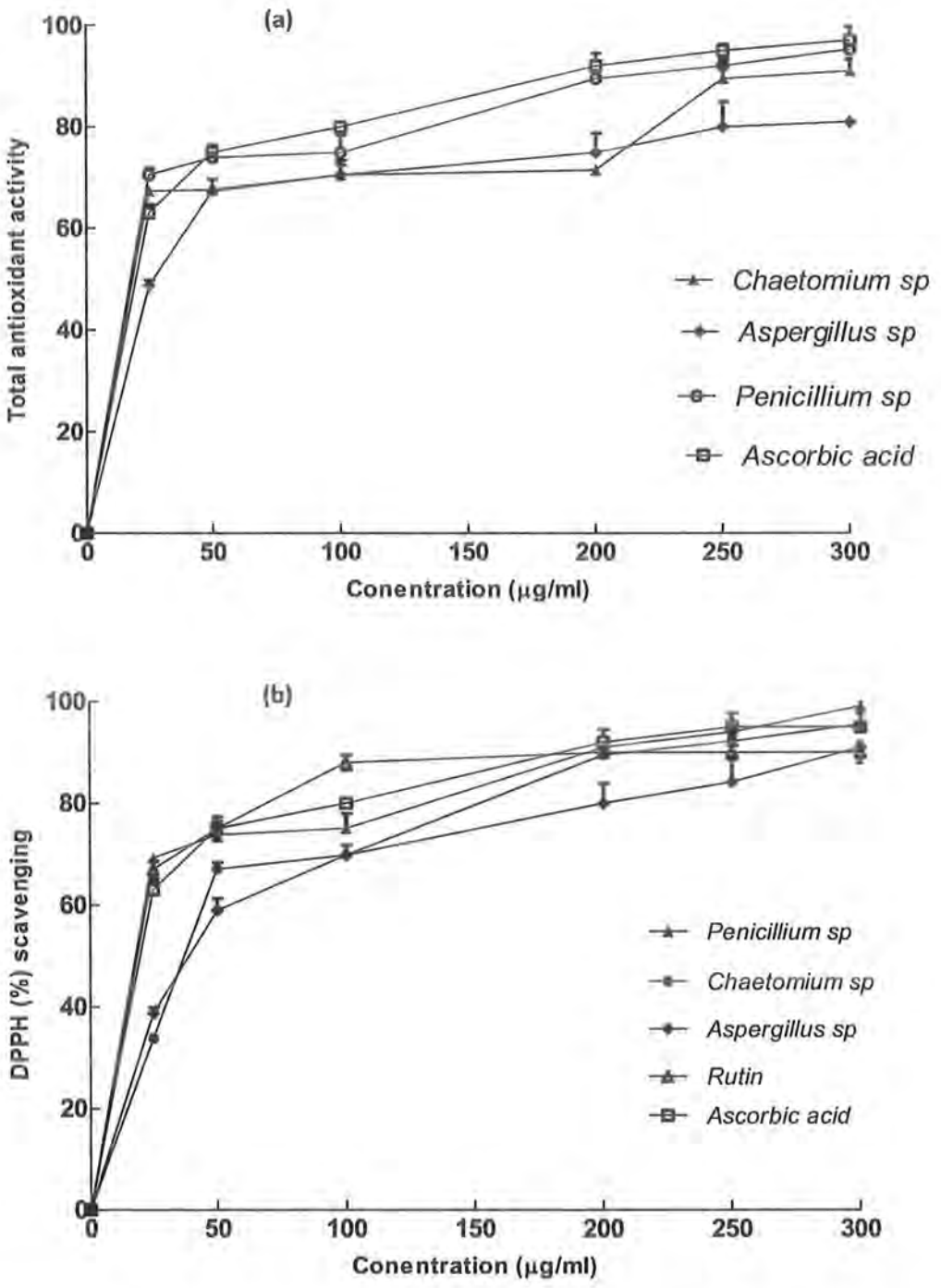


Fig 4.26: Total antioxidant (a) and DPPH free radical scavenging (b) activity (%) of the three pigment producing fungi

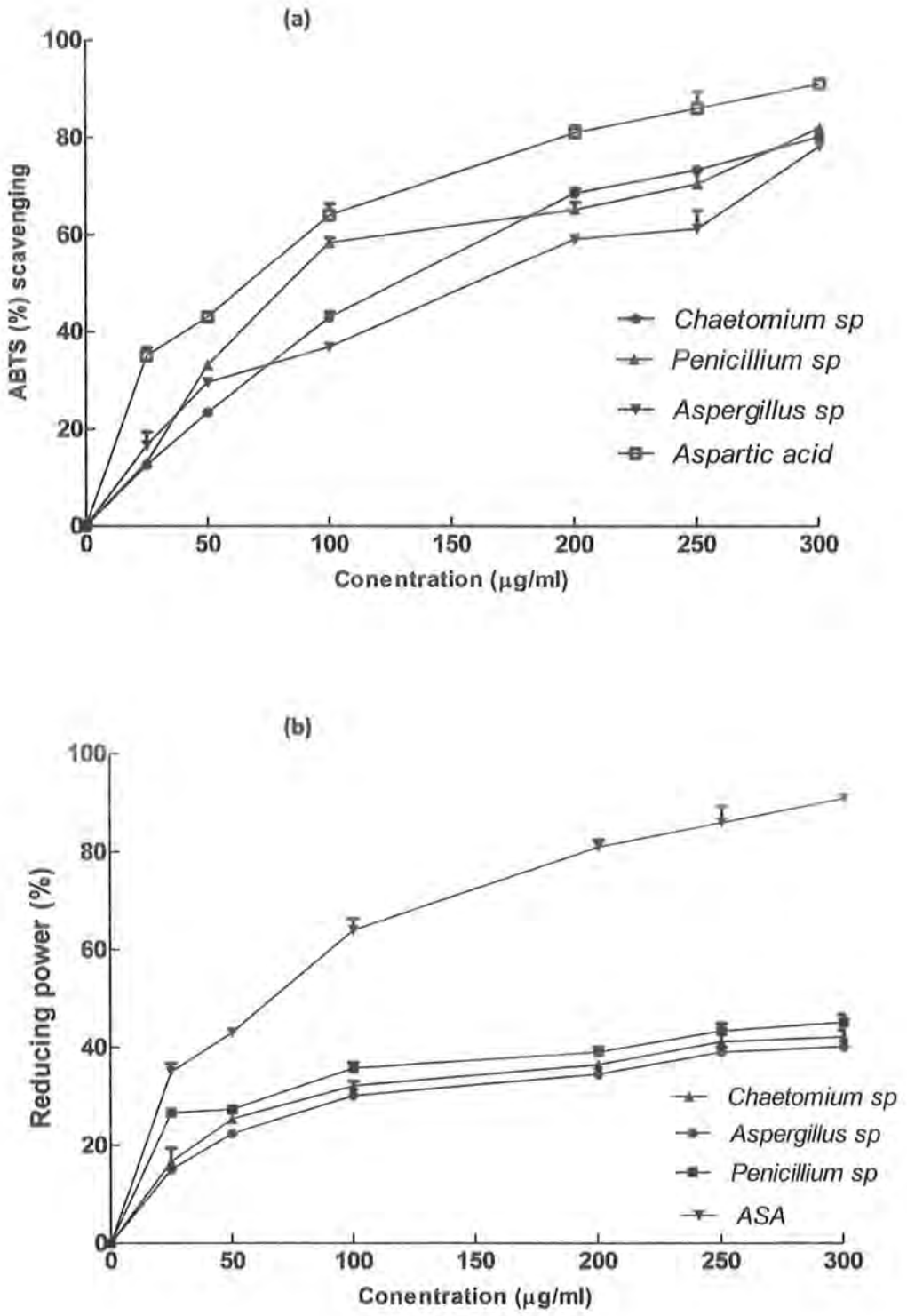


Fig 4.27: Total ABTS scavenging activity (a) and reducing power (%) (b) of the three pigment producing fungi

4.8 Toxicity studies of the pigmented filtrates of fungi

4.8.1 *Phytotoxicity assay*

Phytotoxicity (Raddish seed assay) results showed that with increase in concentration and with passage of time, there was slight increase in toxicity. After 5 days, highest phytotoxicity was seen at highest concentration of pigmented filtrate i.e., 10 mg/ml with maximum cytotoxicity in case of *Aspergillus fumigatus* with seed inhibition of 40% followed by *Penicillium verruculosum* (36%) and (30%) in *Chaetomium strumarium* (Table 4.5). Whereas, least phytotoxicity was observed at lowest concentration i.e. 2 mg/ml where seed inhibition ranged from 12-16% which was comparable to control (12%). The shoots showed better growth (3.5-4.9 cm) after culture filtrate treatment as compared to distilled water (2.4 cm).

Table. 4.5: Phytotoxicity (raddish seeds assay) at different concentrations of pigmented methanolic filtrates of three fungi with their resepective % seed inhibition and germination.

Fungi	Pigmented filtrate (Methanol)	Seed germination (%) (n=75)	Seed inhibition (%) (n = 75)	Shoot length (ave.) (cm)
		After 5 days	After 5 days	After 5 days
***Penicillium* spp.**	50mg/5ml	64	36	3.783
	25mg/5ml	84	16	4.533
	10mg/5ml	88	12	4.920
***Aspergillus* spp.**	50mg/5ml	60	40	3.533
	25mg/5ml	80	20	4.533
	10mg/5ml	84	16	4.920
***Chaetomium* spp.**	50mg/5ml	70	30	3.533
	25mg/5ml	86	14	4.533
	10mg/5ml	88	12	4.920
	Distilled water (5ml)	88	12	2.465

4.8.2 *Cytotoxicity assay*

There was a direct relation between the increase in pigment concentration, time and cytotoxicity (Brine shrimp assay) (Table 4.6). After 24 hours, the LD_{50} value was highest in *Aspergillus fumigatus* i.e., 3827.6 followed by 1380 in *Penicillium verruculosum* > 651.9 in *Chaetomium strumarium.* The % mortality increased with increase in pigment

concentration and it ranged from 13-40 for *Aspergillus fumigatus* followed by 23-46.6 for *Penicillium verruculosum* > 23.3-53 for *Chaetomium strumarium.*

After 48 hours of incubation, cytotoxicity in *Chaetomium strumarium* increased leading to lowest LD_{50} (21.5) ppm followed by 99 ppm for *Aspergillus fumigatus.* Whereas least cytotoxicity in terms of highest LD_{50} i.e., 314 ppm was seen in *Penicillium verruculosum.* The range of % mortality after 48 hours was; *Aspergillus fumigatus* for 33-73; 40-53 in case of *Penicillium verruculosum* while, it was 43-73 in *Chaetomium strumarium* LD_{50} was obtained through finni software.

Table 4.6: Illustration of % age mortality of brine shrimps and respective LD_{50} value at different concentrations of pigmented methanolic filtrates of the three fungi

Pigmented filtrate	% Mortality			
	10 ppm	100 ppm	1000 ppm	LD_{50} (ppm)
Aspergillus spp. 24 hrs	13.33	33.33	40.00	3827.625
Penicillium spp. 24 hrs	23.333	40.00	46.66	1380.22
Chaetomium spp. 24 hrs	23.33	36.66	53.33	651.970
Aspergillus spp. 48 hrs	33.33	43.33	73.33	99.4840
Penicillium spp. 48 hrs	40.00	46.66	53.33	314.190
Chaetomium spp. 48 hrs	43.33	63.33	73.33	21.5410

Kala pani Forest and *Taxus* plant of Pakistan proved to be an important habitat of important fungi. Fungi have an immense potential of producing bioactive compounds. Each fungus has its own requirements of pH, temperature and different nutritional parameters for production of specific products. In order to scale up any product for industrial processes, various physiochemical processes and stability of the product needs to be investigated as each industrial process have its specified requirements. In this study, various parameters were successfully optimized i.e., pH 5.5, temperature 25-30°C glucose 20-40g/L and yeast extract in PDB media and manifold increase in pigments concentration is attained at the end. Moreover, the stability of the pigments at various temperatures and pH was investigated for its future applications in the most suitable industry. Pigments of *Chaetomium strumarium* can be used in future as pH indicator also

due to their loss of color at acidic pH and regain of color at basic pH. Moreover, the pigments produced by *Penicillium verruculosum* are the most stable at wide range of temperatures and pH making its red pigment applicable in variety of industries.

The fungi are playing different roles in their habitats by producing different bioactive compounds that might have protective function including competitive advantage for their survival. The current study was designed in order to explore the biological potential of the three pigment producing fungi. These fungi showed good antimicrobial and antioxidant potential. The natural pigments will have an additional medicinal advantage i.e., antibiotic and anticancer (free radical scavenging activity) and antibacterial clothing when used in various industries (tablet coating, food colorants and textile industry). The low phytotoxicity and cytotoxicity of the pigment producing fungi in the current study showed their least harmful effects to environment and eukaryotic cells (Brine shrimp).The presence of the generalized groups of bioactive compounds further excited towards exploration of potentiality of fungi for specific compounds.

CHAPTER 5

5. Results

Considering in view the pigments producing ability of these fungi, further research was carried out to explore chemical composition of the pigmented culture filtrates of the three fungi. All the three fungi were subjected to bulk pigment production in modified PDA media. The carbon nitrogen ratio (C: N) optimized previously was used in culture media of each fungi. This ratio was 4:1 in *Penicillium verruculosum*, 8:1 in *Aspergillus fumigatus* while 10:1 in case of *Chaetomium strumarium*. The pigments were extracted in Methanol (3x). The organic layer was filtered through filter paper and filtrate obtained was further dried on rotary evaporator (40°C). The pigment extracted from the culture media of each fungus was processed through TLC, Preparatory TLC and column chromatography (C8, Sephadex). Further, the various fractions obtained through chromatography were analyzed through LCMS, LCMS/MS, proton NMR and XRD. The molecular ion peaks (m/z) and respective wavelengths obtained in LCMS and LCMSMS were vigorously searched in literature and different known libraries to match the respective compounds. Further, the different fragments formed by the breakdown of molecular ion peak (M^+) in LCMS and LCMSMS were justified by the logical fragmentation pattern in ChemDraw software.

5.1 Characterization of colored metabolites/pigments through Chromatography Techniques

5.1.1 *Penicillium verruculosum*

Fungus *Penicillium verruculosum* yielded 20gm/4L of pigmented culture filtrate after bulk production. This dried culture filtrate was subjected to column chromatography technique using two different stationary phase (SP) i.e., Silica (SP1) employing solvent system SS1 and SS2 and second stationary phase Sephadex (LH20)(SP2) employing solvent system SS3 to obtain various fractions that contained various colored metabolites and bioactive compounds. Moreover, crystals of a pure yellow compound were also obtained after fractionation. Combined fractions B-e7 and B-e12 run on preparatory TLC (SST1) showed colored bands of different intensities (Fig. 5.1). LCMS of the red band (Fig. 5.1) revealed that it contained Monascorubrine (Fig. 5.3). While, the yellow band (Fig. 5.1) was related to Monascin and it was confirmed

through fragmentation pattern obtained through LCMS/MS (Fig. 5.2). LCMS of the brownish red band below red (Fig.5.1) showed an intense peak (m/z) of Glutamyl-Monascorubrine (Fig. 5.4). The purplish band (Fig. 5.1) at the base of the preparatory TLC has m/z value of 383 and its wavelength and fragmentation pattern in LCDAD/MS predicted it to be an analogue of Monascorubrine (Fig. 5.5). LCMS of B-d fraction indicated presence of Scirpentriol (Fig. 5.11) and Verrucine A (Fig. 5.10).

LCMS of fractions E-a and E-c showed presence of intense peak of 509 and 625 m/z depicting De-methyl Calcimycin (Fig. 5.9) and Citrinadin (Fig 5.8) respectively. Sub-fractions of Fc (1-15) i.e.; F.c4-F.c8 and F.c9-12 contained intense peaks at 613 and 583 m/z depicting Orevactaene (Fig. 5.7) and Pyripyropene respectively (Fig. 5.6). The remaining sub fractions in B, E and F exhibited different m/z values and due to unavailability of their LCMS libraries their corresponding structures/compounds remain unexplored. These include B.e1-B.e6 (460 m/z) (Fig. 5.13), B.e13-B.e15 (394 m/z) (Fig. 5.22), F.c1-F.c3 (456.11 m/z) (Fig. 5.18), F.c12-c15 (613.24) (Fig. 5.19), (327 and 412 m/z) (Fig. 5.17 and Fig. 5.21), E.d (379 and 498.38 m/z) (Fig. 5.12 and 5.16), E.e (512, 271 m/z) (Fig. 5.20 and 5.15) and E.f (213.08 m/z) (Fig. 5.14).

Table 5.1: LCDADMS profile of pigment producing fungi *Penicillium verruculosum*

Penicillium verruculosum				
Retention time	**Fraction**	**m/z value**	**The ions identified annotated m/z signals**	**Figure**
6.49	B.e1_B.e6	460	Unknown	(Fig. 5.13)
9.9	B.e13_B.e15	394	Unknown	(Fig. 5.22)
11.46	F.c1_F.c3	456	Unknown	(Fig. 5.18)
13.32	F.c13-c15	613	Unknown	(Fig. 5.19)
16.16	F.c13-c15	327	Unknown	(Fig. 5.17)
19.44	F.c13-c15	412	Unknown	(Fig. 5.21)
14.57	E.d	379	Unknown	(Fig. 5.12)
15.49	E.d	498	Unknown	(Fig. 5.16)
14.45	E.e	512	Unknown	(Fig. 5.20)
8.78	E.e	271	Unknown	(Fig. 5.15)
9.96	E.f	213	Unknown	(Fig. 5.14)
13.08	B.e7-12	359	Monascin	(Fig. 5.2)
14.38	B.e7-12	382	Monascorubrin	(Fig. 5.3)
13.55	B.e7-12	483	Glutamyl monascorubrin	(Fig. 5.4)
19.47	B.e7-12	383	Analogue of monascorubrin	(Fig. 5.5)
12.45	F.c 9-12	583	Pyripyropene	(Fig. 5.6)
13.23	F.c 4-8	613	Orevactaene	(Fig. 5.7)
15.89	E.a	509	N. De methyl Calcimycin	(Fig. 5.9)
13.81	E.c	625	Citrinadin	(Fig. 5.8)
14.18	B.d	283	Scirpentriol	(Fig. 5.11)
6.63	B.d	377	Verrucine A	(Fig. 5.10)

Fig 5.1: Preparatory TLC plate showing colored metabolites in 10 days old culture filtrate of *P. verruculosum* SG (Fraction Be.7-Be.12)

5.1.1.1 Monascin

A yellow band was obtained on preparatory TLC (Fig. 5.1) of combined fraction B.e7-B.e12. This yellow band was scratched from TLC plate and further purified and extracted with solvent system SST1. The extracted pigment was then dried and dissolved in Methanol for further processing through LCMS and LCMSMS (Fig. 5.2a; b). An intense peak having mass to charge ratio (m/z) 359 and absorbance pattern matching well with compound Monascin was found. The fragmentation pattern of this compound in LCMSMS showed a prominent fragment of m/z 196 which was justified through ChemDraw (Fig. 5.2c).

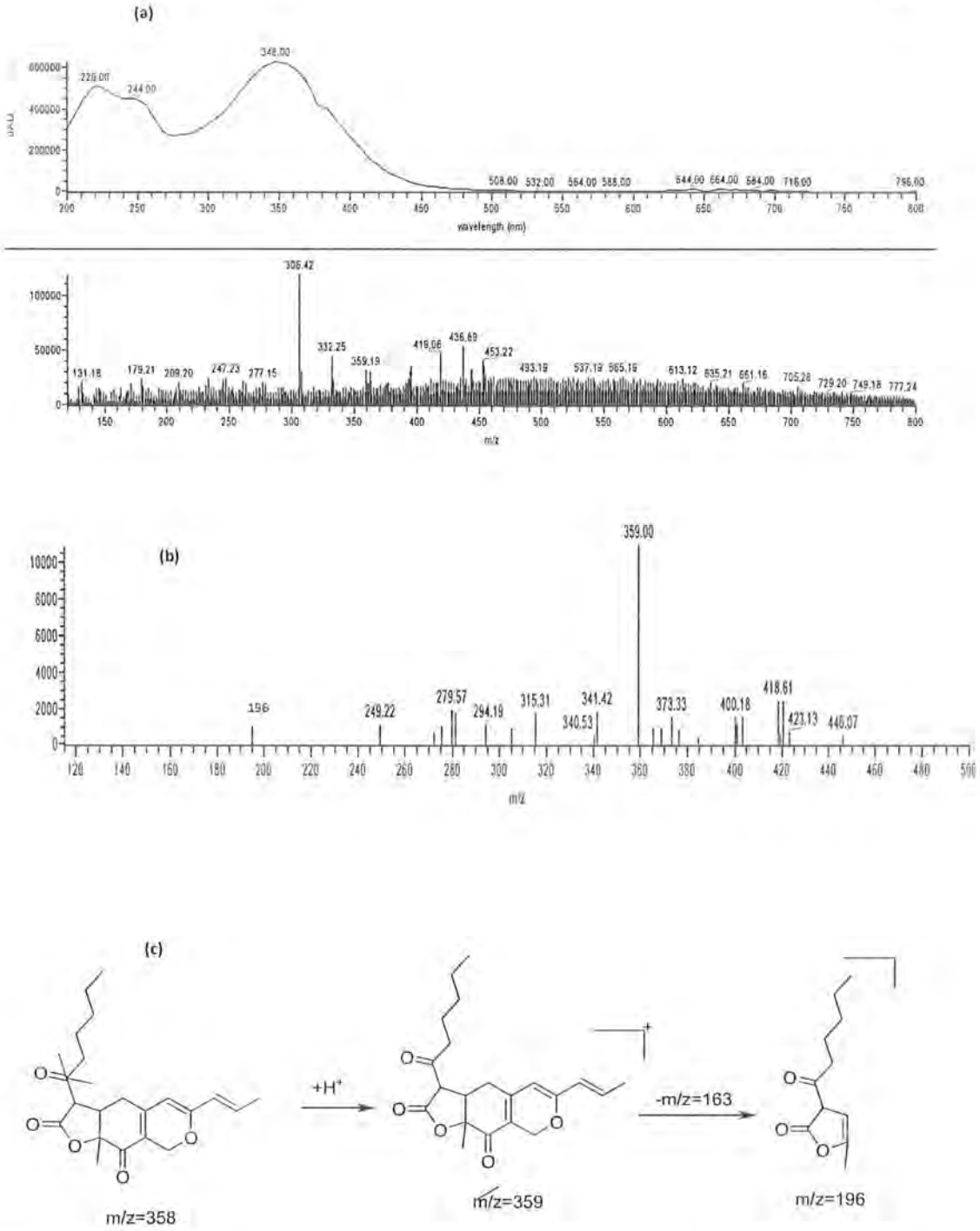


Fig 5.2: Fragmentation pattern of Monascin (c) [showing presence of molecular ion peak 359 m/z and a major fragment at 196 m/z (b)] predicting its chemical structure following LCMS (a) and LCMS/MS (b)

5.1.1.2 Monascorubrin

The red prominent band obtained on preparatory TLC (Fig. 5.1) of combined fractions B.e7-B.e12 was scratched and further extracted in appropriate solvents i.e., SST2. This red band was processing through LCMS (5.3a) by dissolving in Methanol. An intense peak of m/z 382 and fragments of m/z 338 and 256 (5.3a; b) confirmed this compound to be Monascorubrin. The wavelength absorbance pattern also matched with that already reported in literature and library (Fig. 5.3)

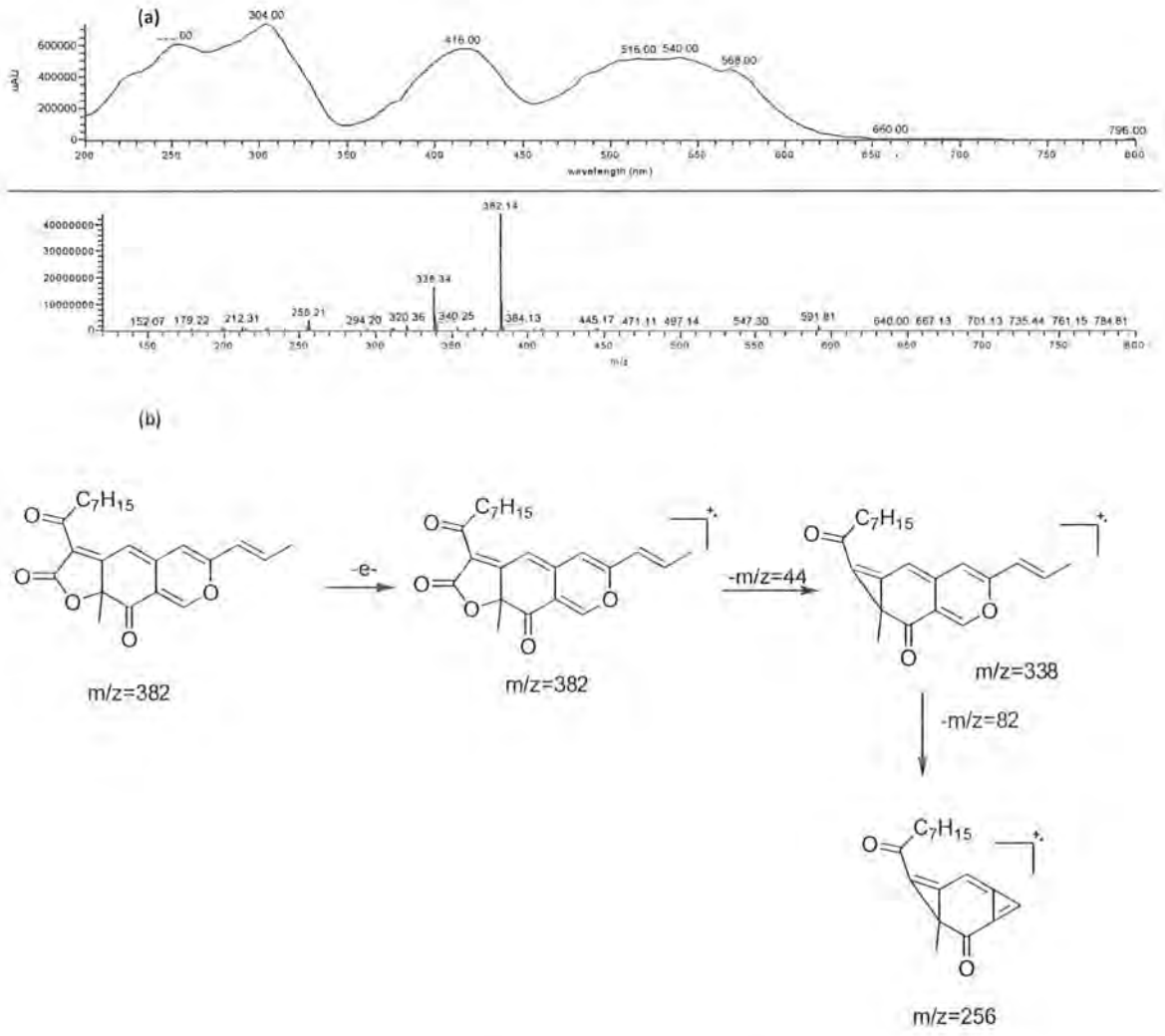


Fig 5.3: Fragmentation pattern of Monascorubrine (b) [(showing the presence of molecular ion peaks at 382 m/z and fragments at 338 and 256 m/z) (a)] predicting its chemical structure and absorbance pattern following LCMS (a).

5.1.1.3 Glutamyl Monascorubrine

The purple band below the Monascorubrin on preparatory TLC (Fig. 5.1) (Fraction B.e7-B.e12) was scratched and extracted with suitable solvent system (SST1). It was further dried and dissolved in methanol to be processed through LCMS where an intense peak of m/z 483 was seen. The compound was found to be Glutamyl Monascorubrine after logical fragmentation done through ChemDraw. The fragments of m/z 442, 425 and the absorbance pattern affirmed the compound identity (Fig. 5.4).

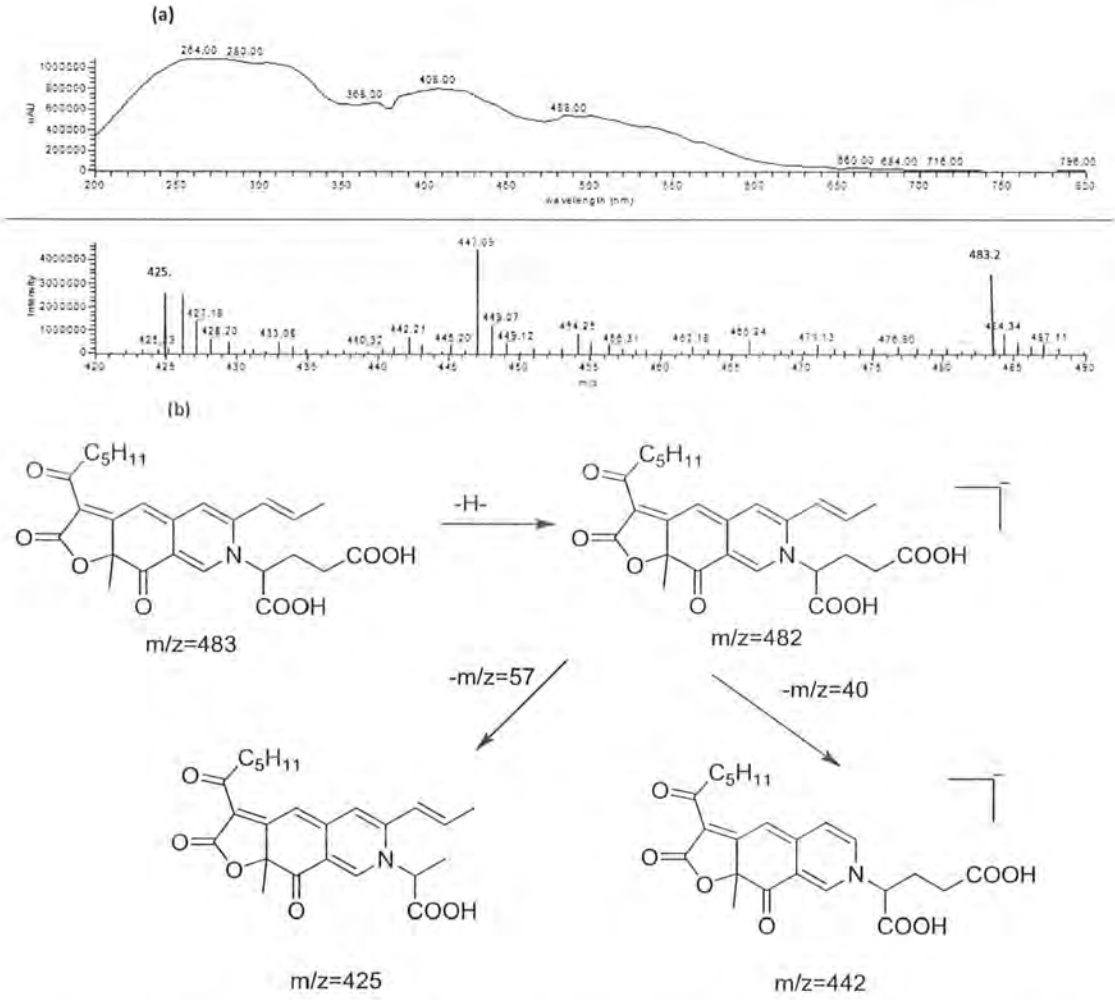


Fig 5.4: Fragmentation pattern of Glutamyl Monascorubrine [(showing the presence of molecular ion peak 483 and fragments 442 and 425 m/z) (b)] predicting its chemical structure following LCDADMS (a).

5.1.1.4 Analogue of Monascorubrine

The purple band at the base of preparatory TLC (Fig. 5.1) was scratched and extracted with solvent system (SST1). It was dried and dissolved in methanol for further processing through LCMS where an intense peak of m/z 383 was seen. The compound was found to be analogue of Monascorubrine as confirmed by fragments of m/z 338 and 256 found previously in Monascorubrine while the absorbance pattern differed from the Monascorubrine suggesting that it might be analogue of Monascorubrine (Fig. 5.5).

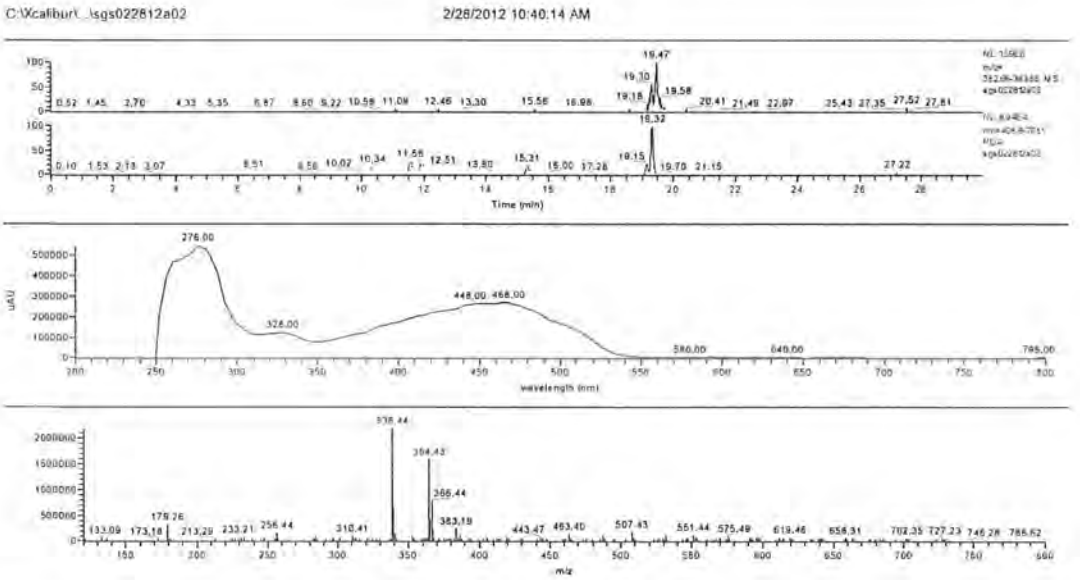


Fig 5.5: Analogue of Monascorubrine having the same molecular ion peak m/z 383 and the fragments at 338 and 256 as monascorubrin but different wavelength pattern.

5.1.1.5 Pyripyropene

Fractions Fc.9-Fc.12 were combined based on TLC analysis and subjected to LCMS where an intense peak of m/z 583 was obtained. Further LCMS-MS of this peak confirmed it to be compound Pyripyropene as determined from the fragments with m/z 564 and 326 (Fig. 5.6).

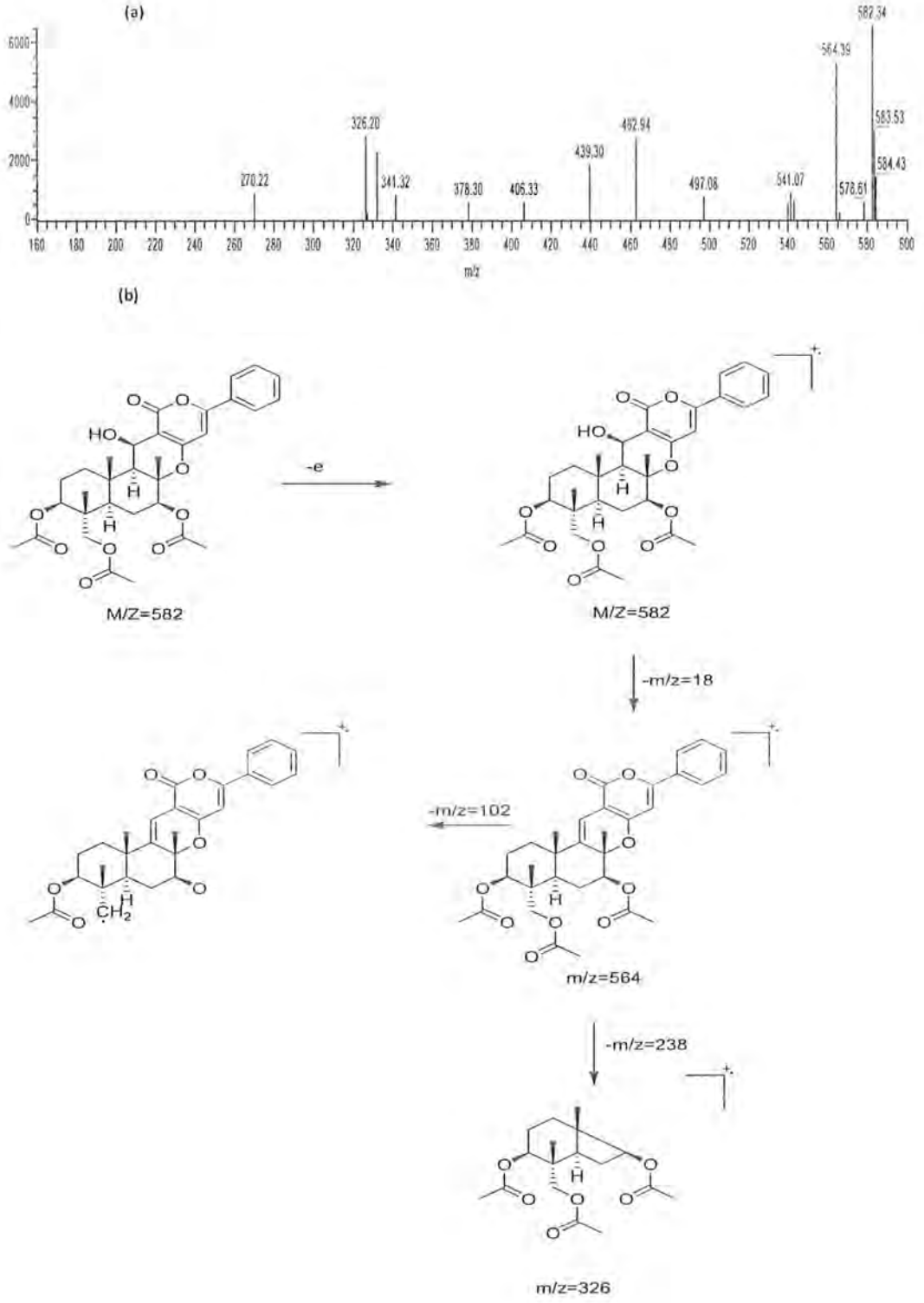


Fig 5.6: Fragmentation pattern (b) of Pyripyropene (Fraction Fc.9-Fc.12) [showing the presence of molecular ion peak 564 m/z and a major fragment at 462 and 326 m/z(a)] predicting its chemical structure following LCMSMS (a).

5.1.1.6 Orevactaene

Fractions Fc.4-Fc.8 containing yellow spot in TLC were combined and subjected to LCMS. An intense peak of m/z 613 was observed in LCMS. This compound was found to be Orevactaene as shown by its wavelength absorbance pattern and presence of M+H 613 and a fragment formed by loss of water i.e., m/z 595 (Fig. 5.7).

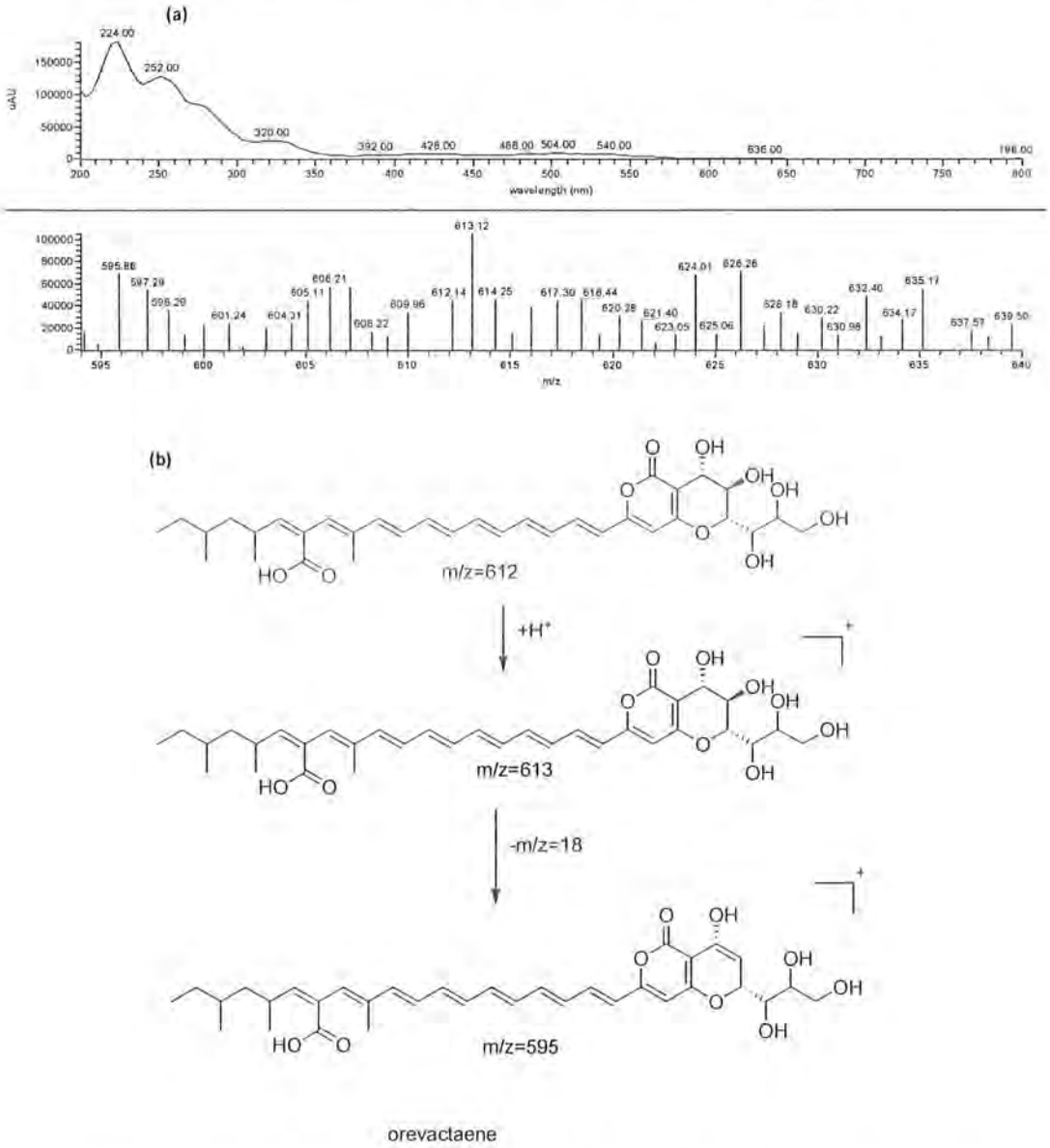


Fig 5.7: Fragmentation pattern of Orevactaene (b) (Fraction Fc.4-Fc.8) [showing the presence of molecular ion peak 613 m/z and a fragment of m/z 595 in LCDAD/MS (a)] predicting its chemical structure.

5.1.1.7 Citrinadin

Fraction E.c was found to contain an intense peak of m/z 625. This peak was later on found to be Citrinadin compound as justified by its fragmentation pattern with fragments of m/z 625, 526 and 481. Moreover the wavelength absorbance also matched well with the above mentioned compound (Fig. 5.8).

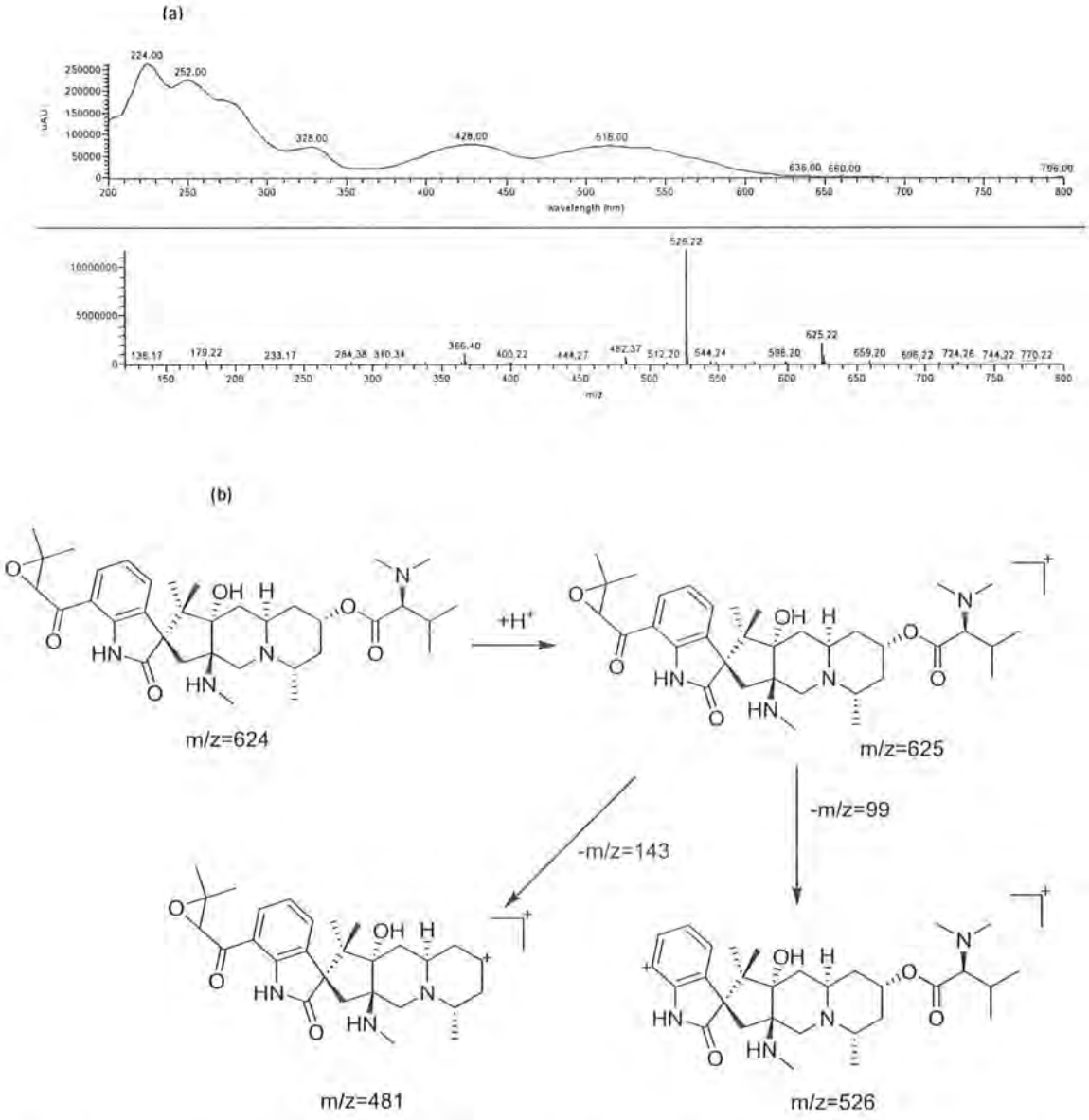
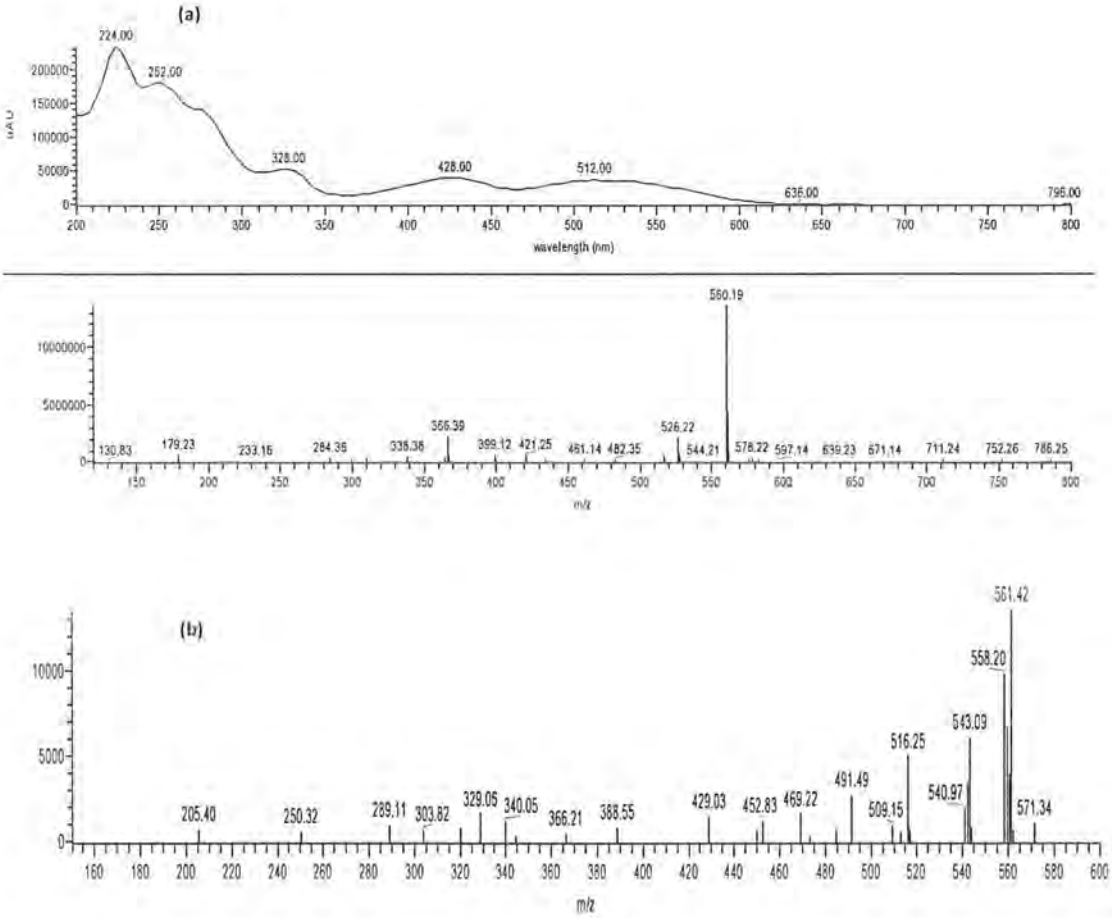


Fig 5.8: Fragmentation pattern of Citrinadin (a) (Fraction E.c) [showing the presence of molecular ion peak 625 m/z and fragments of 526, 481 m/z in LCDADMS (b)] predicting its chemical structure.

5.1.1.8 N-Demethyl-Calcimycin

The fraction E.a showed an intense peak of m/z 509 in LCMS and further LCMSMS of this peak was done for detailed fragmentation pattern and compound confirmation. The fragments of m/z 509, 388 and 289 were found in LCMSMS and justified by ChemDraw (Fig. 5.9).



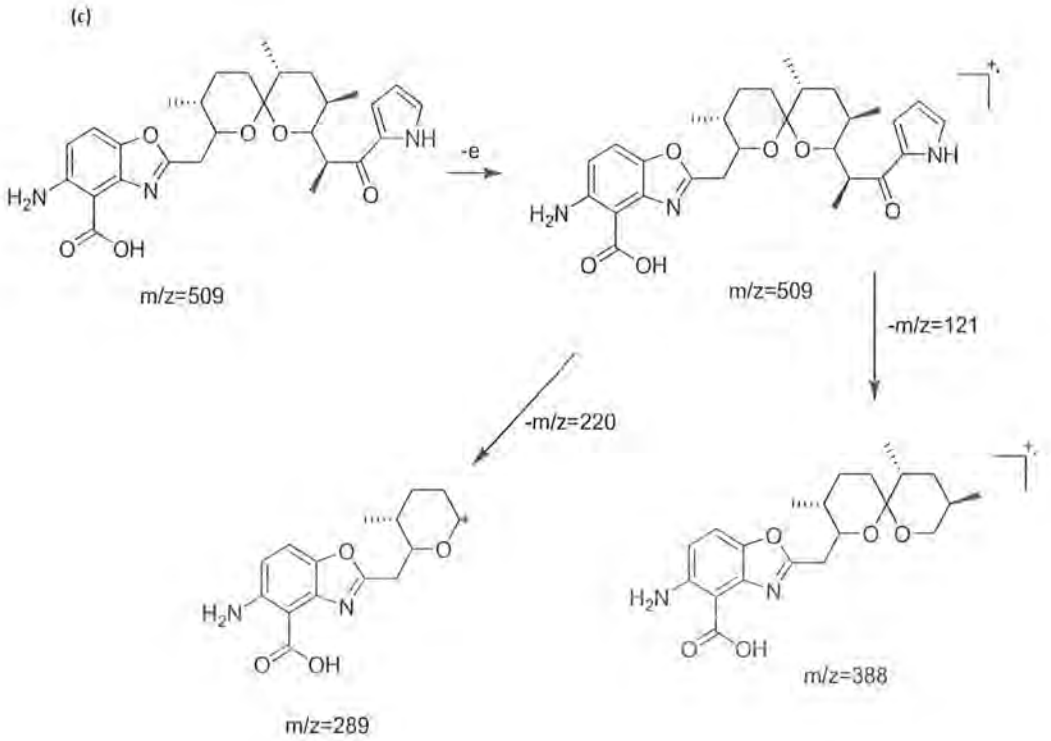


Fig 5.9: Fragmentation pattern of Calcimycin (c) (Fraction E.a) showing the presence of molecular ion peak 283 m/z in LCMS (a) and fragments of 247, 184 m/z in LCMSMS (b) predicting its chemical structure.

5.1.1.9 Verrucine A

Fraction B.d when subjected to LCMS showed presence of m/z 376 and its fragments i.e., 377, 360 and 331 as well as absorbance pattern confirmed this peak to be Verrucine A (Fig. 5.10).

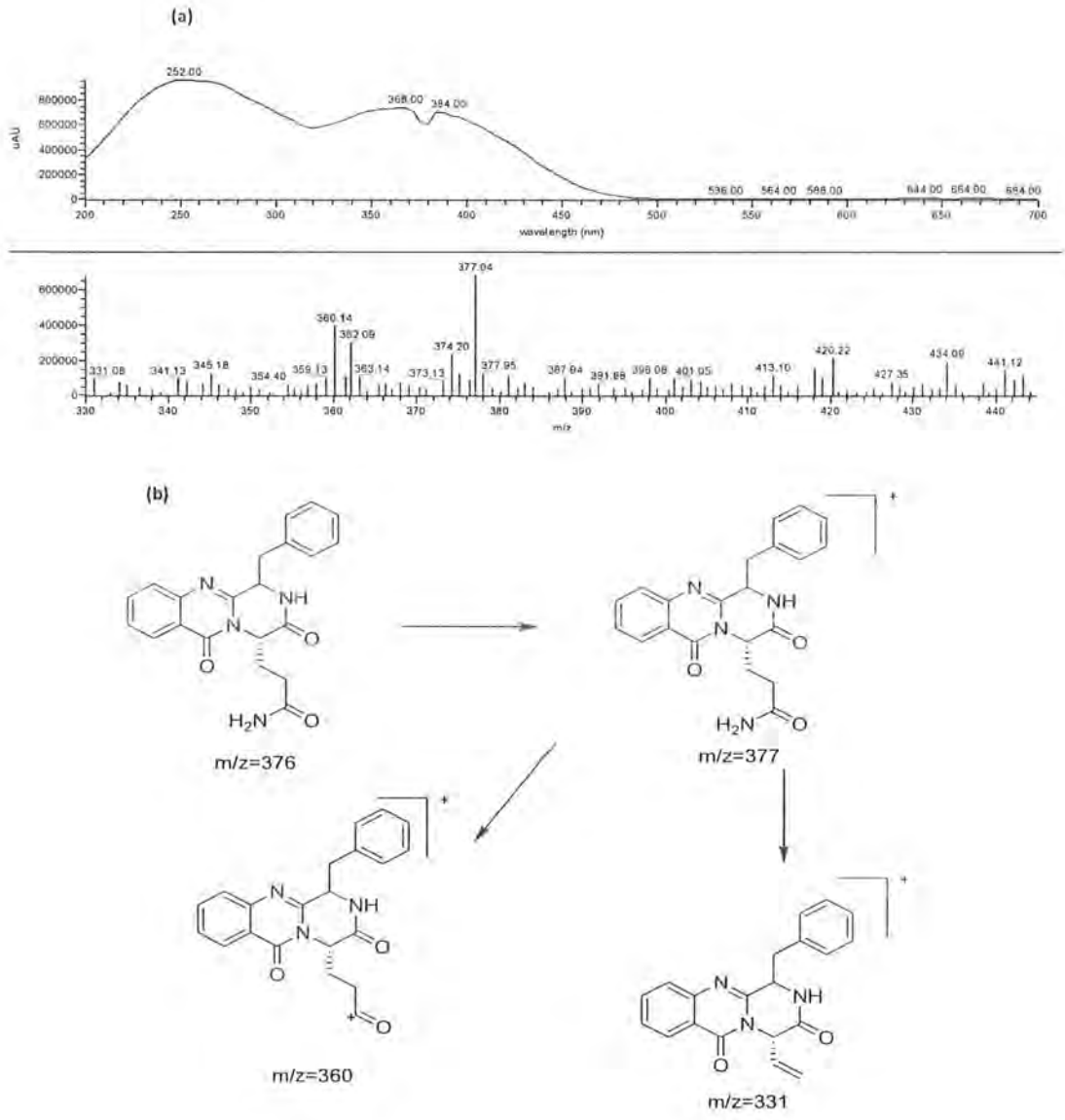


Fig 5.10: Fragmentation pattern (b) of Verrucine A (Fraction B.d) [showing the presence of molecular ion peak 377 m/z and fragments of 360, 331 m/z in LCDADMS (a)] predicting its chemical structure.

5.1.1.10 Scirpentriol

Fraction B.d was found to contain an intense peak of m/z 282 which when subjected to LCMSMS showed the fragments with m/z 247 and 184 which corresponds to the structure of Scirpentriol (Fig. 5.11).

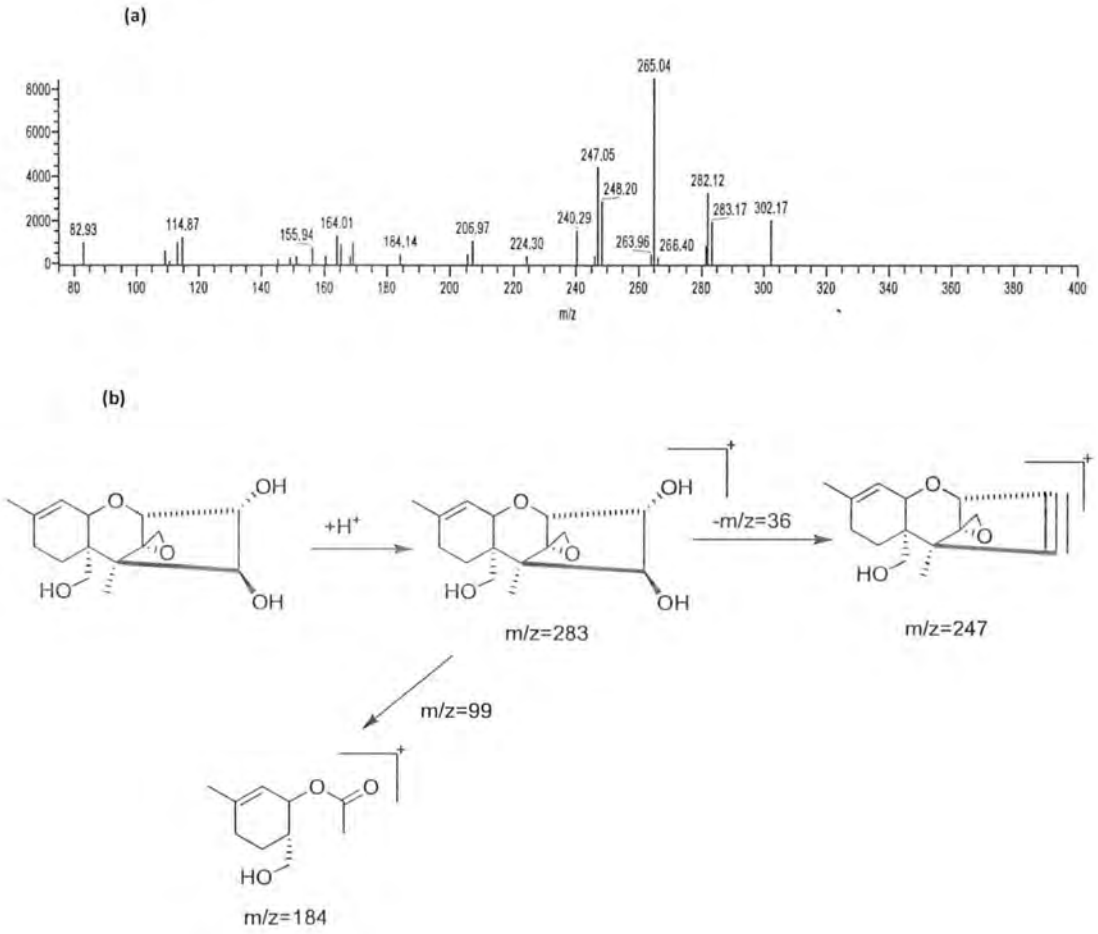


Fig 5.11: Fragmentation pattern of Scirpentriol (b) (Fraction B.d) [(showing the presence of molecular ion peak 283 m/z and fragments of 247, 184 m/z in LCMSMS) (a)] predicting its chemical structure.

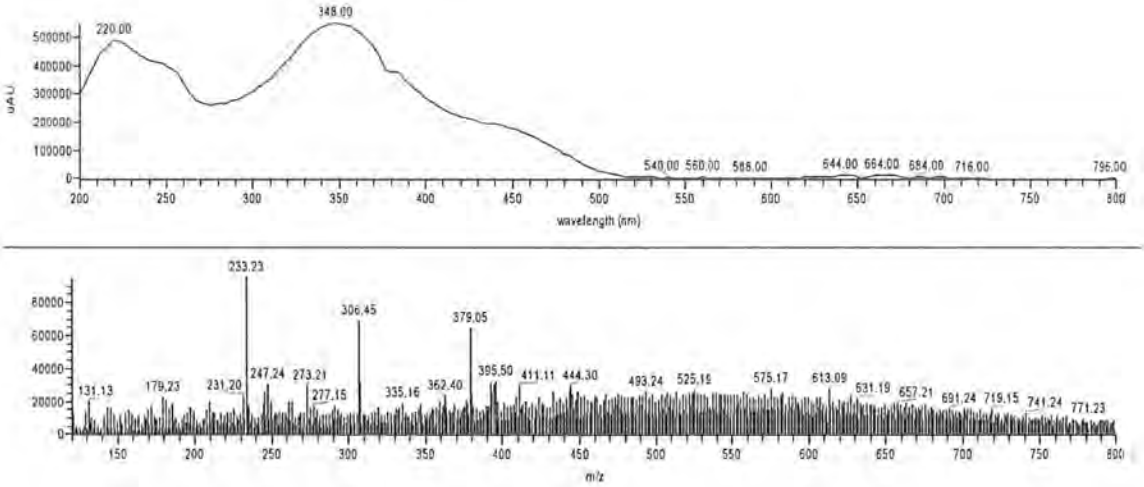


Fig 5.12: Fraction E.d obtained after processing of methanolic extract of *Penicillium verruculosum* contained a peak of 379 m/z in LCMS (Compound unknown).

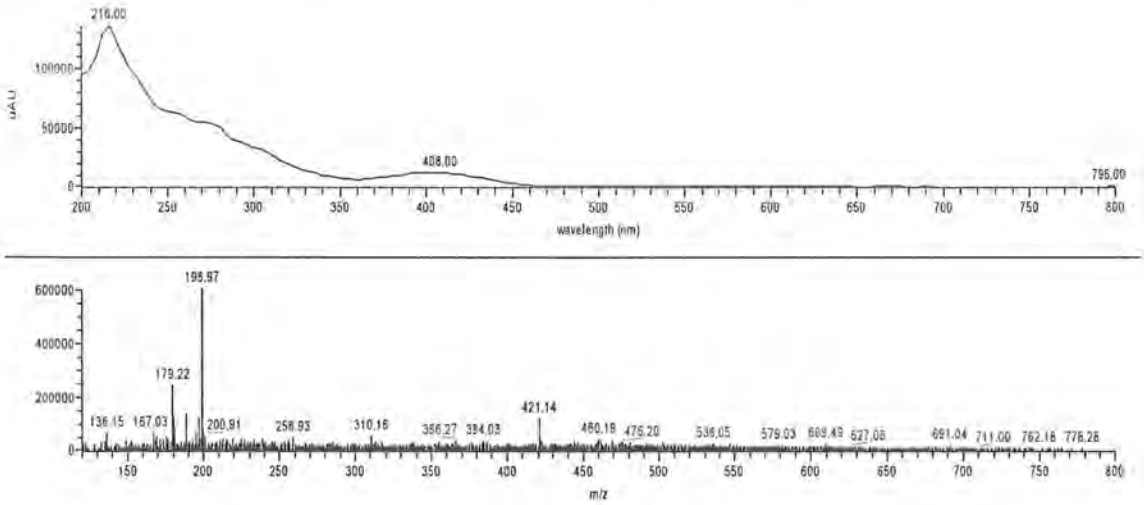


Fig 5.13: Fraction B.e 1-B.e 6 obtainied after processing methanolic extract of *Penicillium verruculosum* were combined and contained a peak of 460 m/z in LCMS (Unknown compound).

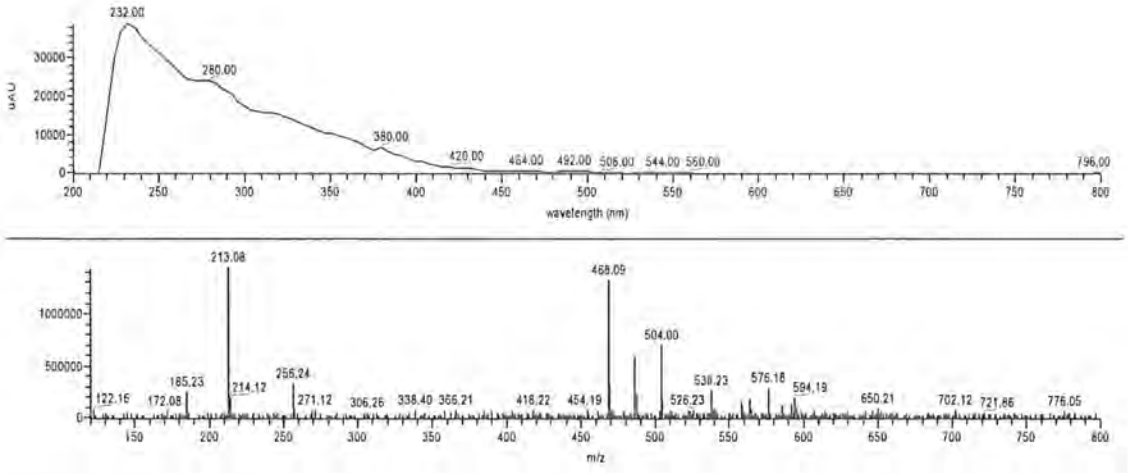


Fig 5.14: Fraction E.f obtained after processing of methanolic pigmented filtrate of *Penicillium verruculosum* contained a peak of 213 m/z in LCMS (unknown compound).

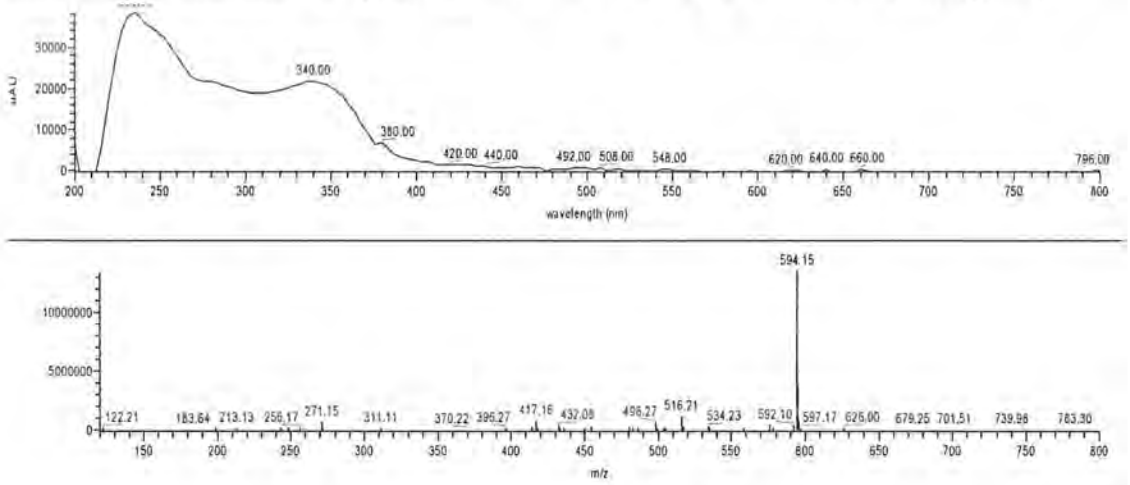


Fig 5.15: Fraction E.e obtained after processing of methanolic pigmented filtrate of *Penicillium verruculosum* contained a peak of m/z 271 in LCMS (unknown compound).

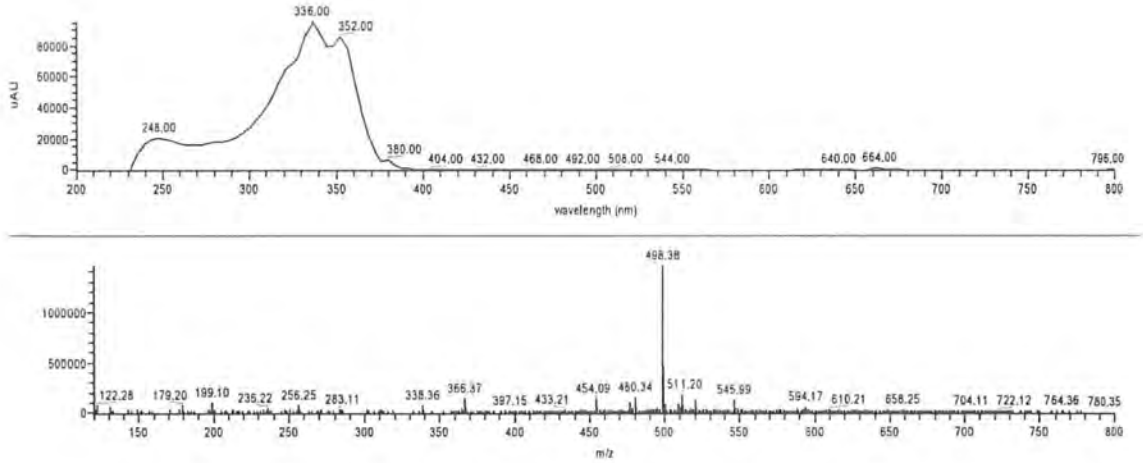


Fig 5.16: Fraction E.d obtained after processing of methanolic pigmented filtrate of *Penicillium verruculosum* contained a peak of 498 m/z in LCMS (Unknown compound).

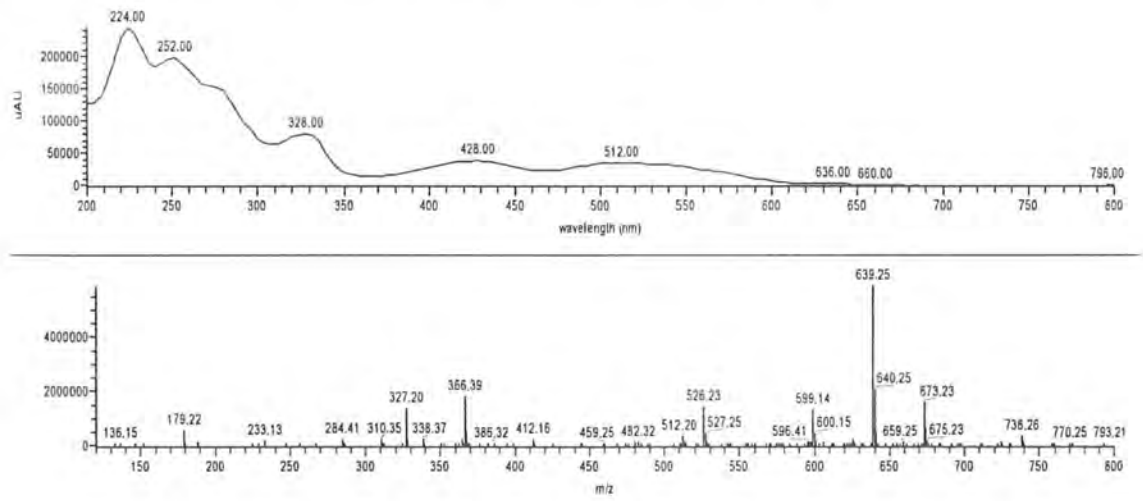


Fig 5.17: Fraction F.c 12-15 obtained after processing of methanolic pigmented filtrate of *Penicillium verruculosum* contained a peak of m/z 327 in LCMS (Unknown compound).

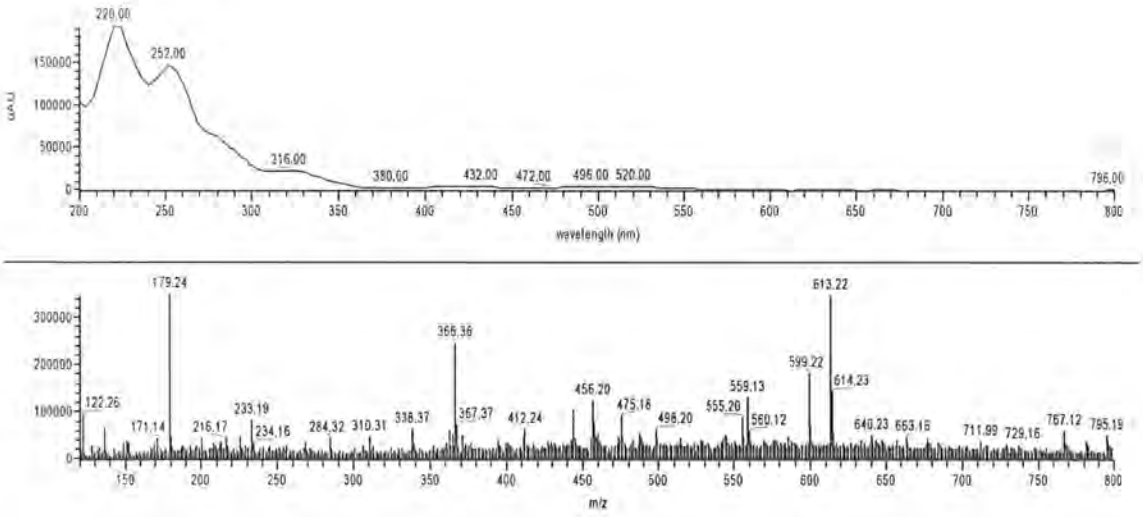


Fig 5.18: Fraction F.c 1-3 obtained after processing of methanolic pigmented filtrate of *Penicillium verruculosum* contained a peak of m/z 456 in LCMS (Unknown compound).

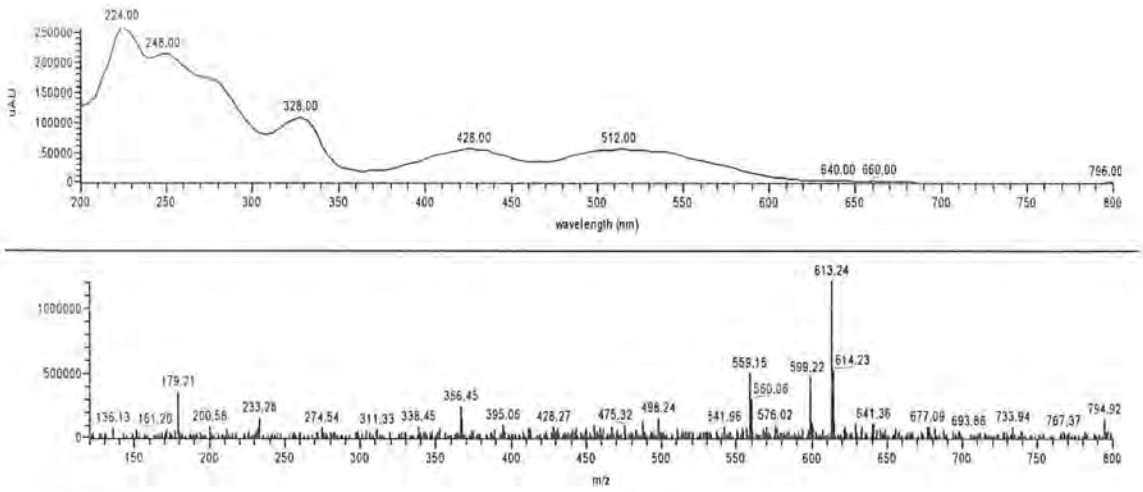


Fig 5.19: Fraction F.c12-15 obtained after processing of methanolic pigmented filtrate of *Penicillium verruculosum* contained a peak of m/z 613 in LCMS (Unknown compound).

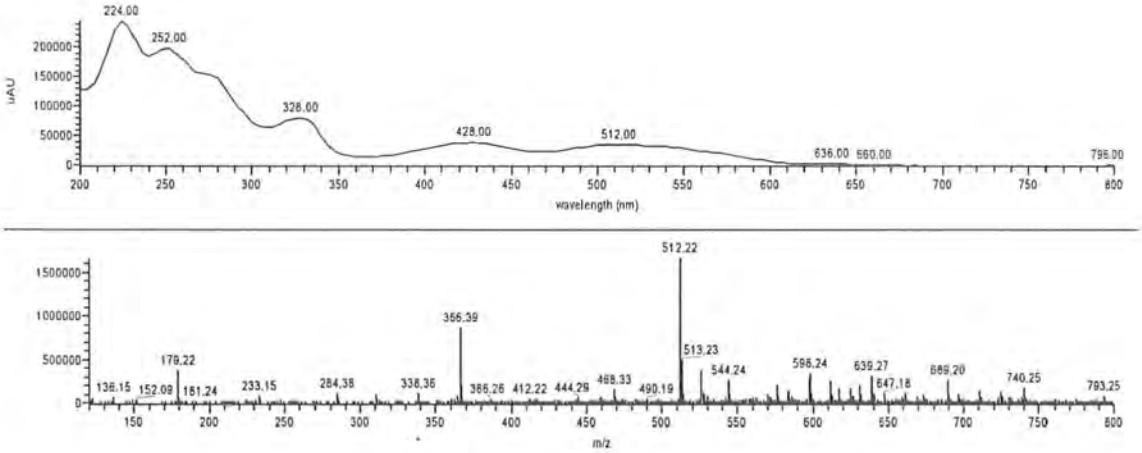


Fig 5.20: Fraction E.e obtained after processing of methanolic pigmented filtrate of *Penicillium verruculosum* contained a peak of m/z 512 in LCMS (Unknown compound).

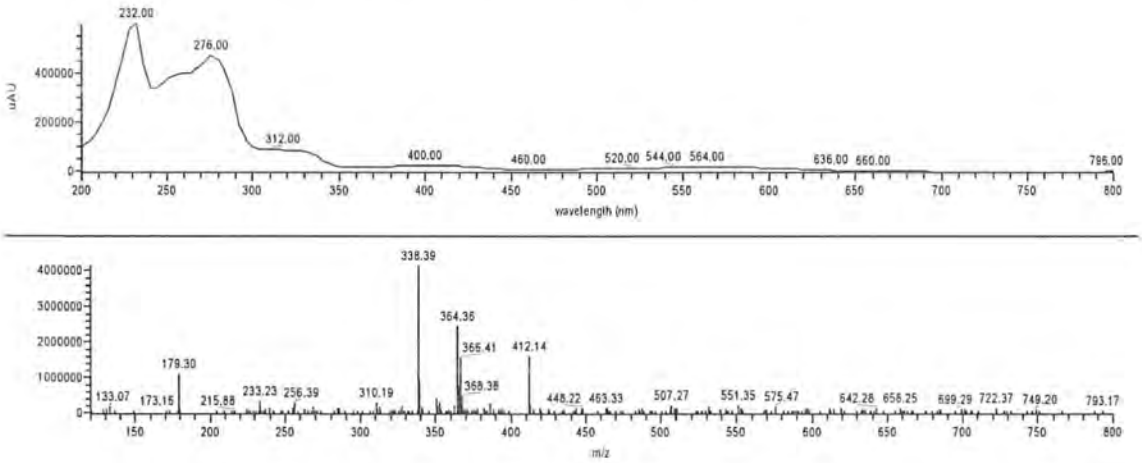


Fig 5.21: Fraction F.c12-15 obtained after processing of methanolic pigmented filtrate of *Penicillium verruculosum* contained a peak of m/z 412 in LCMS (Unknown compound).

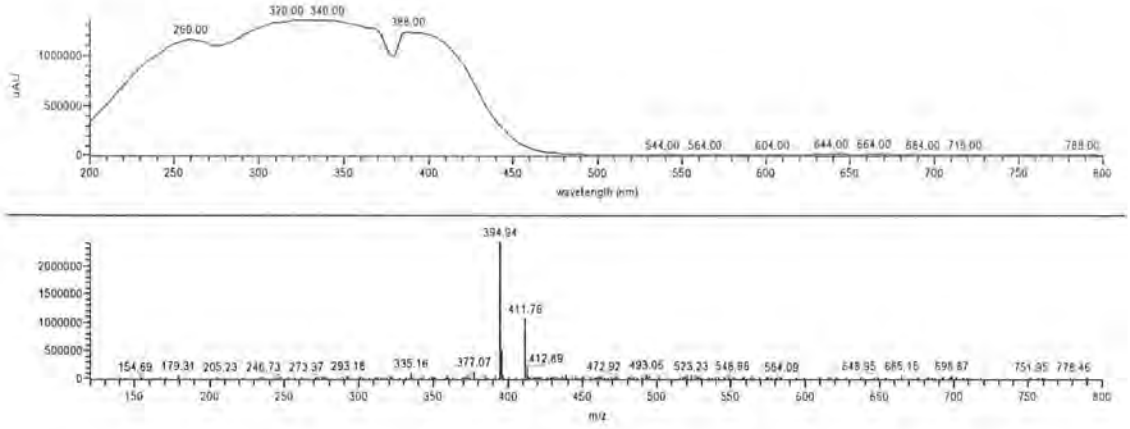


Fig 5.22: Fraction B.e13-15 obtained after processing of methanolic pigmented filtrate of *Penicillium verruculosum* contained a peak of m/z 394 in LCMS (Unknown compound).

5.1.2 Cytotoxicity Assays (Cell line assay)

Cytotoxicity assays of different fractions i.e., B, E F and sub-fractions of culture filtrate of *P. verruculum* were carried out with 5 different normal and cancerous cell lines (Table 5.2; A27-A43). Moreover, the different colored bands scratched from preparatory TLC contained important *Monascus* azophilone group of pigments were also subjected to these assays to define their toxicity limits and it was found that these fractions and colored bands showed KA3IT cancerous cell line inhibition at a concentration which was below IC_{50} value for normal cell lines. This gives the possibility of using these colored metabolites and this filtrate to be promising source of future anticancer compounds.

IC_{50} for KA3IT ranged from 5 (Monascorubrin, Orevactaene) to 160 µg/ml (Mixture). All the normal cell lines showed lowest IC_{50} value for Scirpentriol, Verrucine A. Other than Scirpentriol, verrucine A, the trend of IC_{50} for NIH3T3 ranging from 35 (Orevactaene) to 120µg/ml (Mixture). It was found that these normal cells NIH3T3 can bypass killing by the drugs resulting in selective killing of its virally transformed cancerous form (KA3IT). IC_{50} for MDCK cell line ranged from 200 µg/ml for mixture and lowest for Scirpentriol, Verrucine A (8±0.88) containing fraction followed by Monascorubrine (34±0.27) (Non significantly differed from Pyripyropene) (Table 5.1). The highest IC_{50} for HSCT6 was shown by mixture i.e., 80µg/ml and lowest in case of analogue of Monascorubrin (34µg/ml) which was

greater than Scirpentriol and Verrucine A (Table 5.2). HEK cell line was also used to determine the IC_{50} values of the fractions and colorants obtained from the culture filtrate. The highest IC_{50} value was found in case of Glutamyl Monascorubrin (100µg/ml) and lowest in B (33µg/ml) but it was higher than Scirpentriol, verrucine A (14± 0.72). Different IC_{50} values showed that these fractions containing important compounds are not toxic to these cell lines except the fraction that contained Scirpentriol and Verrucine A. Inhibition of the KA3IT which is a cancerous cell line was checked with different colored fractions (B, E, F) and colored bands. It was found that these different fractions have the ability to inhibit this cell line and hence have anticancer activity with LD_{50} ranging from 5-25µg/ml as shown in fig. 5.23.

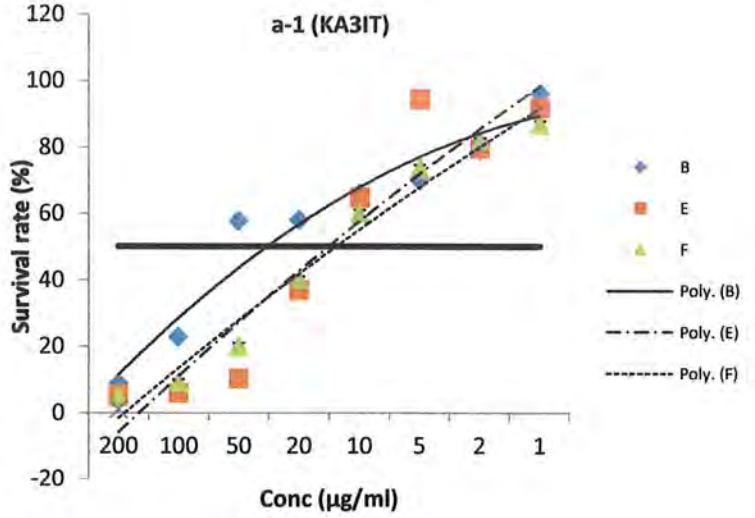
Table 5.2: Cytotoxic effect (IC_{50}) of different compounds and major fractions of pigmented culture filtrate of *Penicillium verruculosum* SG against different normal and cancer cell lines (LSD)

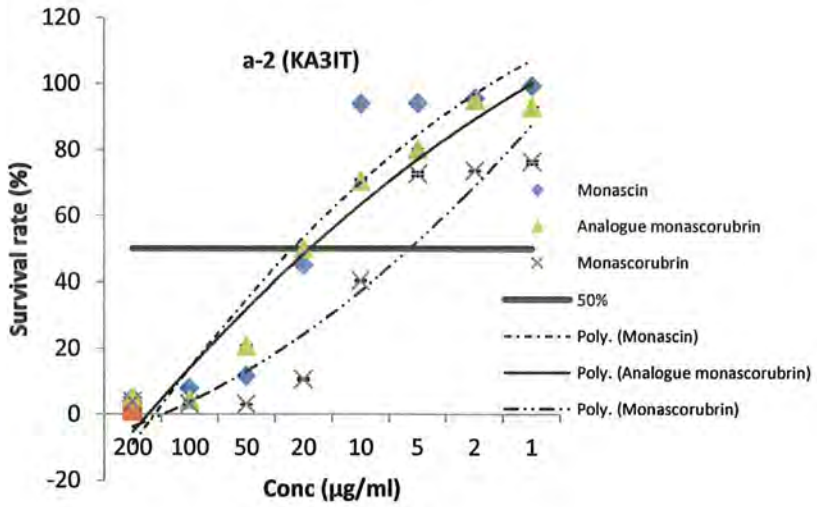
(Average values sharing a common letter do not differ significantly, other differ significantly ($p < 0.05$)

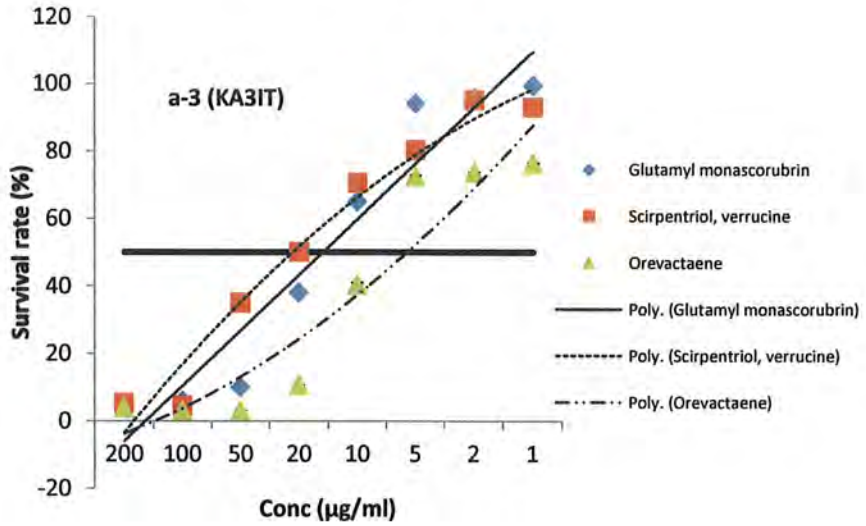
Fungal Filtrate		Mammalian cell lines											
Main fractions	Sub-fractions	KA3IT (µg/ml)		MDCK (µg/ml)		HSCT6 (µg/ml)		HEK (µg/ml)		NIH3T3 (µg/ml)		Combine effect	
		Ave.±SD (n = 3)	R	Ave.±SD (n = 3)	R	Ave.±SD (n = 3)	R	Ave.±SD (n = 3)	R	Ave.±SD (n = 3)	R	Ave. (n = 5)	R
B		20± 0.38	D	60± 0.84	B	38± 0.72	F	33± 0.87	H	48± 0.54	E	**39.8±15**	B
F		15± 0.27	F	50±0.52	C	50± 0.18	D	45± 0.45	F	55± 0.89	C	**43±16**	B
E		10± 0.89	G	40± 0.9	E	54± 0.88	C	50± 0.18	E	65± 0.36	B	**43.8±20**	B
	Scirpentriol, Verrucine A	22± 1.63	C	8± 0.88	G	8± 0.44	I	14± 0.72	I	7± 0.59	H	**11.8±6**	C
	Monascin	25±0.89	B	47±0.54	D	50±0.89	D	75±0.53	B	40± 0.89	F	**47.4±18**	B
	Analogue of monascorubrine	18± 0.84	E	50± 0.17	C	32± 0.89	H	60± 0.82	D	35± 0.72	G	**39±16**	B
	Monascorubrine	5+ 0.89	I	34± 0.45	F	37± 0.35	F	46± 0.88	F	50± 0.76	D	**34.4±17**	BC
	Glutamyl monascorubrine	16± 0.34	EF	35± 0.99	F	50± 1.2	D	100± 1.5	A	50± 0.48	DE	**50.2±31**	BC
	Oreavactaene	5± 0.44	I	45± 0.89	D	40± 0.76	E	47± 0.09	F	35± 0.47	G	**34.4±17**	BC
	Pyripyropene	8± 0.63	H	34± 0.27	F	70± 0.82	B	50± 0.26	E	50± 0.79	DE	**42.4±23**	B
	Calcimycin	18± 0.99	E	39± 0.53	E	40± 0.87	E	74± 0.18	B	36± 0.54	G	**41.4±20**	B
	Citrinadin	16 ± 1.4	F	50± 0.68	C	35± 0	G	40± 0.79	G	40± 1.78	F	**36.2±12**	B
Crude culture filtrate		160± 0.8	A	200± 1.7	A	80± 2.7	A	70± 1.8	C	120± 2.6	A	**126±54**	A
	Ave. (n = 13)	35.57±40.7		63.71±45.8		47.44±17.8		55.2±21.8		53.6±25.52		45.4±25.4	
	R	B		A		A		A		A			

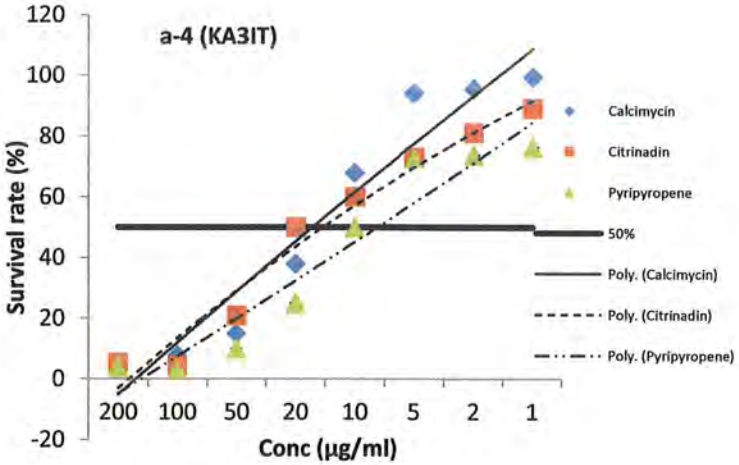
Foot notes: Vertically ranking associated with IC_{50} of different fractions with values against different cell lines. Horizontally ranking depict average of IC_{50} values from all cell lines. IC_{50} was calculated by formula= Treated cell conc. (OD)/untreated cell conc. (OD)*100.

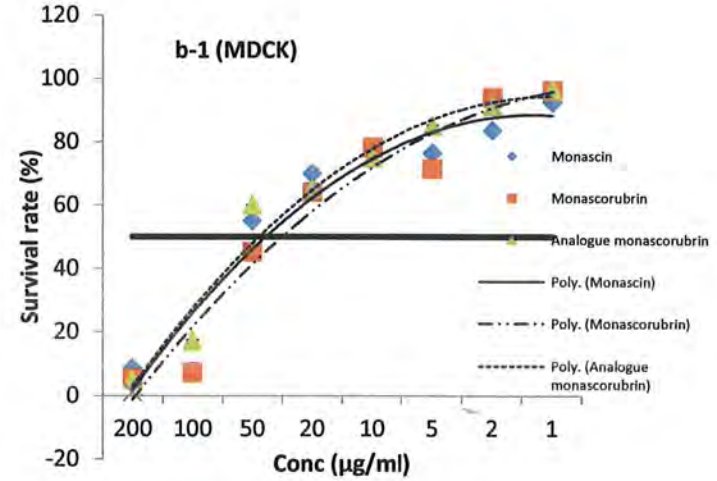
LSD = Least Square Difference; R = Rank order; SD = Standard deviation; Ave = Average; NIH3T3 = cells mouse embryonic fibroblasts; HSCT6 cells = rat hepatic stellate cell line; HEK293 = cell line derived from human embryonic kidney cells grown in tissue culture; MDCK Line = Madin-Darby Canine Kidney Epithelial Cells; KA3IT = virally transformed cancerous cell line.

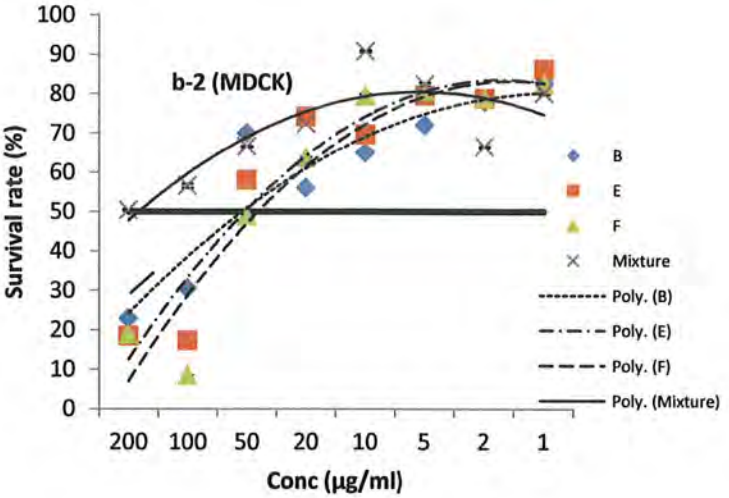

a-1 (KA3IT)
Survival rate (%)
120
100
80
60
40
20
0
-20
200 100 50 20 10 5 2 1
Conc (μg/ml)
B
E
F
Poly. (B)
Poly. (E)
Poly. (F)

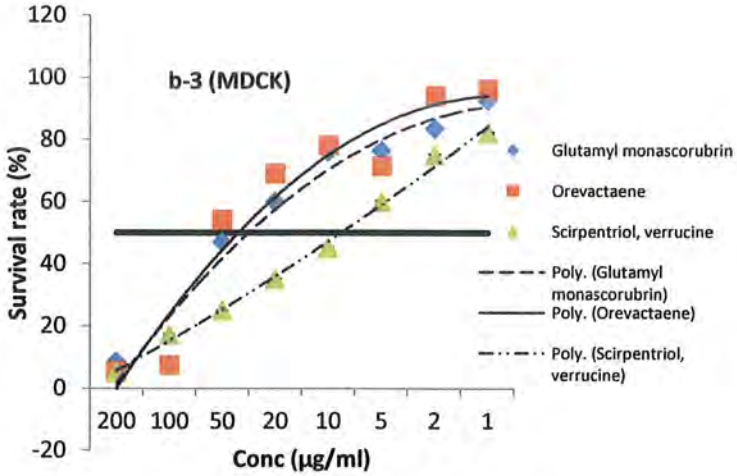

a-2 (KA3IT)
Survival rate (%)
120
100
80
60
40
20
0
-20
200 100 50 20 10 5 2 1
Conc (μg/ml)
Monascin
Analogue monascorubrin
Monascorubrin
50%
Poly. (Monascin)
Poly. (Analogue monascorubrin)
Poly. (Monascorubrin)

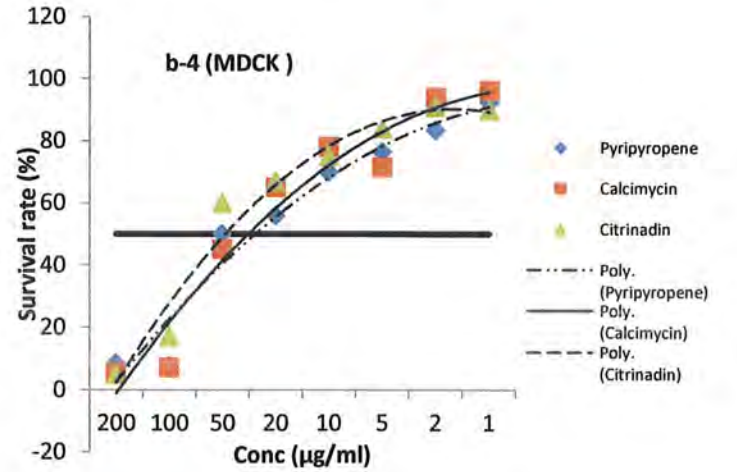

a-3 (KA3IT)
Survival rate (%)
120
100
80
60
40
20
0
-20
200 100 50 20 10 5 2 1
Conc (μg/ml)
Glutamyl monascorubrin
Scirpentriol, verrucine
Orevactaene
Poly. (Glutamyl monascorubrin)
Poly. (Scirpentriol, verrucine)
Poly. (Orevactaene)

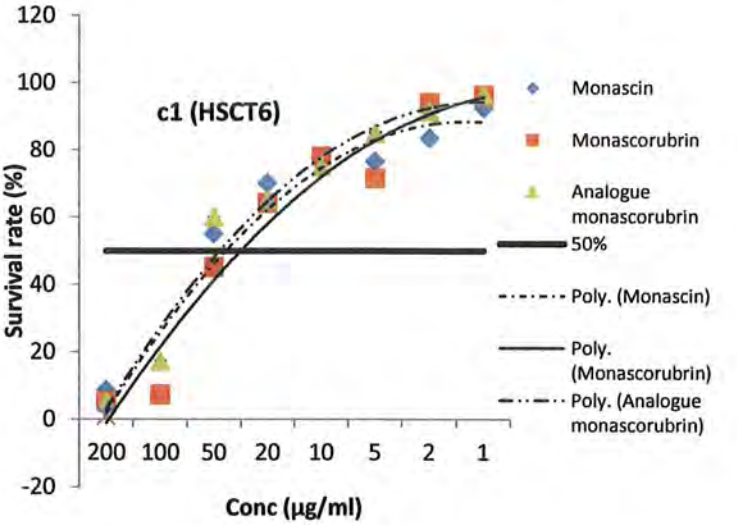

a-4 (KA3IT)
Survival rate (%)
120
100
80
60
40
20
0
-20
200 100 50 20 10 5 2 1
Conc (μg/ml)
Calcimycin
Citrinadin
Pyripyropene
50%
Poly. (Calcimycin)
Poly. (Citrinadin)
Poly. (Pyripyropene)

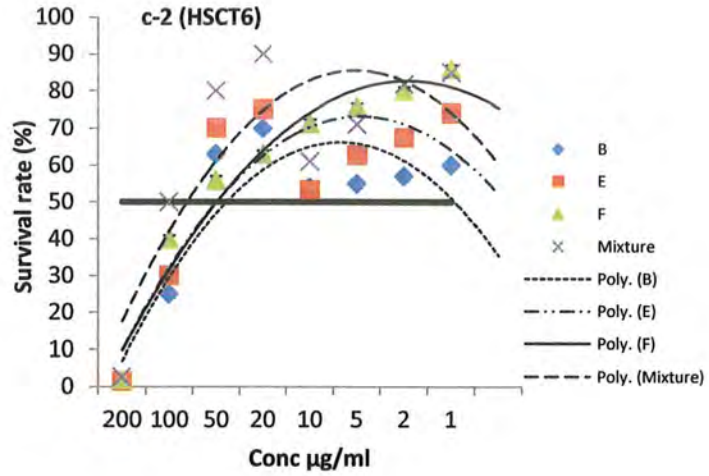

b-1 (MDCK)
Survival rate (%)
120
100
80
60
40
20
0
-20
200 100 50 20 10 5 2 1
Conc (μg/ml)
Monascin
Monascorubrin
Analogue monascorubrin
Poly. (Monascin)
Poly. (Monascorubrin)
Poly. (Analogue monascorubrin)

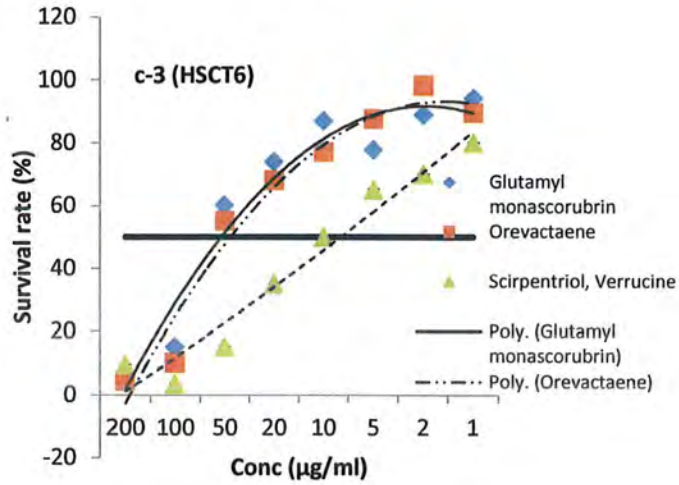

b-2 (MDCK)
Survival rate (%)
100
90
80
70
60
50
40
30
20
10
0
200 100 50 20 10 5 2 1
Conc (μg/ml)
B
E
F
Mixture
Poly. (B)
Poly. (E)
Poly. (F)
Poly. (Mixture)

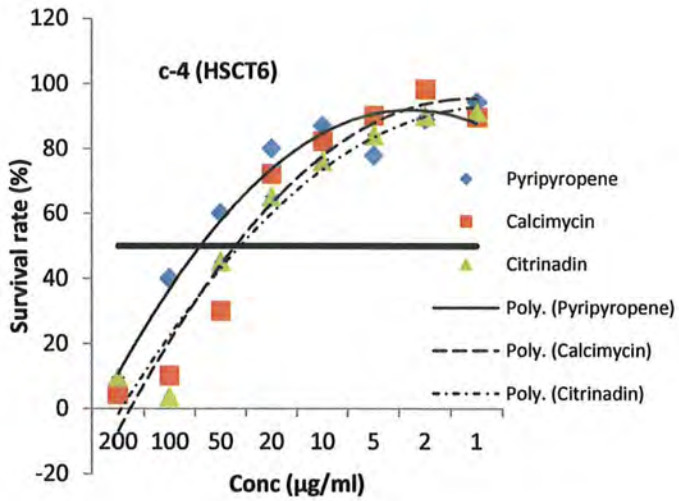

b-3 (MDCK)
Survival rate (%)
120
100
80
60
40
20
0
-20
200 100 50 20 10 5 2 1
Conc (μg/ml)
Glutamyl monascorubrin
Orevactaene
Scirpentriol, verrucine
Poly. (Glutamyl monascorubrin)
Poly. (Orevactaene)
Poly. (Scirpentriol, verrucine)

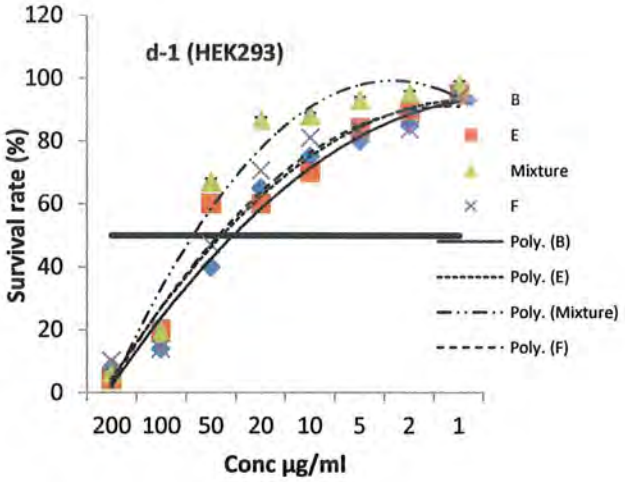

b-4 (MDCK)
Survival rate (%)
120
100
80
60
40
20
0
-20
200 100 50 20 10 5 2 1
Conc (μg/ml)
Pyripyropene
Calcimycin
Citrinadin
Poly. (Pyripyropene)
Poly. (Calcimycin)
Poly. (Citrinadin)

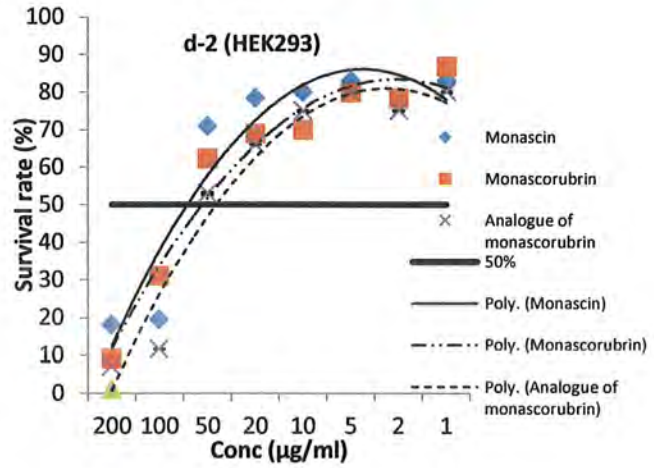

c1 (HSCT6)
Survival rate (%)
120
100
80
60
40
20
0
-20
200 100 50 20 10 5 2 1
Conc (μg/ml)
Monascin
Monascorubrin
Analogue monascorubrin
50%
Poly. (Monascin)
Poly. (Monascorubrin)
Poly. (Analogue monascorubrin)

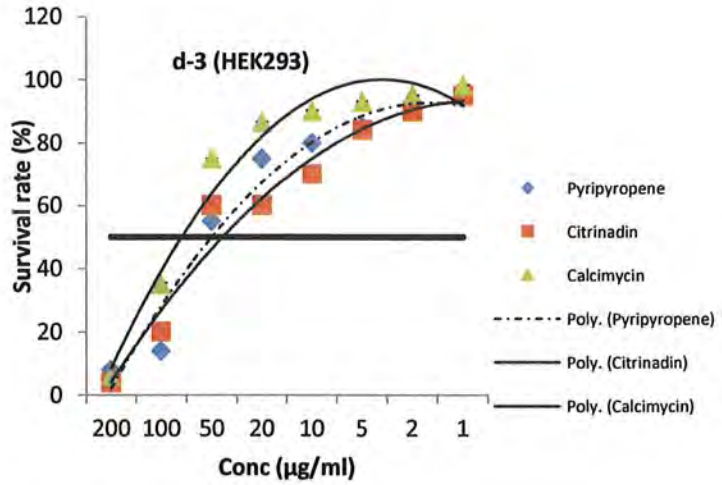

c-2 (HSCT6)
Survival rate (%)
100
90
80
70
60
50
40
30
20
10
0
200 100 50 20 10 5 2 1
Conc µg/ml
B
E
F
Mixture
Poly. (B)
Poly. (E)
Poly. (F)
Poly. (Mixture)

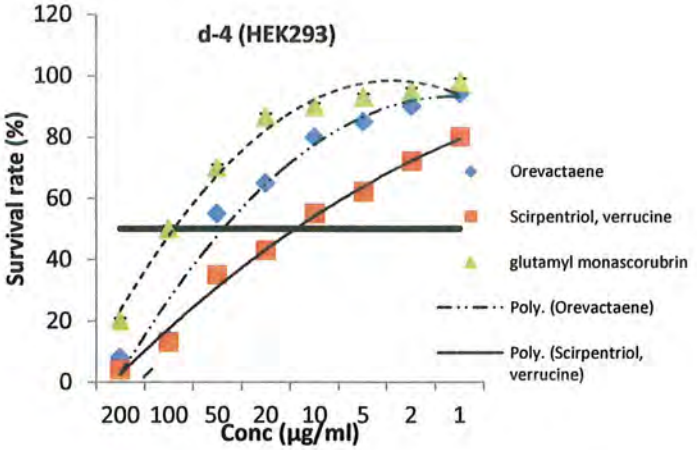

c-3 (HSCT6)
Survival rate (%)
120
100
80
60
40
20
0
-20
200 100 50 20 10 5 2 1
Conc (µg/ml)
Glutamyl monascorubrin
Orevactaene
Scirpentriol, Verrucine
Poly. (Glutamyl monascorubrin)
Poly. (Orevactaene)

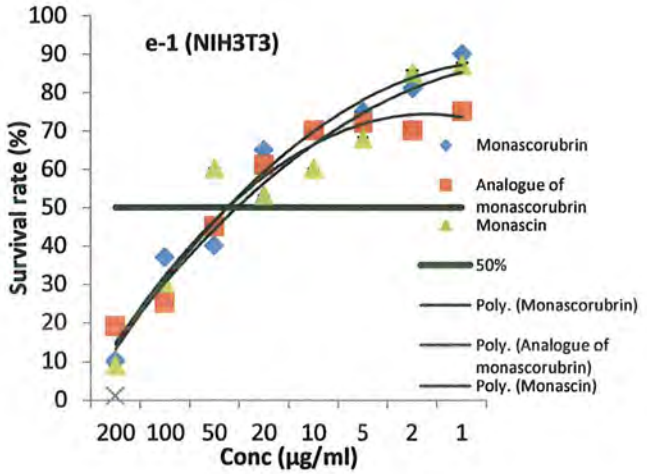

c-4 (HSCT6)
Survival rate (%)
120
100
80
60
40
20
0
-20
200 100 50 20 10 5 2 1
Conc (µg/ml)
Pyripyropene
Calcimycin
Citrinadin
Poly. (Pyripyropene)
Poly. (Calcimycin)
Poly. (Citrinadin)

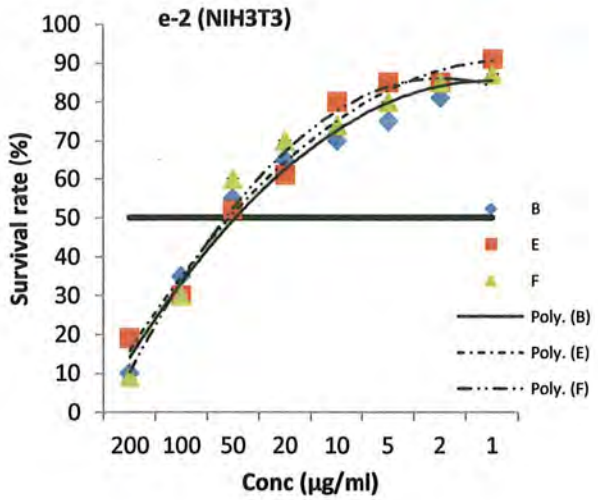

d-1 (HEK293)
Survival rate (%)
120
100
80
60
40
20
0
200 100 50 20 10 5 2 1
Conc µg/ml
B
E
Mixture
F
Poly. (B)
Poly. (E)
Poly. (Mixture)
Poly. (F)


d-2 (HEK293)
Survival rate (%)
100
90
80
70
60
50
40
30
20
10
0
200 100 50 20 10 5 2 1
Conc (µg/ml)
Monascin
Monascorubrin
Analogue of monascorubrin
50%
Poly. (Monascin)
Poly. (Monascorubrin)
Poly. (Analogue of monascorubrin)


d-3 (HEK293)
Survival rate (%)
120
100
80
60
40
20
0
200 100 50 20 10 5 2 1
Conc (µg/ml)
Pyripyropene
Citrinadin
Calcimycin
Poly. (Pyripyropene)
Poly. (Citrinadin)
Poly. (Calcimycin)


d-4 (HEK293)
Survival rate (%)
120
100
80
60
40
20
0
200 100 50 20 10 5 2 1
Conc (μg/ml)
Orevactaene
Scirpentriol, verrucine
glutamyl monascorubrin
Poly. (Orevactaene)
Poly. (Scirpentriol, verrucine)


e-1 (NIH3T3)
Survival rate (%)
100
90
80
70
60
50
40
30
20
10
0
200 100 50 20 10 5 2 1
Conc (μg/ml)
Monascorubrin
Analogue of monascorubrin
Monascin
50%
Poly. (Monascorubrin)
Poly. (Analogue of monascorubrin)
Poly. (Monascin)


e-2 (NIH3T3)
Survival rate (%)
100
90
80
70
60
50
40
30
20
10
0
200 100 50 20 10 5 2 1
Conc (μg/ml)
B
E
F
Poly. (B)
Poly. (E)
Poly. (F)

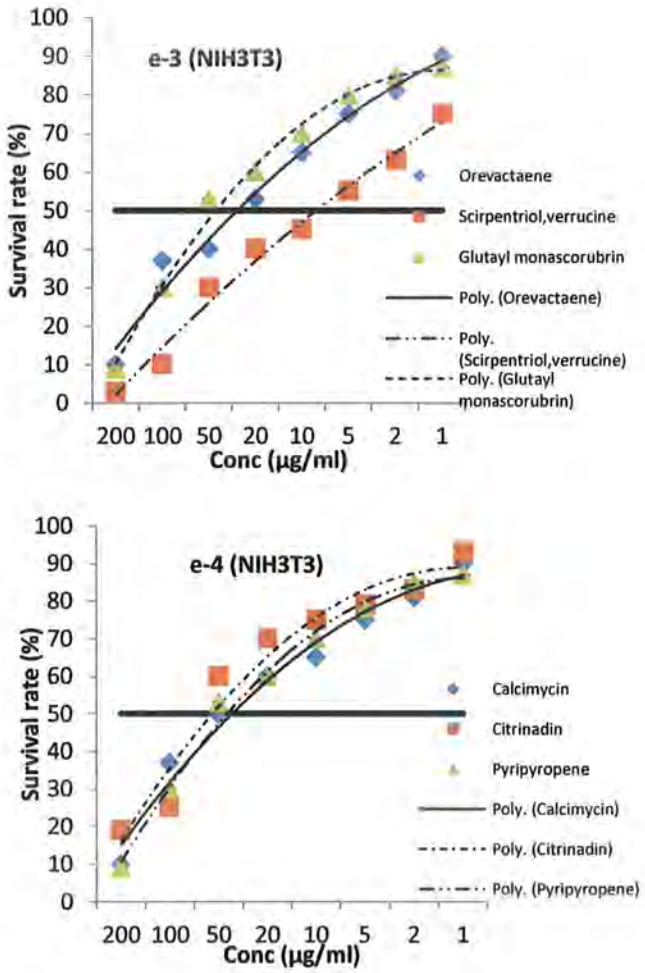


Fig 5.23: Cytotoxicity assays against five different mammalian cell lines i.e., KA3IT (a; Table A27-A30), MDCK (b; Table A31-A33), HSCT6 (c; A34-A36), HEK293 (d; Table A37-A39) and NIH3T3 (d; Table A40-A43) by the different fractions of *Penicillium verruculosum*.

5.1.3 *Chaetomium Strumarium*

A 20g/10 L of the pigmented filtrate of *Chaetomium strumarium* was obtained from modified PDA (C: N 10:1) and further processed through column chromatography technique by using two stationary phases i.e., SP1 (Silica) and SP2 (LH20). Fractions F, E and C containing colored spots when observed through TLC were selected for further processing and analysis. Purple Fractions F-c3-F.c5 obtained after Sephadex column were combined and their preparatory TLC was done in solvent system SST1 mentioned previously. The purple band was scratched and its LCMS analysis showed an intense peak of M^+ 507 m/z in LCMS with major fragments having m/z 184 and

144 corresponding to the structure of Cochliodinol (Fig. 5.24 and 5.25). Fraction C-b was subjected to Sephadex and its 15 sub-fractions were further analyzed by TLC and LCMS as follows; Fraction C-b1-4 obtained and combined was found to contain an intense peak of M^+ m/z 513 which when subjected to LCMSMS showed the fragments with m/z 447, 440, 182 and 138 which correspond to the structure of Dihydromaltophytin (Fig. 5.26). Another red colored spot was observed in TLC of Fraction C-b5-10 which were then combined and their preparatory TLC was done in solvent system SST1 and the red band obtained was scratched for LCMS and was found to contain an intense peak of 382 m/z and the fragments with m/z 179, 158 and 143 which correspond to the structure of Monascorubramine (Fig. 5.27 and 5.28).

Fraction C-b 11-15 were combined after TLC analysis were subjected to LCMS and an intense peak of m/z 402 with major fragments of m/z 328, 282 and 168 was found which corresponded to the structure of Alizarin (Fig. 5.29). A Fraction F-c 6-9 contained two intense peaks, one of m/z 505 which when subjected to LCMSMS showed the fragments with m/z 367 and 303 corresponding to the structure of Tryptoquialanine A (Fig. 5.30) whereas second intense peak of m/z 518 which when subjected to LCMSMS showed the major fragment with m/z 302 which correspond to the structure of Tryptoquialanine B (Fig. 5.31).

Fraction Fc-1-2 was found to contain an intense peak of m/z 436 which when subjected to LCMS showed the fragments with m/z 418 and 359 corresponding to the structure of Paxilline (Fig. 5.32). Whereas, other fractions contained some peaks of compounds with specific molecular weights (m/z) and these were not found in libraries to elucidate compound structures and names. The respective molecular ion peaks observed in LCMS and LCMSMS included 517m/z (Fig. 33), 431m/z (Fig. 34), 378 (Fig. 35), 453 (Fig. 36), 379 (Fig. 37), 677 (MSMS) (Fig. 38), 430 (MSMS) (Fig. 39), 419 (MSMS) (Fig. 40), 654 (MSMS) (Fig. 41), 672 (MSMS) (Fig. 42) 625 (LCMSMS) (Fig. 43) in the fractions Fc-10 , E.a, E.b, E.c, E.e E.f, E.g, F.a-b, F.d, F.e, F.f respectively.

Table 5.3: LCDADMS profile of pigment producing fungi *Chaetomium strumarium*

Chaetomium strumarium				
Retention time	**Fraction**	**m/z value**	**The ions identified annotated m/z signals**	**Figure**
19.02	F.c3-5	507	Cochliodinol	(Fig. 5.25)
11.07	C.b1-4	512	Dihydromaltophytin	(Fig. 5.26)
8.18	C.b5-10	382	Monascorubramine	(Fig. 5.28)
13.36	C.b11-15	402	Alizarin	(Fig. 5.29)
9.98	F.c6-9	505	Tryptoquialanine A	(Fig. 5.30)
10.67	F.c6-9	518	Tryptoquialanine B	(Fig. 5.31)
3.13	F.c1-2	436	Paxilline	(Fig. 5.32)
20.4	F.c10	517	Unknown	(Fig. 5.33)
6.87	E.a	431	Unknown	(Fig. 5.34)
14.43	E.b	478	Unknown	(Fig. 5.35)
13.24	E.c	453	Unknown	(Fig. 5.36)
14.40	E.e	379	Unknown	(Fig. 5.37)
18.06	E.f	677	Unknown	(Fig. 5.38)
12.49	E.g	430	Unknown	(Fig. 5.39)
13.16	F.a-b	419	Unknown	(Fig. 5.40)
4.14	F.d	654	Unknown	(Fig. 5.41)
6.74	F.e	672	Unknown	(Fig. 5.42)
13.81	F.f	625	Unknown	(Fig. 5.43)

5.1.3.1 Cochliodinol

The fractions F-c3-F.c5 were combined and their preparatory TLC was run in SST1 solvent system. The purple band on TLC plate was scratched (Fig. 5.24) and extracted in SST1 again to remove impurities. The sample was dried and dissolved in Methanol for LCMS analysis. An intense peak of 504 m/z was obtained with two major fragments of m/z 144 and 184 confirming this molecular ion peak to be of compound Cochliodinol (Fig. 5.25).

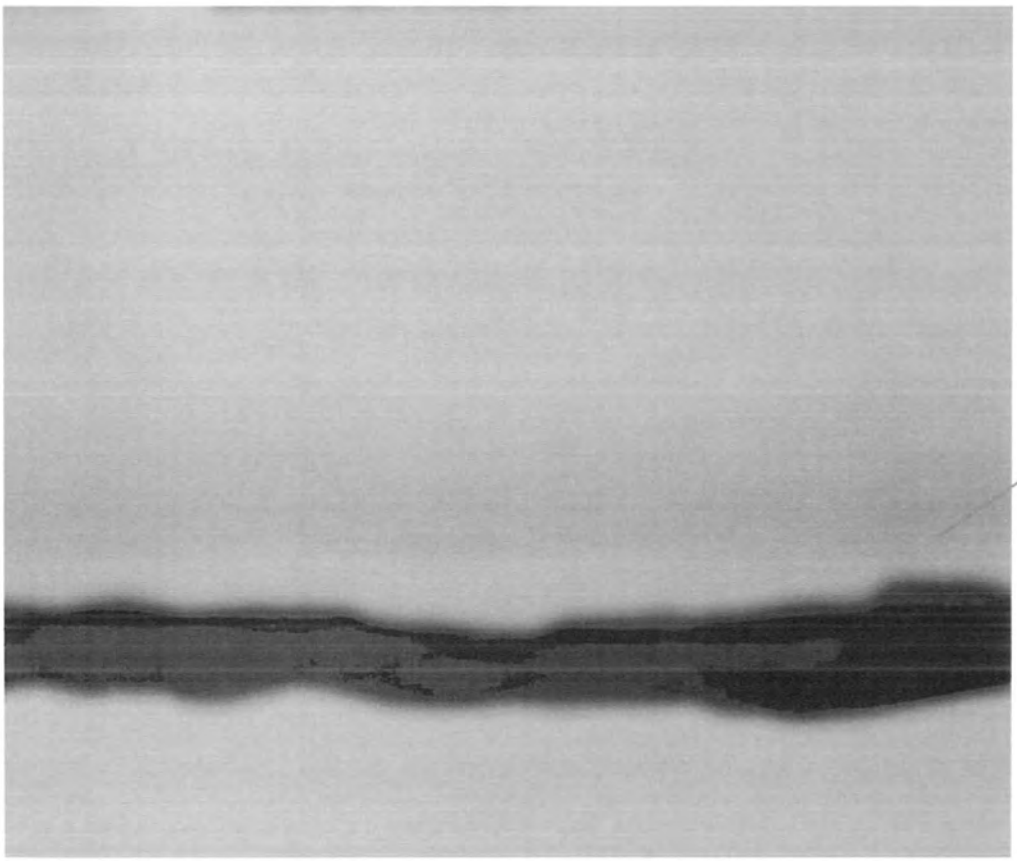


Fig 5.24: Preparatory TLC plate showing visible purple band of Cochliodinol (F-c3-F.c5)

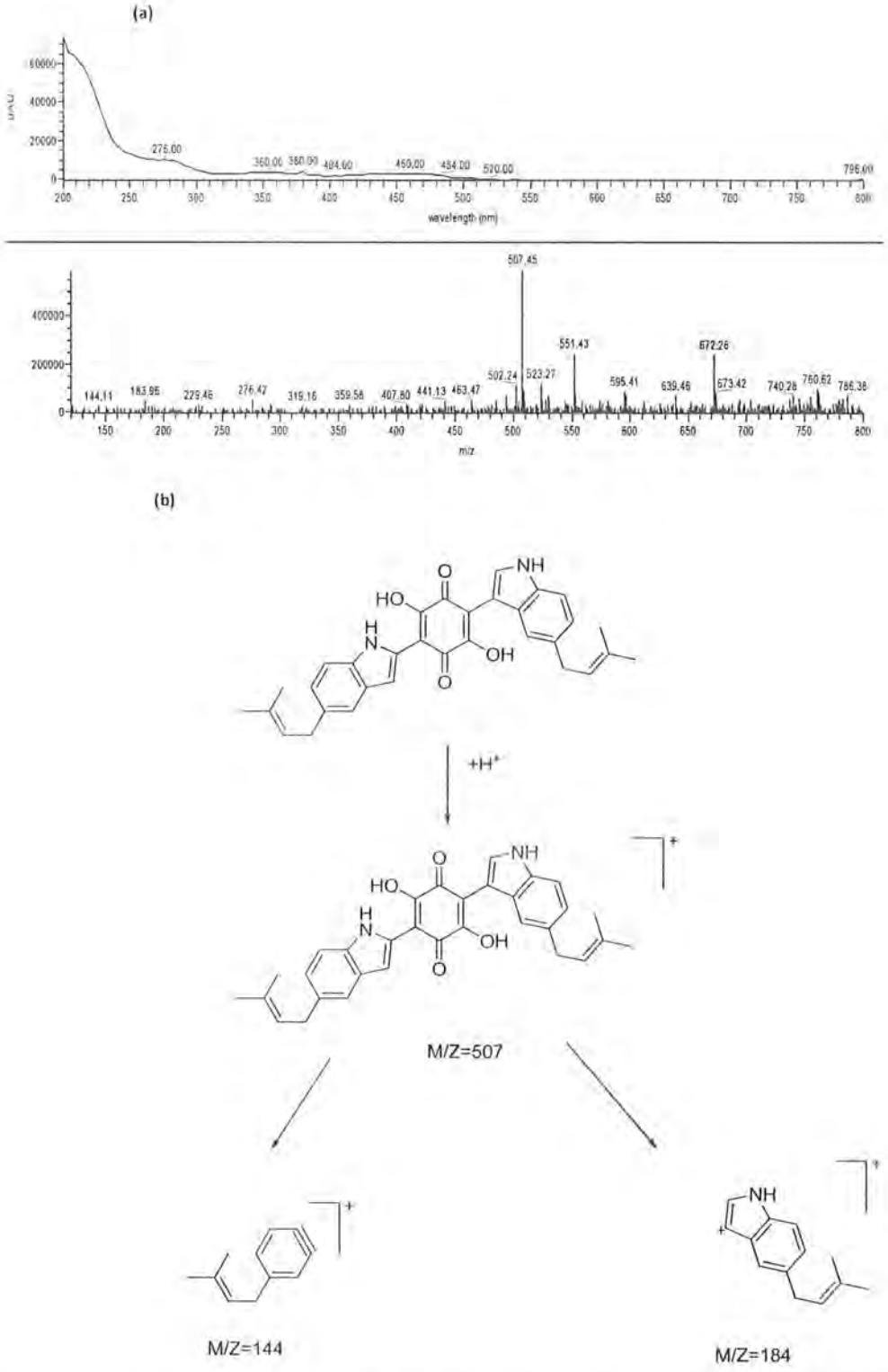


Fig 5.25: Fragmentation pattern of Cochliodiol (b) (FractionF-c3-F.c5) [(Molecular ion peak of M^+ 507 m/z and major fragments having m/z 184 and 144 in LCMS) (a)] corresponding to its structure.

5.1.3.2 Dihydromaltophytin

Fractions C-b1-4 were analyzed on LCMS/MS and showed presence of molecular ion peak 477 m/z which was formed by the loss of two water molecules from Dihydromaltphytin structure i.e., 512 m/z and the major fragments at 440, 182 and 138 confirmed its structure as Dihydromaltophytin (Fig. 5.26).

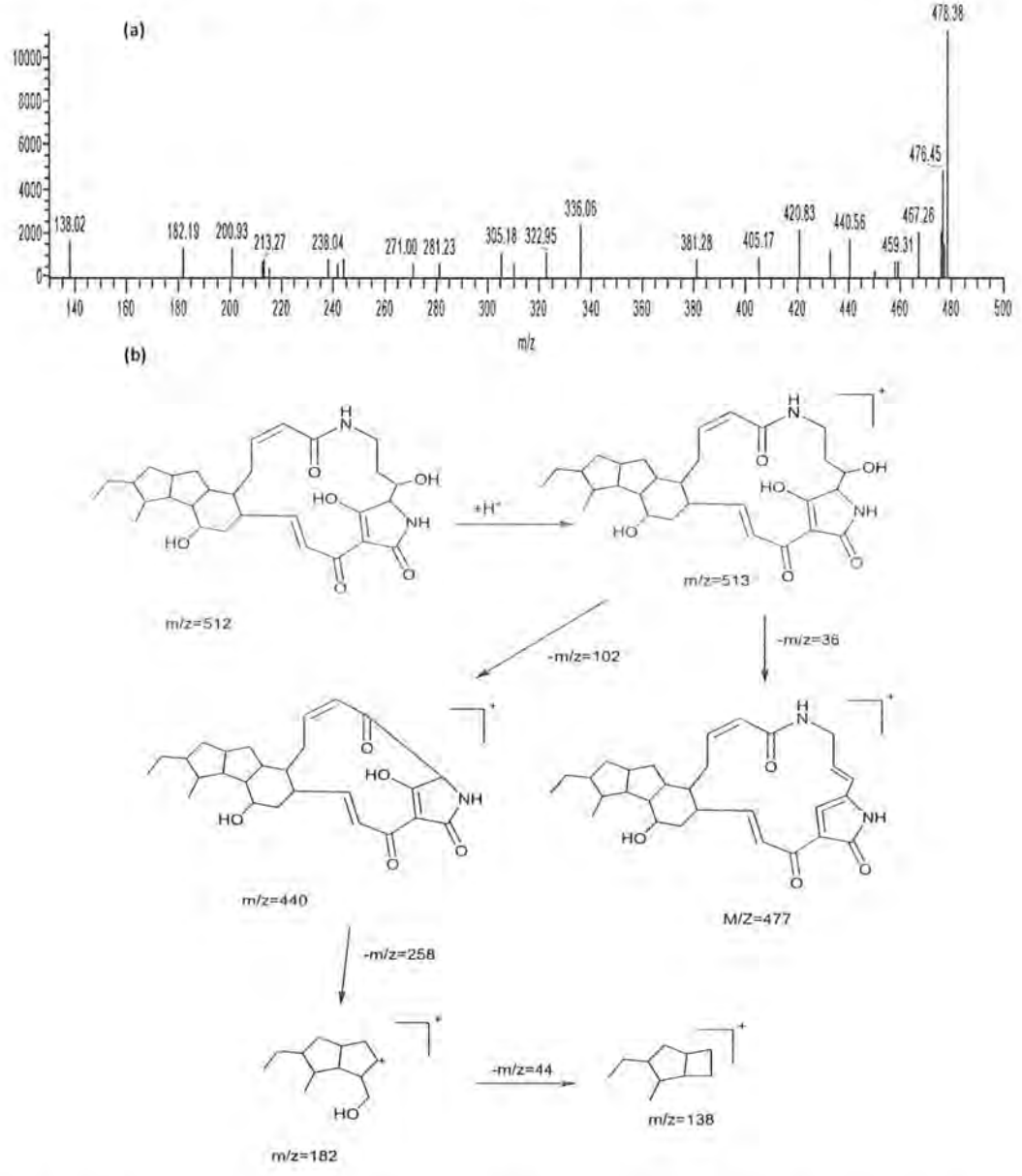


Fig 5.26: Fragmentation pattern of Dihydromaltophytin (b) (Fraction C-b1-4) [(Molecular ion peak of m/z 477 and fragments with m/z 447, 440, 182 and 138 in LCMSMS) (a)] corresponding to its structure.

5.1.3.3 Monascorubramine

Fractions C.b5-10 were combined after TLC analysis to chase red spot visible in all these fractions and their preparatory TLC (SST2) was done and after that the red band (Fig. 5.27) was scratched and analyzed on LCMS. It showed an intense peak of molecular ion 382 m/z and the fragments at 179, 158 and 143. This confirmed its structure to be Monascorubramine (Fig. 5.28).

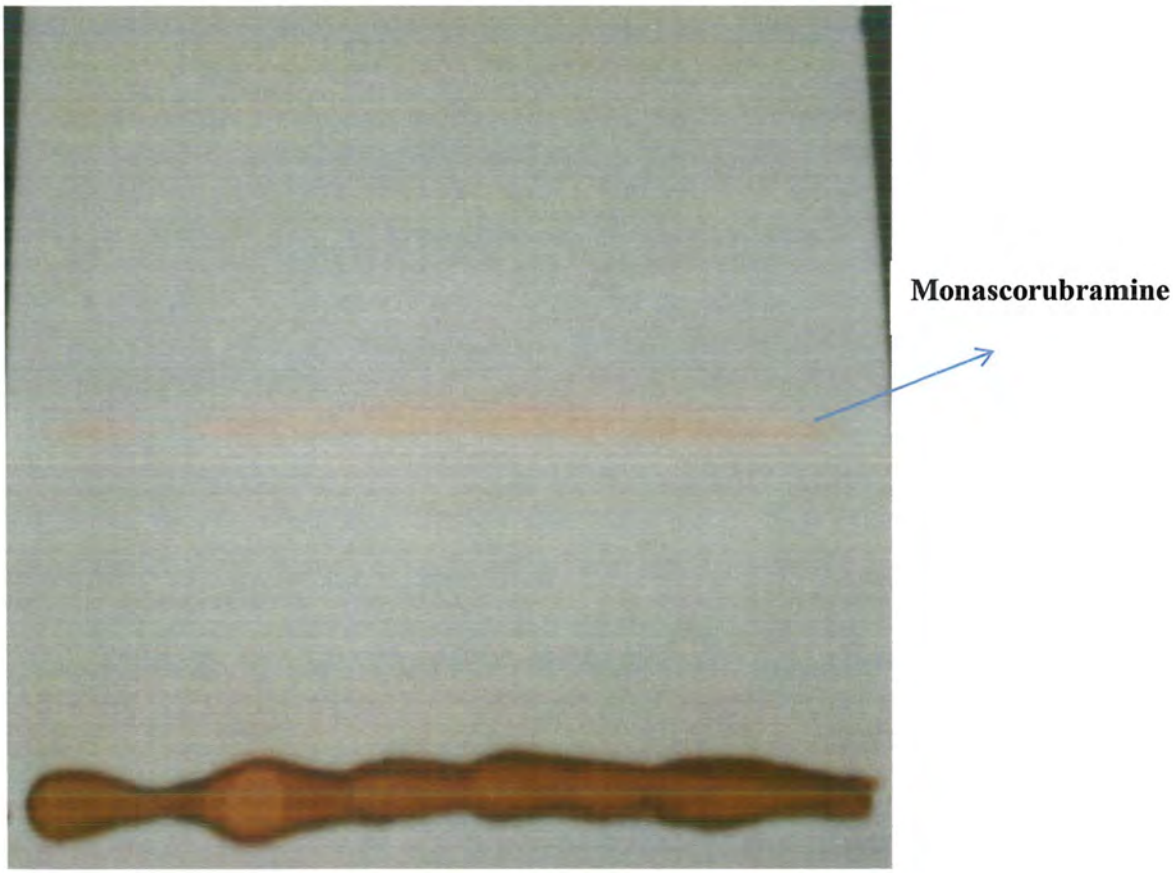


Fig 5.27: Preparatory TLC showing the presence of reddish band of Monascorubramine (C-b5-10)

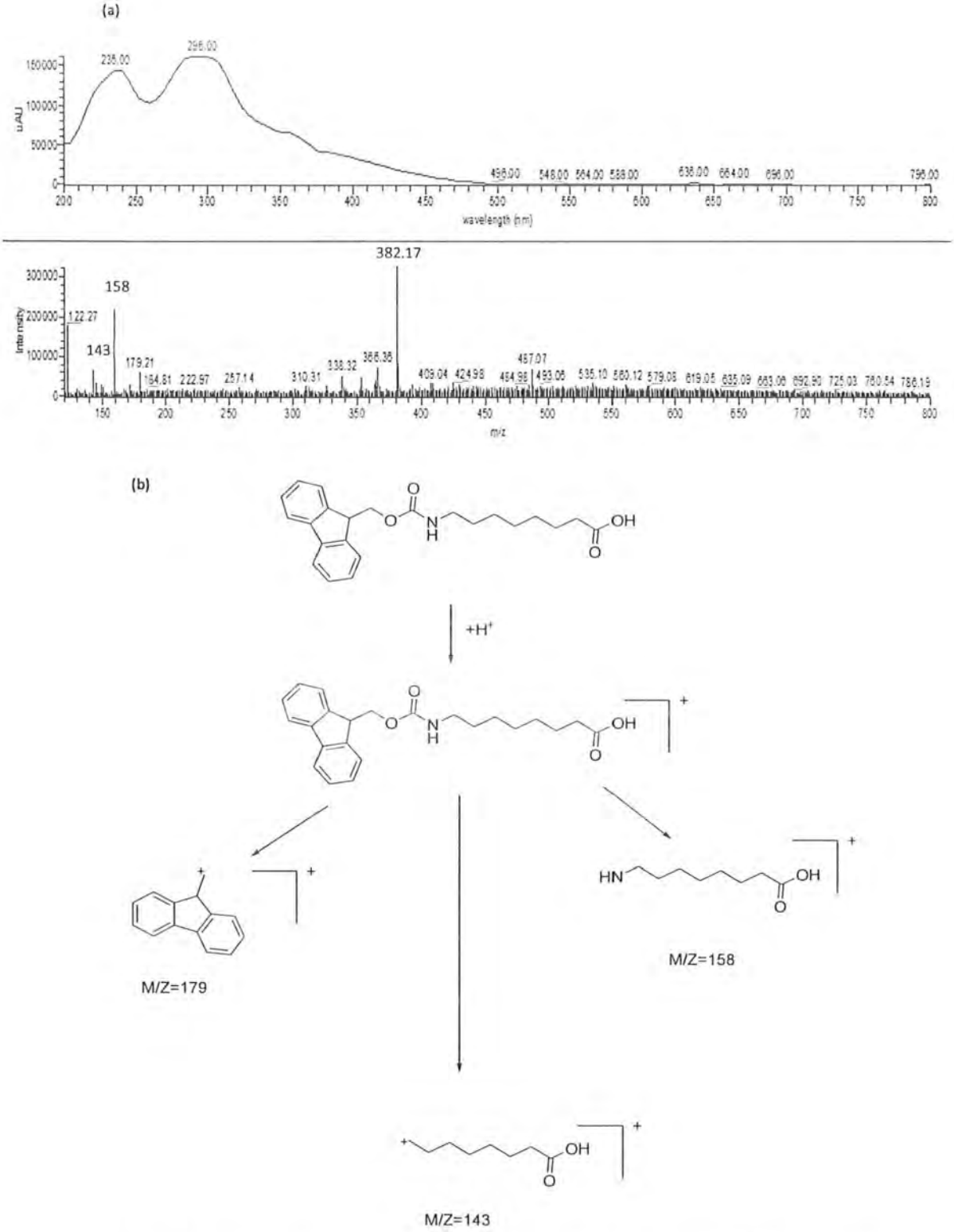


Fig 5.28: Fragmentation pattern of Monascorubramine (b) (Fraction C-b5-10) [(A molecular ion of 382 m/z and different fragments with m/z 179, 158 and 143 in LCMS) (a)] corresponding to its structure.

5.1.3.4 Alizarin

Fractions C-b 11-15 when combined and subjected to LCMS were found to contain an intense peak of m/z 402 and fragments with m/z 328, 282 and 168 which corresponds to the structure of Alizarin (Fig. 5.29).

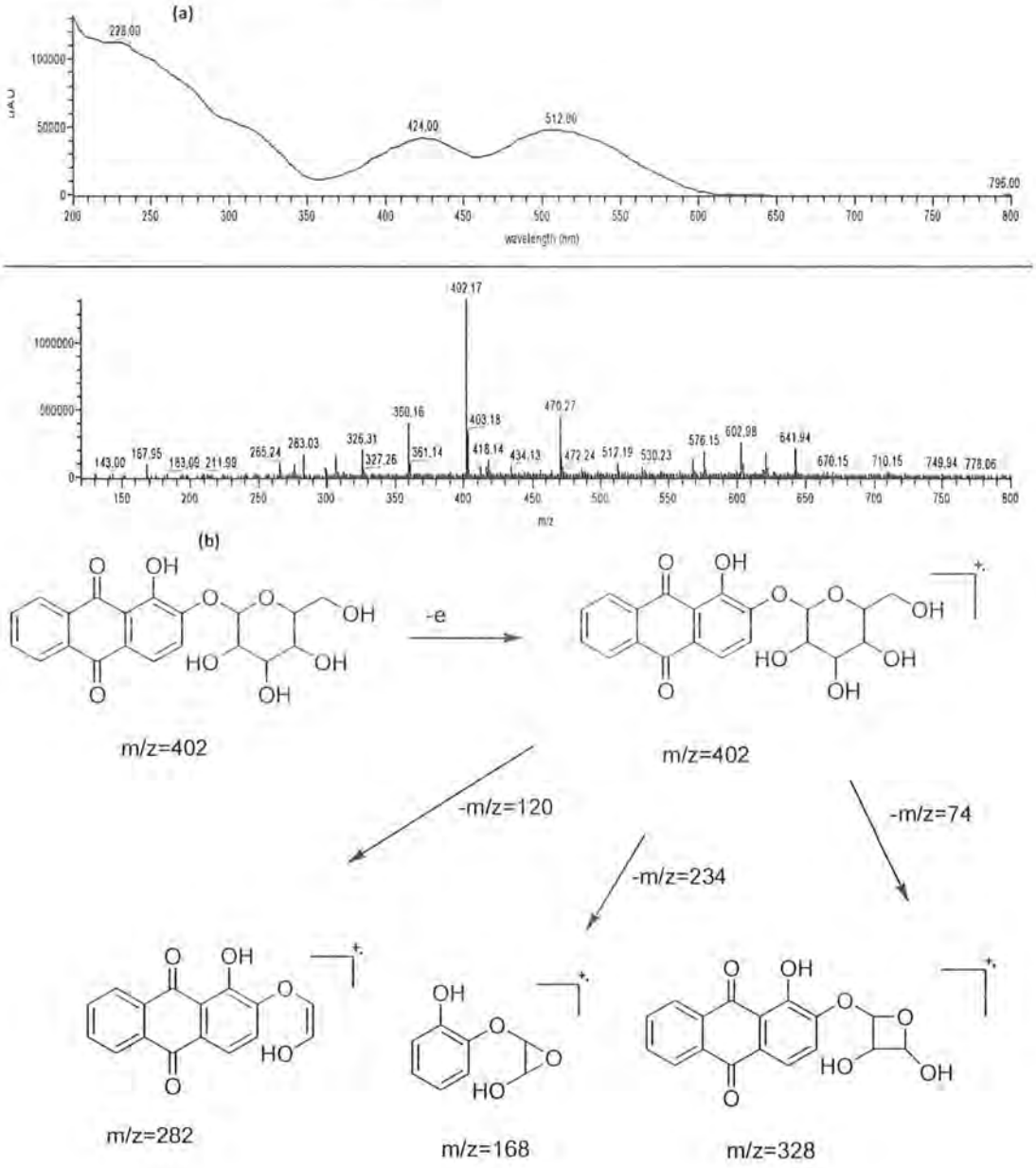


Fig 5.29: Fragmentation pattern of Alizarin (b) (C-B 11-15) [(showing the presence of molecular ion peak 402 m/z and fragments of 328, 282 and 168 m/z in LCMS) (a)] predicting its chemical structure.

5.1.3.5 Tryptoquialanine A.

Fraction F.c 6-9 was found to contain an intense peak of m/z 505 which when subjected to LCMSMS showed the fragments with m/z 367 and 303 which correspond to the structure of Tryptoquialanine A (Fig. 5.30).

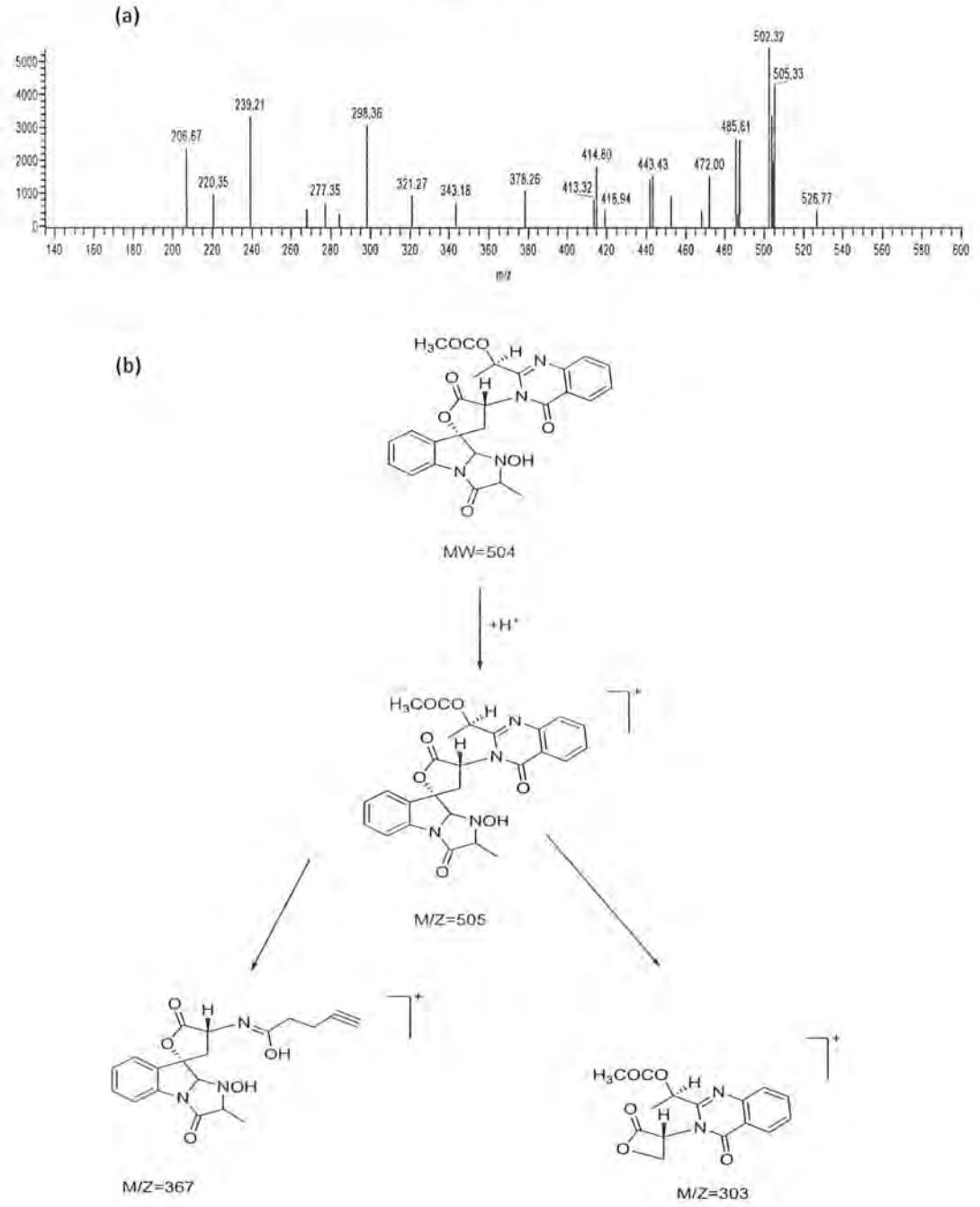


Fig 5.30: Fragmentation pattern of Tryptoquialanine B (F.c 6-9) [showing molecular ion peak 505 m/z and two major fragments m/z 367 and 303 in LCMS(a)] predicting its structure.

5.1.3.6 Tryptoquialanine B

Fraction F.c6-9 when combined and processed through LCMSMS was found to contain an intense peak of m/z 518 which when subjected to LCMSMS showed the major fragment with m/z 302 corresponding to the structure of Tryptoquialanine B (Fig. 5.31).

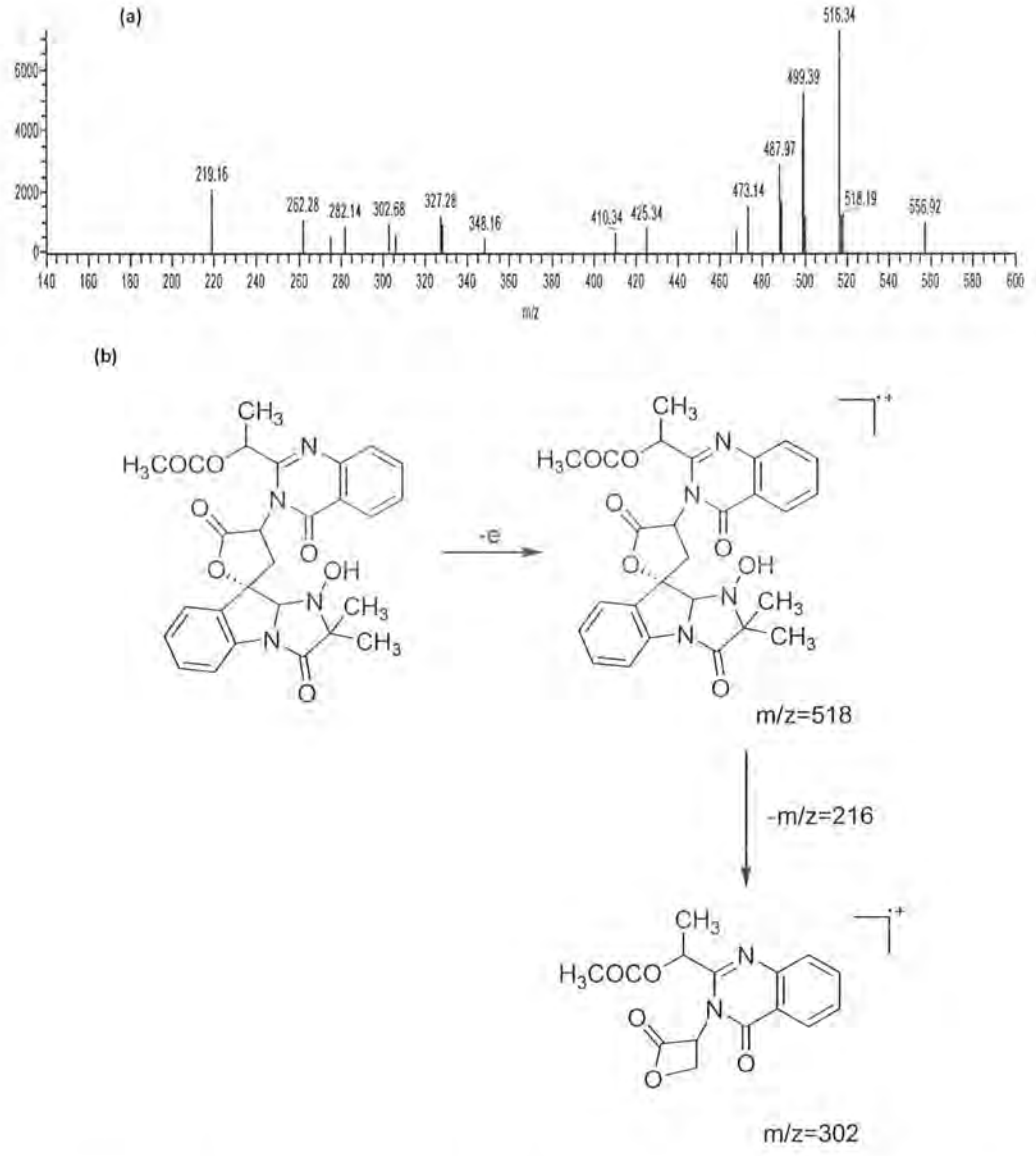


Fig 5.31: Fragmentation pattern of Tryptoquialanine B (b) (F.c6-9) [(showing presence of molecular ion peak m/z 518 and the major fragment m/z 302 in LCMSMS) (a)] predicting its structure.

5.1.3.7 Paxilline

Fraction Fc 1-2 was found to contain an intense peak of m/z 436 which when subjected to LCMS showed the fragments with m/z 418 and 359 corresponding to structure of Paxilline (Fig. 5.32).

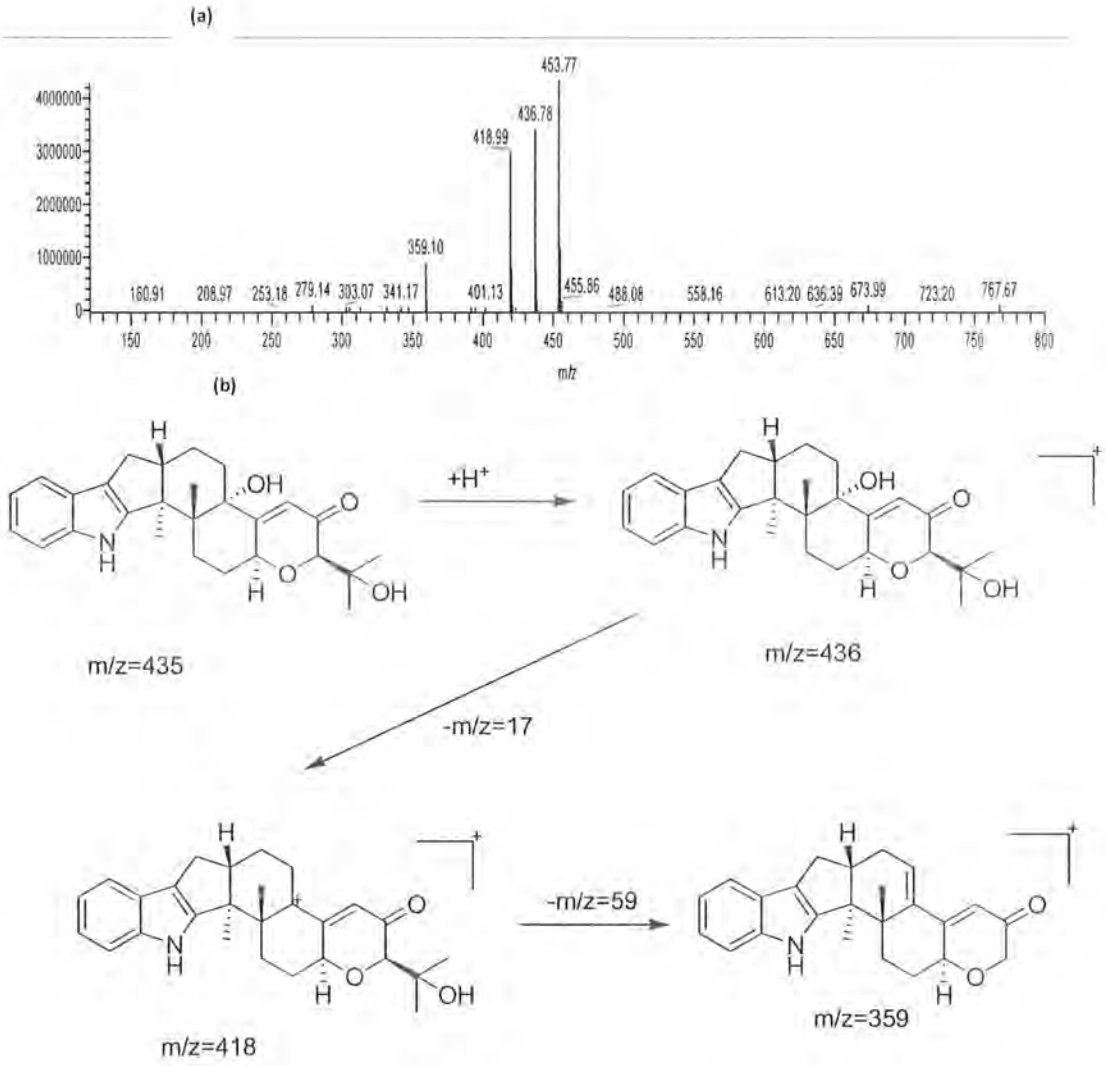


Fig 5.32: Fragmentation pattern of Paxilline (Fraction Fc1-2) (showing presence of molecular ion peak 436 m/z and fragments 418 and 359 m/z when subjected to LCMS) predicting its chemical structure.

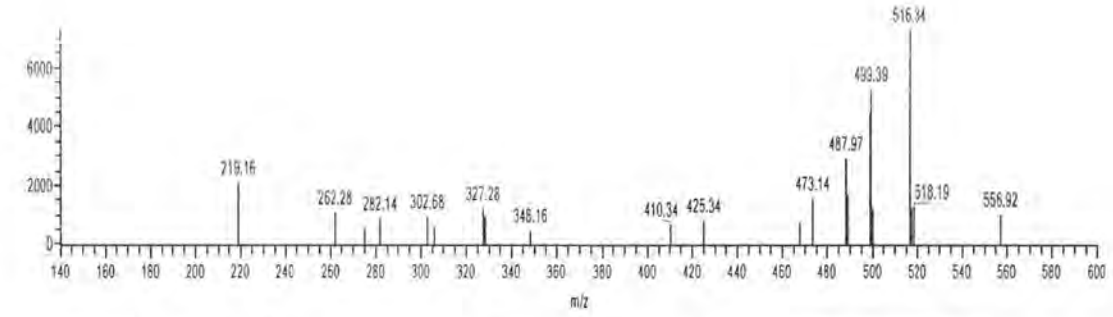


Fig 5.33: Fraction F.c10 obtained after processing of methanolic pigmented filtrate of *Chaetomium strumarium* contained a peak of 517m/z in LCMSMS (Unknown compound).

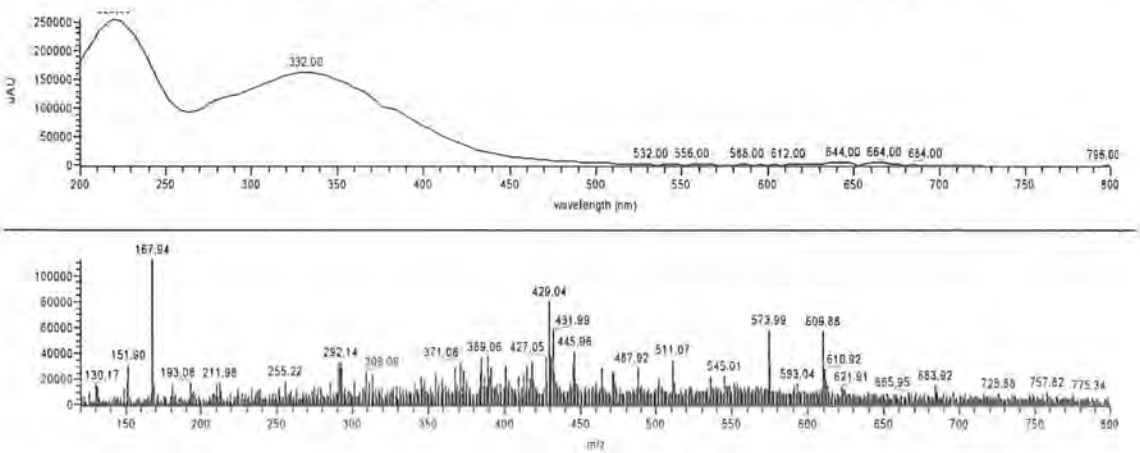


Fig 5.34: Fraction E.a obtained after processing of methanolic pigmented filtrate of *Chaetomium strumarium* contained a peak of m/z 431 in LCMS (Unknown compound).

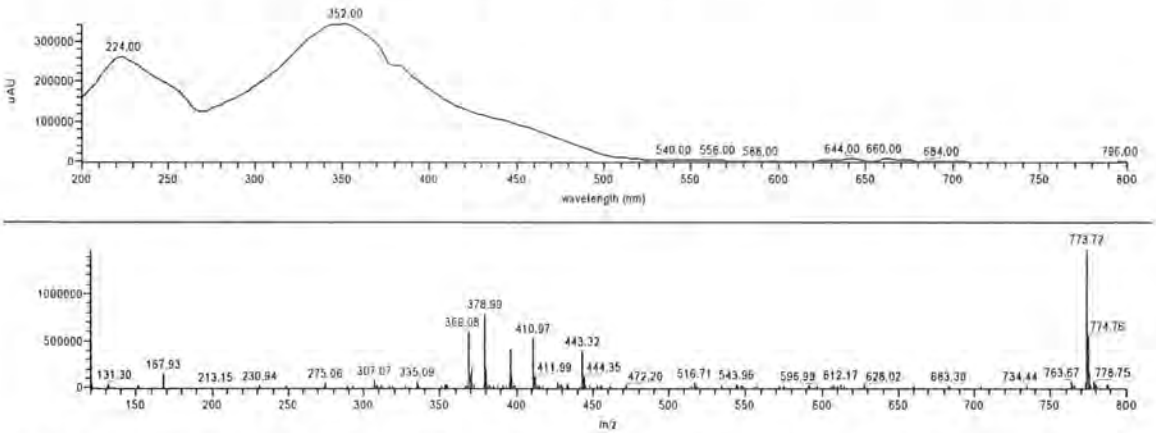


Fig 5.35: Fraction E.b obtained after processing of methanolic pigmented filtrate of *Chaetomium strumarium* contained a peak of m/z 378 in LCMSMS (Unknown compound).

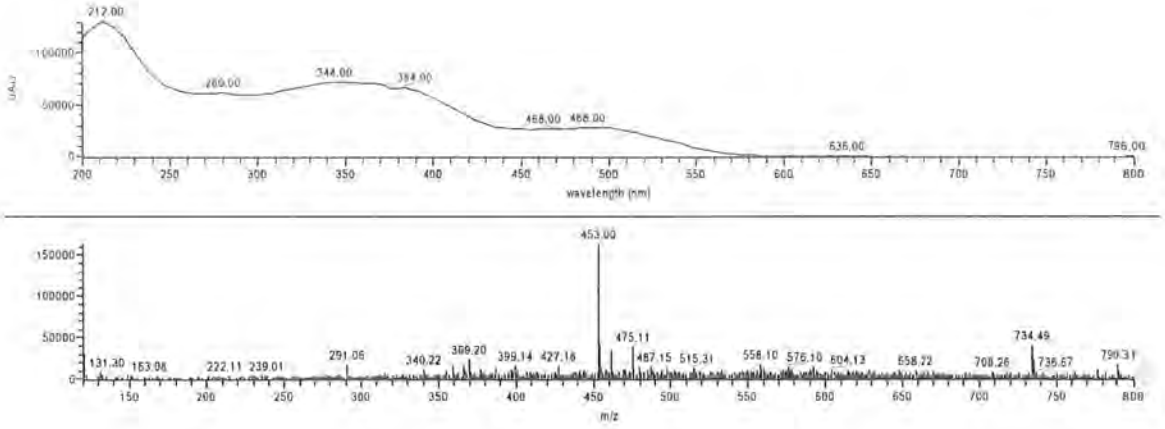


Fig 5.36: Fraction E.c obtained after processing of methanolic pigmented filtrate of *Chaetomium strumarium* contained a peak of m/z 453 in LCMS (Unknown peak).

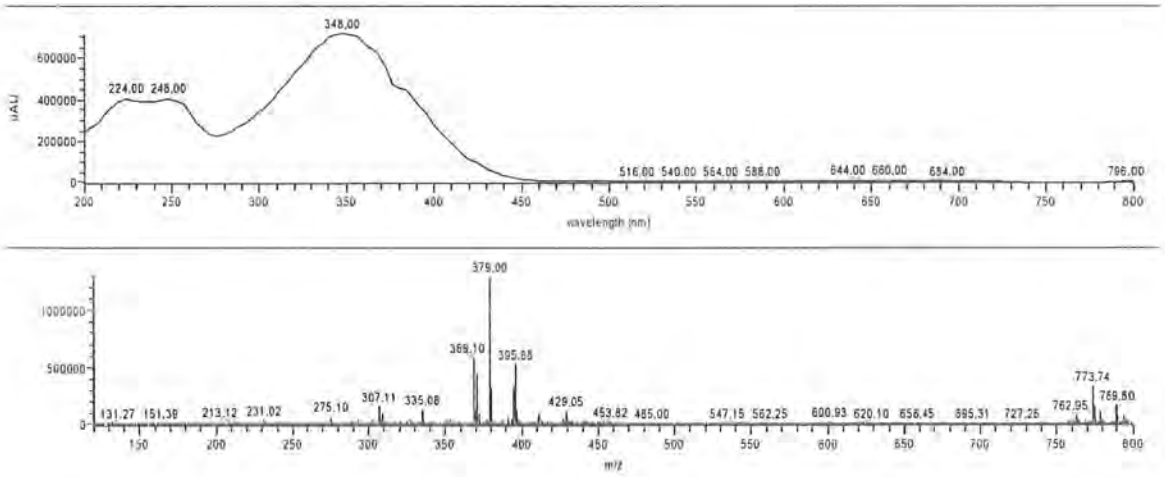


Fig 5.37: Fraction E.e obtained after processing of methanolic pigmented filtrate of *Chaetomium strumarium* contained a peak of m/z 379 in LCMS (Unknown compound).

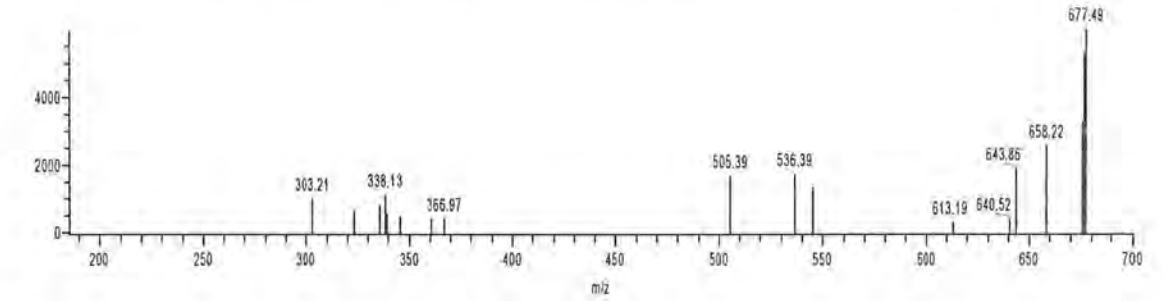


Fig 5.38: Fraction E.f10 obtained after processing of methanolic pigmented filtrate of *Chaetomium strumarium* contained peak of m/z 677 in LCMSMS (Unknown compound).

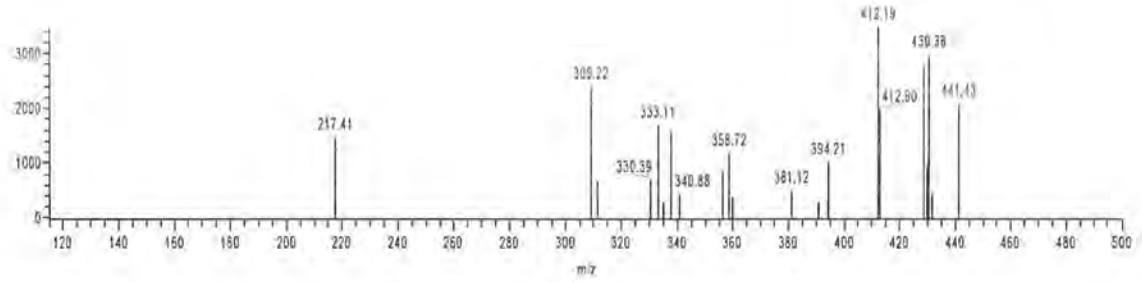


Fig 5.39: Fraction E.g obtained after processing of methanolic pigmented filtrate of *Chaetomium strumarium* contained a peak of m/z 430 in LCMSMS (Unknown compound).

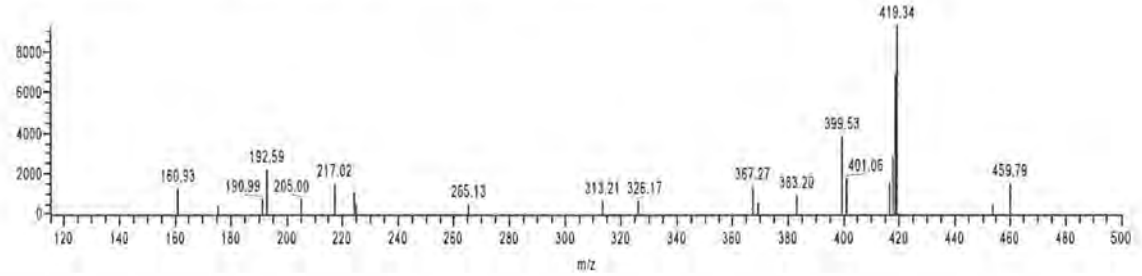


Fig 5.40: Fraction F.a-b obtained after processing of methanolic pigmented filtrate of *Chaetomium strumarium* contained a peak of m/z 419 in LCMSMS (Unknown compound).

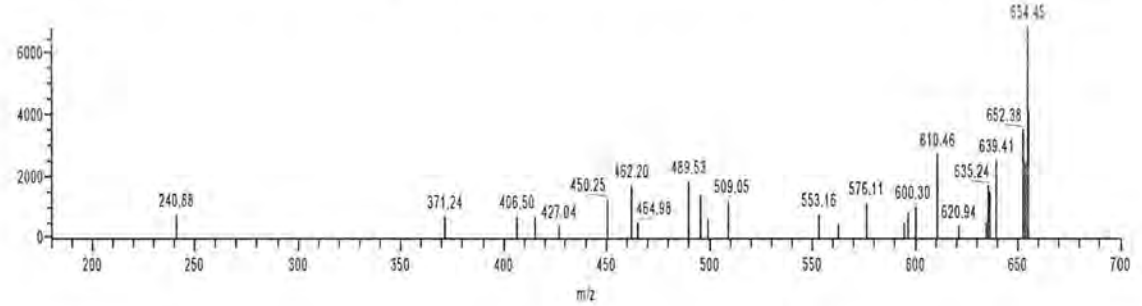


Fig 5.41: Fraction F.d obtained after processing of methanolic pigmented filtrate of *Chaetomium strumarium* contained a peak of m/z 654 in LCMSMS (Unknown compound).

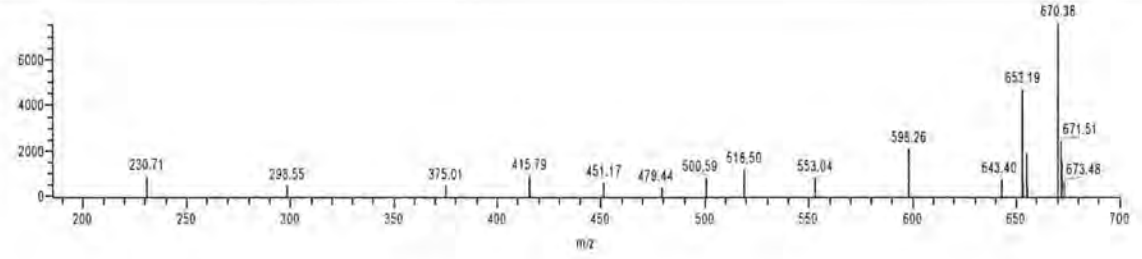


Fig 5.42: Fraction F.e obtained after processing of methanolic pigmented filtrate of *Chaetomium strumarium* contained a peak of m/z 672 in LCMSMS (Unknown compound).

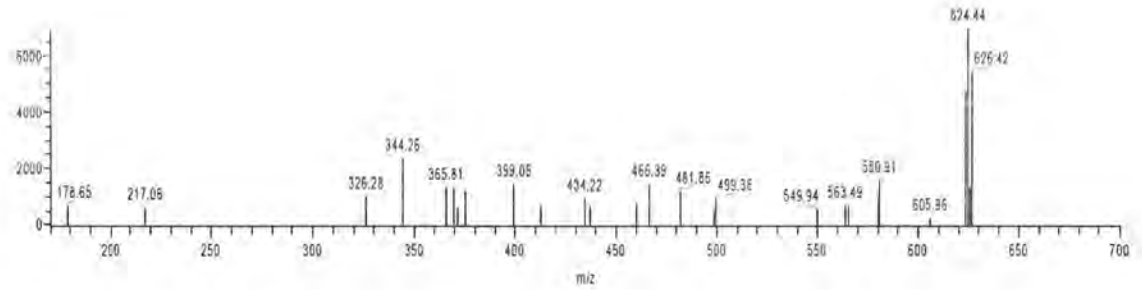


Fig 5.43: Fraction F.f obtained after processing of methanolic pigmented filtrate of *Chaetomium strumarium* contained a peak of m/z 625 in LCMSMS (Unknown compound).

5.1.4 Mammalian cell line cytotoxicity assays of *Chaetomium strumarium* fractions

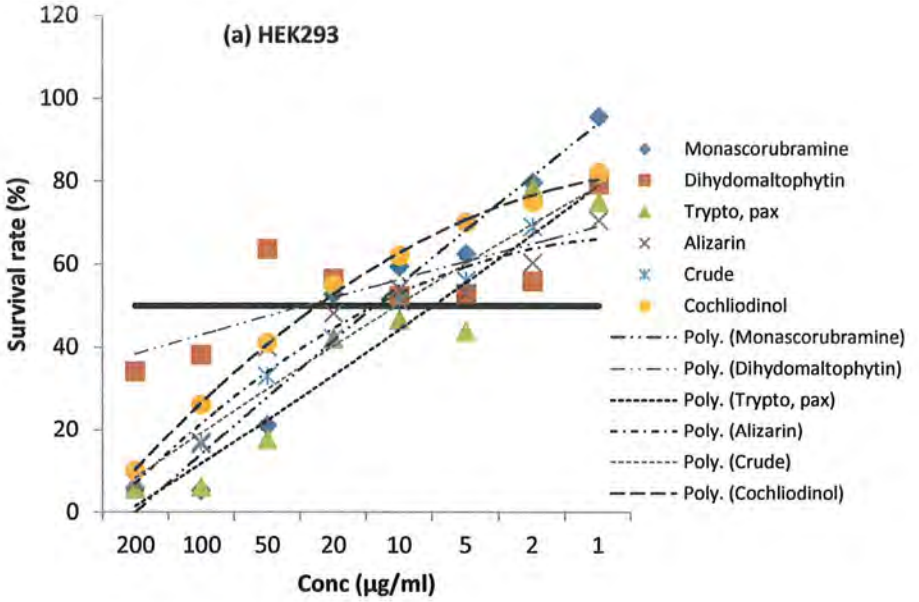
The IC_{50} value showed by various fractions of *Chaetomium strumarium* against KA3IT ranged from 1-50µg/ml whereas, it differed non-significantly from IC_{50} values of all other cell lines except HSCT6 cell line (Table 5.4; A17, A20, A22, A23 & A26). Overall, Dihydropmaltophytin containing fraction had highest IC_{50} (45.2±24.4) value against all cell lines showing its least toxic nature and it differed significantly from Alizarin [(differed non-significantly from Monascorubramine (32±16.4), Cochliodinol (29.2±22.7) and Crude pigmented filtrate (28±20.4)]. Lowest IC_{50} was found to be in fraction containing Nivalenol/Paxilline (32±16.4). The fraction containing Cochliodinol and crude pigmented filtrate were most effective against the cancerous cell line KA3IT and their IC_{50} values differed significantly from Nivalenol/paxilline containing fraction (20.0± 0.34) [(Non-significantly differed from Alizarin (20+ 0.89) and Dihydromaltophytin (20±0.84)]. In case of MDCK cell line, least cytotoxicity was shown by Dihydromaltpphytin containing fraction (73± 0.17) followed by Alizarin (50± 0.45) (differed non significantly from crude) which differed significantly from cytotoxicity of Nivalenol/Paxilline (21.0±0.99) (non-significantly differed from Cochliodinol and Monascorubramine). In case of cytotoxicity to HEK cell line, least cytotoxicity was shown by Dihydromaltophytin (63±0.82) containing fraction followed by Cochliodinol (55± 0.72) >Monascorubramine (20±0.53) >Crude (10± 0.09) (non-significantly differed from Alizarin)>Nivalenol/Paxilline (1.0± 1.5). The cytotoxicity shown against NIH3T3 was least in case of Cochliodinol (50.± 0.59) (non significantly differed from Dihydromaltophytin, Crude and Alizarin) followed by Nivalenol/Paxilline (21.0± 0.48)>Monascorubramine (20± 0.89) (Table 5.4; Fig. 5.44).

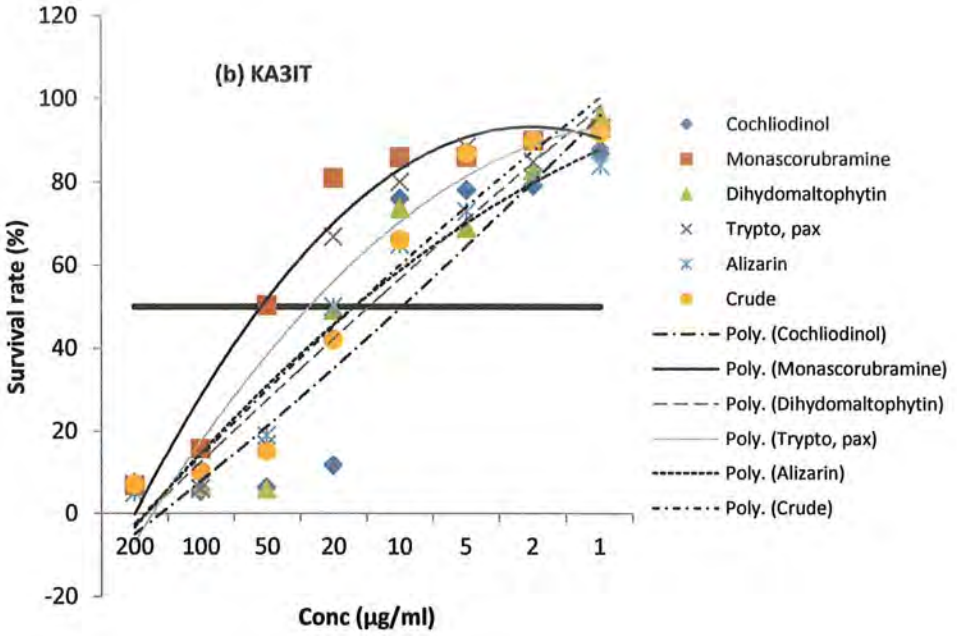
Table 5.4: Cytotoxic effect (IC_{50}) of different compounds and major fractions of culture filtrate of *Chaetomium Strumarium* SG against different normal and cancer cell lines (LSD) (Average values sharing a common letter do not differ significantly, other differ significantly ($p < 0.05$))

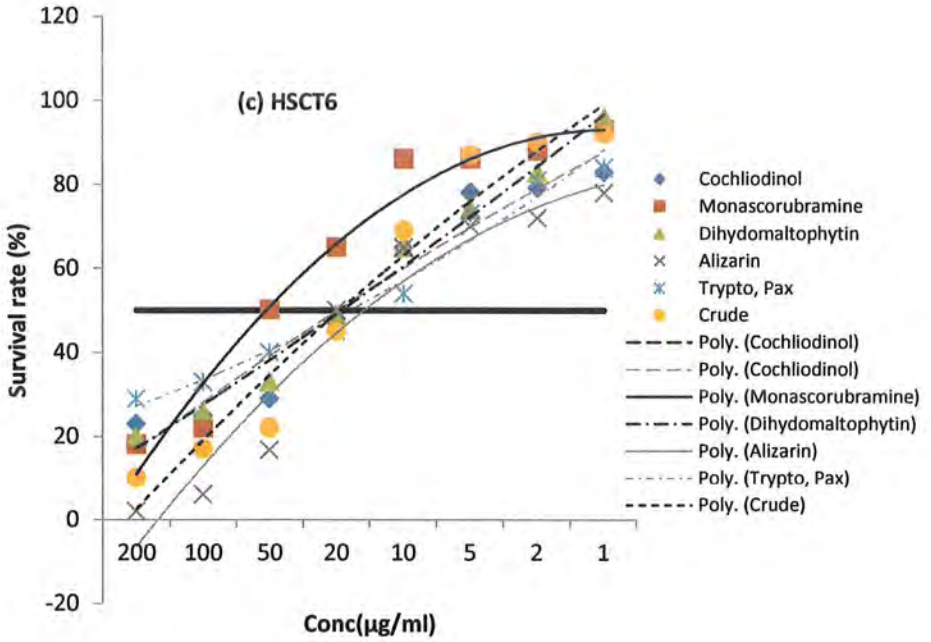
Fungal Filtrate		Mammalian cell lines											
Main fractions	Sub-fractions	KA3IT (µg/ml)		MDCK (µg/ml)		HSCT6 (µg/ml)		HEK (µg/ml)		NIH3T3 (µg/ml)		Combine effect	
		Ave.±SD (n = 3)	R	Ave.±SD (n = 3)	R	Ave.±SD (n = 3)	R	Ave.±SD (n = 3)	R	Ave.±SD (n = 3)	R	Ave. (n = 5)	R
F C	Cochliodinol	10± 1.63	C	20± 0.88	C	20± 0.44	B	55± 0.72	B	50 ± 0.59	A	29.2±22.7	B
	Monascorubramine	50±0.89	A	20±0.54	C	50±0.89	A	20±0.53	C	20± 0.89	B	32±16.4	B
	Dihydromaltophytin	20± 0.84	B	73± 0.17	A	20± 0.89	B	63± 0.82	A	50± 0.72	A	45.2±24.8	A
	Alizarin	20± 0.89	B	50± 0.45	B	20± 0.35	B	10± 0.88	D	50± 0.76	A	36.0±19.4	B
	Tryptoquialanine/Paxilline	20.0± 0.34	B	21.0± 0.99	C	10± 1.2	C	1.0± 1.5	E	21.0± 0.48	B	10.6±9.76	C
Crude culture filtrate		10± 0.44	C	50±0.89	B	20± 0.76	B	10± 0.09	D	50± 0.47	A	28±20.4	B
	Ave. (n = 13) R	23.3±17 B		39.6±26.4 A		23.3±13.6 B		26.5±26.0 B		40.1±15.23 A		30.16±11.6	

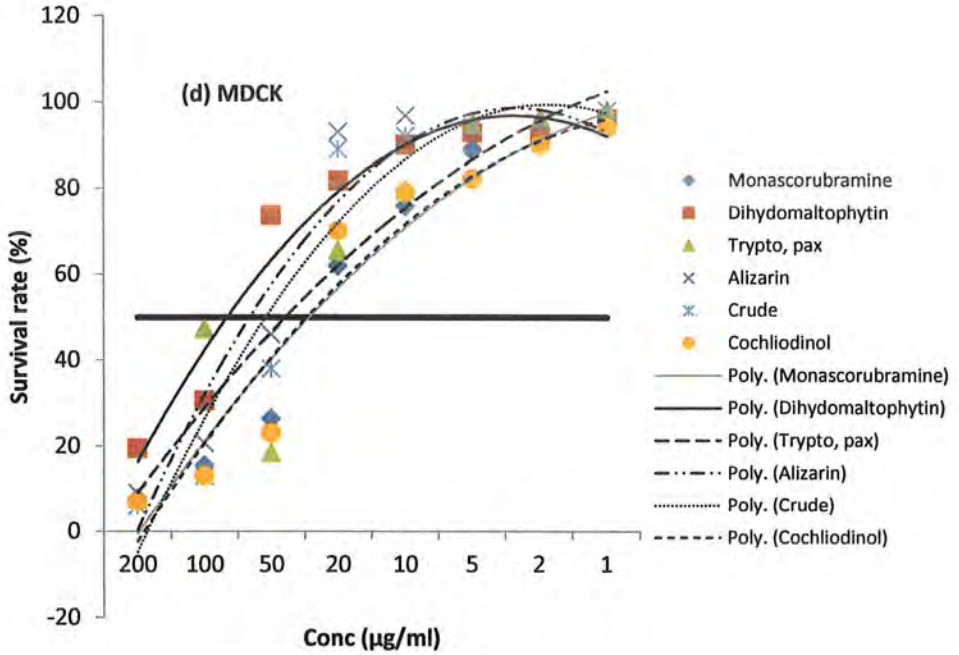
Foot notes: Vertically ranking associated with IC_{50} of different fractions with values against different cell lines. Horizontally ranking depict average of IC_{50} values from all cell lines. IC_{50} was calculated by formula= Treated cell conc (OD)/untreated cell conc. (OD)*100.

LSD = Least Square Difference; R = Rank order; SD = Standard deviation; Ave = Average; NIH3T3 = cells mouse embryonic fibroblasts; HSCT6 cells = rat hepatic stellate cell line; HEK293 = cell line derived from human embryonic kidney cells grown in tissue culture; MDCK Line = Madin-Darby Canine Kidney Epithelial Cells; KA3IT = virally transformed cancerous cell line.


(a) HEK293
Survival rate (%)
120
100
80
60
40
20
0
200 100 50 20 10 5 2 1
Conc (µg/ml)
Monascorubramine
Dihydomaltophytin
Trypto, pax
Alizarin
Crude
Cochliodinol
Poly. (Monascorubramine)
Poly. (Dihydomaltophytin)
Poly. (Trypto, pax)
Poly. (Alizarin)
Poly. (Crude)
Poly. (Cochliodinol)


(b) KA3IT
Survival rate (%)
120
100
80
60
40
20
0
-20
200 100 50 20 10 5 2 1
Conc (µg/ml)
Cochliodinol
Monascorubramine
Dihydomaltophytin
Trypto, pax
Alizarin
Crude
Poly. (Cochliodinol)
Poly. (Monascorubramine)
Poly. (Dihydomaltophytin)
Poly. (Trypto, pax)
Poly. (Alizarin)
Poly. (Crude)


(c) HSCT6
Survival rate (%)
120
100
80
60
40
20
0
-20
200
100
50
20
10
5
2
1
Conc(µg/ml)
Cochliodinol
Monascorubramine
Dihydomaltophytin
Alizarin
Trypto, Pax
Crude
Poly. (Cochliodinol)
Poly. (Cochliodinol)
Poly. (Monascorubramine)
Poly. (Dihydomaltophytin)
Poly. (Alizarin)
Poly. (Trypto, Pax)
Poly. (Crude)


(d) MDCK
Survival rate (%)
120
100
80
60
40
20
0
-20
200
100
50
20
10
5
2
1
Conc (µg/ml)
Monascorubramine
Dihydomaltophytin
Trypto, pax
Alizarin
Crude
Cochliodinol
Poly. (Monascorubramine)
Poly. (Dihydomaltophytin)
Poly. (Trypto, pax)
Poly. (Alizarin)
Poly. (Crude)
Poly. (Cochliodinol)

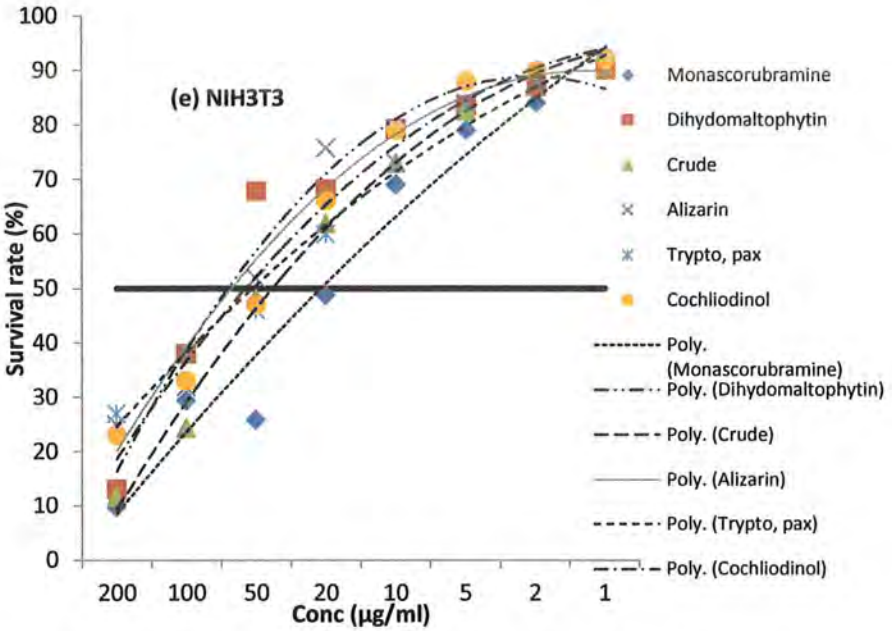


Fig 5.44: Cytotoxicity (IC_{50}) of different fractions *of Chaetomium strumarium* containing important colored and bioactive compounds against five different cell lines i.e., HEK293 (a; Table A17), KA3IT (b; Table A22), HSCT6 (c; Table A20), MDCK (d; Table A23), NIH3T3 (e; Table A26)

5.1.4 *Aspergillus fumigatus*

An about 15 gm/15L of pigmented culture filtrate of fungi *Aspergillus fumigatus* was extracted in Methanol and dried on rotary evaporator. This dried pigmented filtrate was then subjected to column chromatography with two stationary phases i.e., SP1 (Silica), SP2 (LH20) to obtain colored metabolite containing fractions. Fraction B, D and C was selected based on TLC as they contained colored spots. Fraction C obtained after first column run was subjected to C18 column and the fraction C-a containing colored spots were subjected to Sephadex column LH20 and the sub fractions obtained from C.a 1-10 were subjected to LCMS after combining them based on TLC. Fraction C-a 1-4 was found to contain an intense peak of m/z 477 which when subjected to LCMSMS showed the fragments with m/z 478 and 354 which correspond to the structure of Pyrrocidine B (Fig. 5.49). Preparatory TLC of the fractions C.a5-C.a10 contained colored bands (Fig. 5.45). The top most band contained PPR-Monascorubramine which was orange in color (Fig. 5.46). The orange band below PPR-Monascorubramine was scratched and processed through LCMS and contained intense peak of M^+ m/z 307 with major fragment i.e., m/z 289 corresponding to the structure of Fusarubin (Fig. 5.47). Fraction C-a11-15 was found to contain two intense peaks. One is of m/z 526 which when subjected to LCMSMS showed the fragments with m/z 482, 465, 430, 400 and 190 corresponding to the structure of Teritrem (Fig. 5.51). Another intense peak of m/z 311 was subjected to LCMSMS showed the fragments with m/z 281 which corresponds to the structure of Nivalenol (Fig. 5.50).

Fraction B-c obtained after normal phase column chromatography was found to contain an intense peak of m/z 509 which when subjected to LCMSMS showed the fragments with m/z 491 and 242 which correspond to the structure of Communosine g (Fig. 5. 48). Other remaining fractions contained unknown peaks viz fraction B.a-c (343 and 394 m/z) (Fig. 5.53), B.d 326 m/z (Fig. 5.54), D.a (429 m/z) (Fig. 5.55), D.b (376) (Fig. 5.56), D.c (411) (Fig. 5.57), D.d (635) (MSMS) (Fig. 5.58), (616) (MSMS) (Fig. 5.59), D.e (418) (MSMS) (Fig. 5.60), (405) (MSMS) (Fig. 5.61).

Table. 5.5: LCDADMS metabolite profiling of pigment producing fungi *Aspergillus fumigatus*

Aspergilus fumigatus				
Retention time	**Fraction**	**m/z value**	**The ions identified annotated m/z signals**	**Figure**
13.67	C.a5-10	471	PP-R, 7-(2-hydroxyethyl)-Monascorubramine	(Fig. 5.46)
14.47	C.a5-10	307	Fusarubin	(Fig. 5.47)
11.8	B.c	509	Communosine g	(Fig. 5.48)
15.85	C.a1-4	477	Pyrrocidine b	(Fig. 5.49)
9.66	Ca11-15	311	Nivalenol	(Fig. 5.50)
15.23	C.a11-15	526	Teritrem	(Fig. 5.51)
12.6	C.a11-15	324	Sterigmatocystin	(Fig. 5.52)
13.32	B.a-c	394	Unknown	(Fig. 5.53)
9.54	B.a-c	343	Unknown	(Fig. 5.53)
8.24	B.d	326	Unknown	(Fig. 5.54)
12.49	D.a	429	Unknown	(Fig. 5.55)
15.33	D.b	376	Unknown	(Fig. 5.56)
10.47	D.c	411	Unknown	(Fig. 5.57)
14.31	D.d	635	Unknown	(Fig. 5.58)
18.29	D.d	616	Unknown	(Fig. 5.59)
13.04	D.e	418	Unknown	(Fig. 5.60)
10.31	D.e	405	Unknown	(Fig. 5.61)

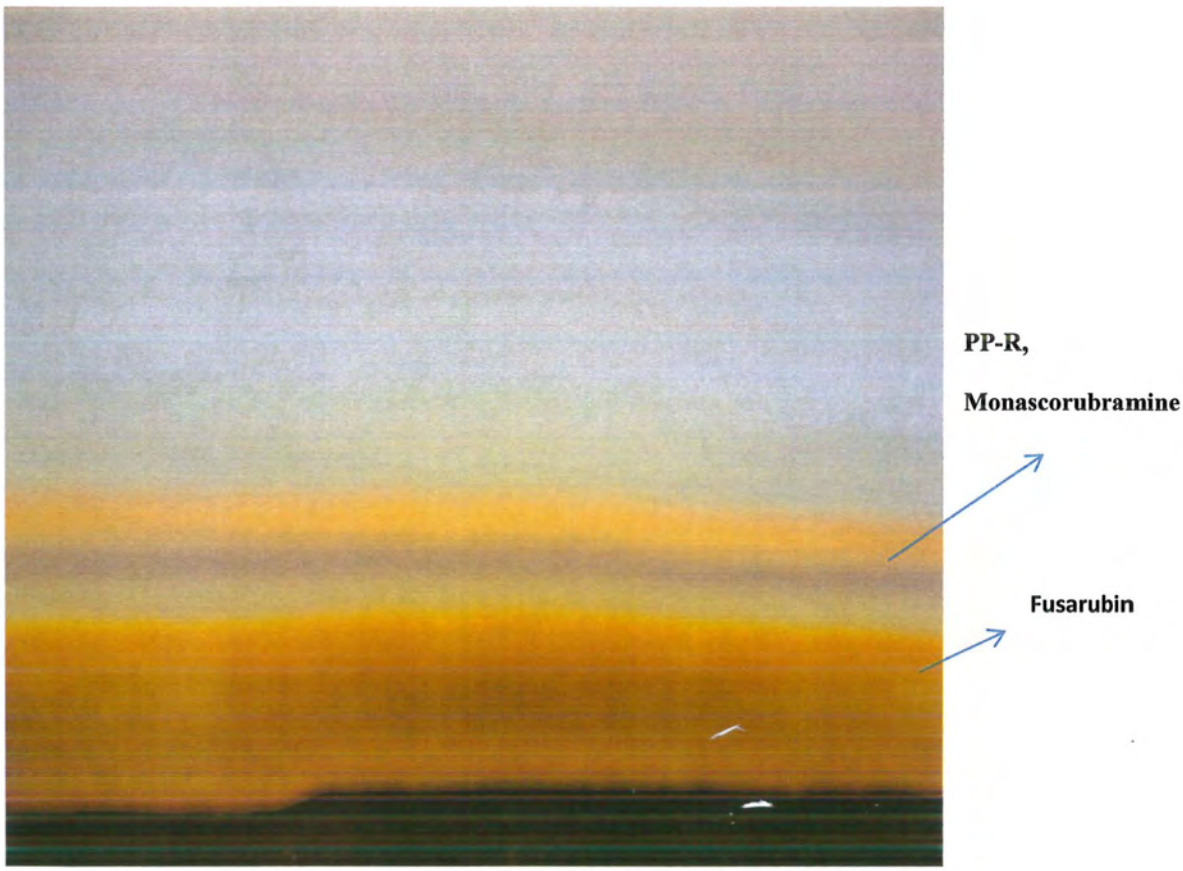


Fig 5.45: Preparatory TLC showing different colored bands of *Aspergillus fumigatus* (C.a5-10)

5.1.4.1 PP-R, 7-(2-hydroxyethyl)-Monascorubramine

Fraction C.a5-10 were combined and their preparatory TLC in solvent system SST1 showed a red band which was scratched from the TLC. Further, it was washed with solvent system SST1 to remove impurities. An intense peak of m/z 471 was seen in LCMSMS with fragments m/z 494 and 291 correspond to the structure of PP-R, 7-(2-hydroxyethyl)-Monascorubramine (Fig. 5.45.and 5.46).

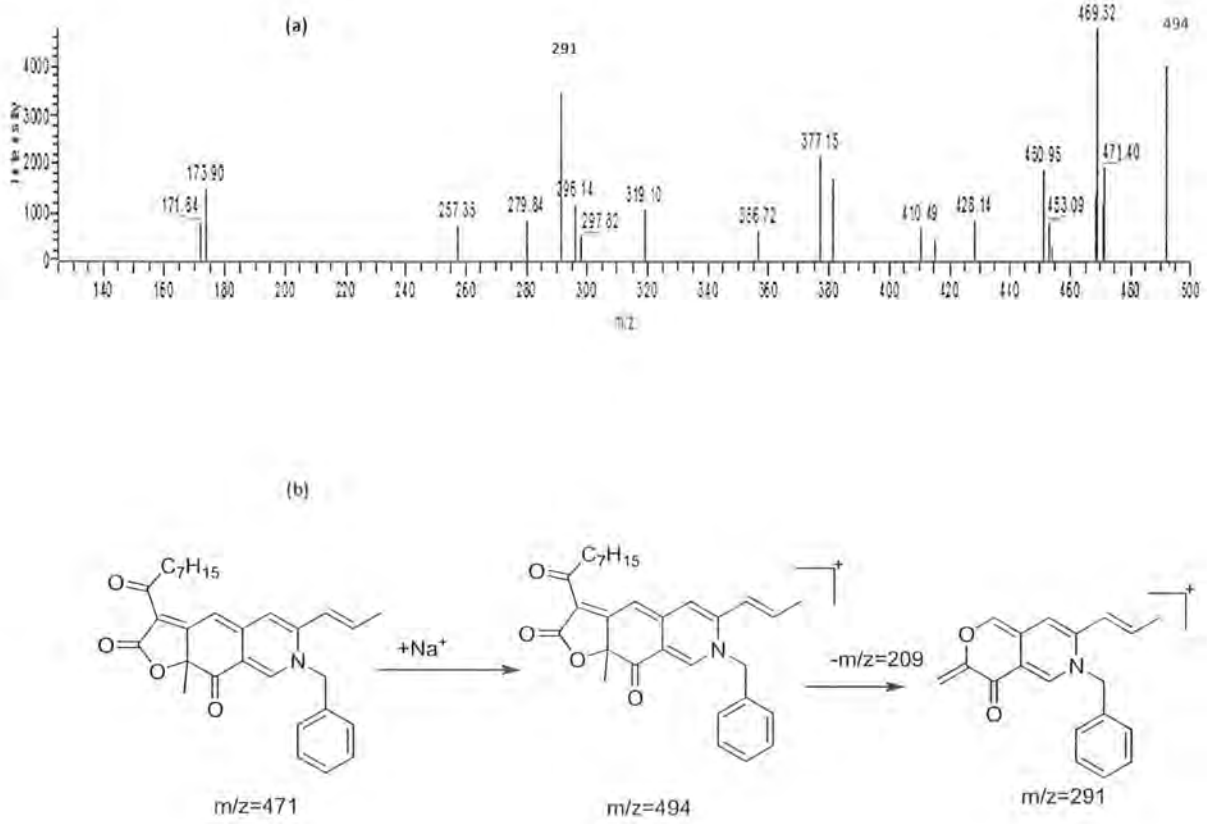


Fig 5.46: Fragmentation pattern of PP-R, 7-(2-hydroxyethyl)-Monascorubramine (b) (C.a5-10) [(showing the presence of molecular ion peak 471 m/z , M+ +Na=494 and major fragment at 291 m/z in LCMS) (a)] predicting its structure.

5.1.4.2 Fusarubin

Fraction C.a5-10 were combined and their preparatory TLC was run in SST1.The band scratched from the TLC and further washed with SST1 solvent system to remove any impurity. This band was found to contain an intense peak of m/z 307 in LCMS and the fragments with m/z 289 corresponded to the structure of Fusarubin (Fig. 5.47).

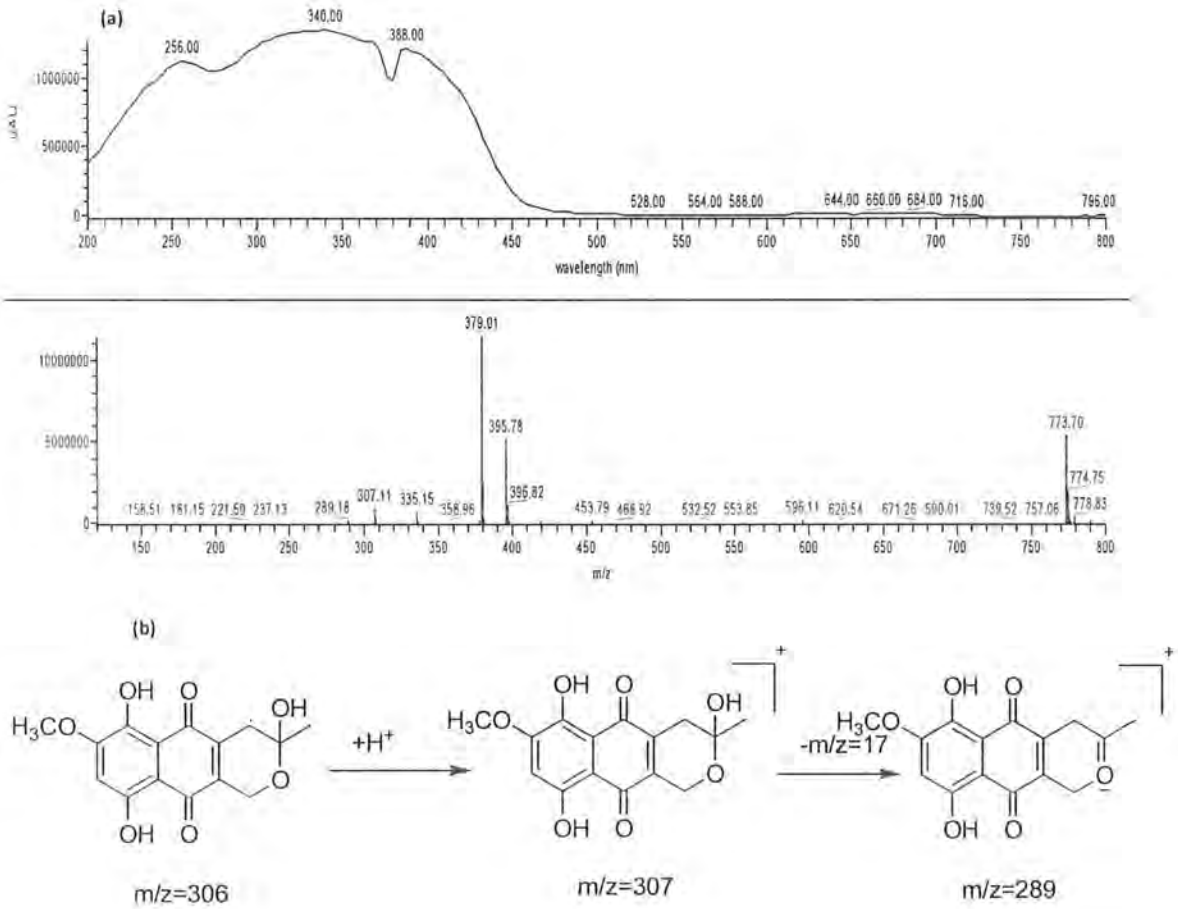


Fig 5.47: Fragmentation pattern of Fusarubin (b) (Fraction C.a5-10) [(showing presence of molecular ion peak m/z 307 and fragment with m/z 289 in LCMS) (a)] predicting its structure.

5.1.4.3 Communosine G

Fraction B-c was found to contain an intense peak of m/z 509 which when subjected to LCMSMS showed the fragments with m/z 491 and 242 which corresponded to the structure of Communosine (Fig. 5.48).

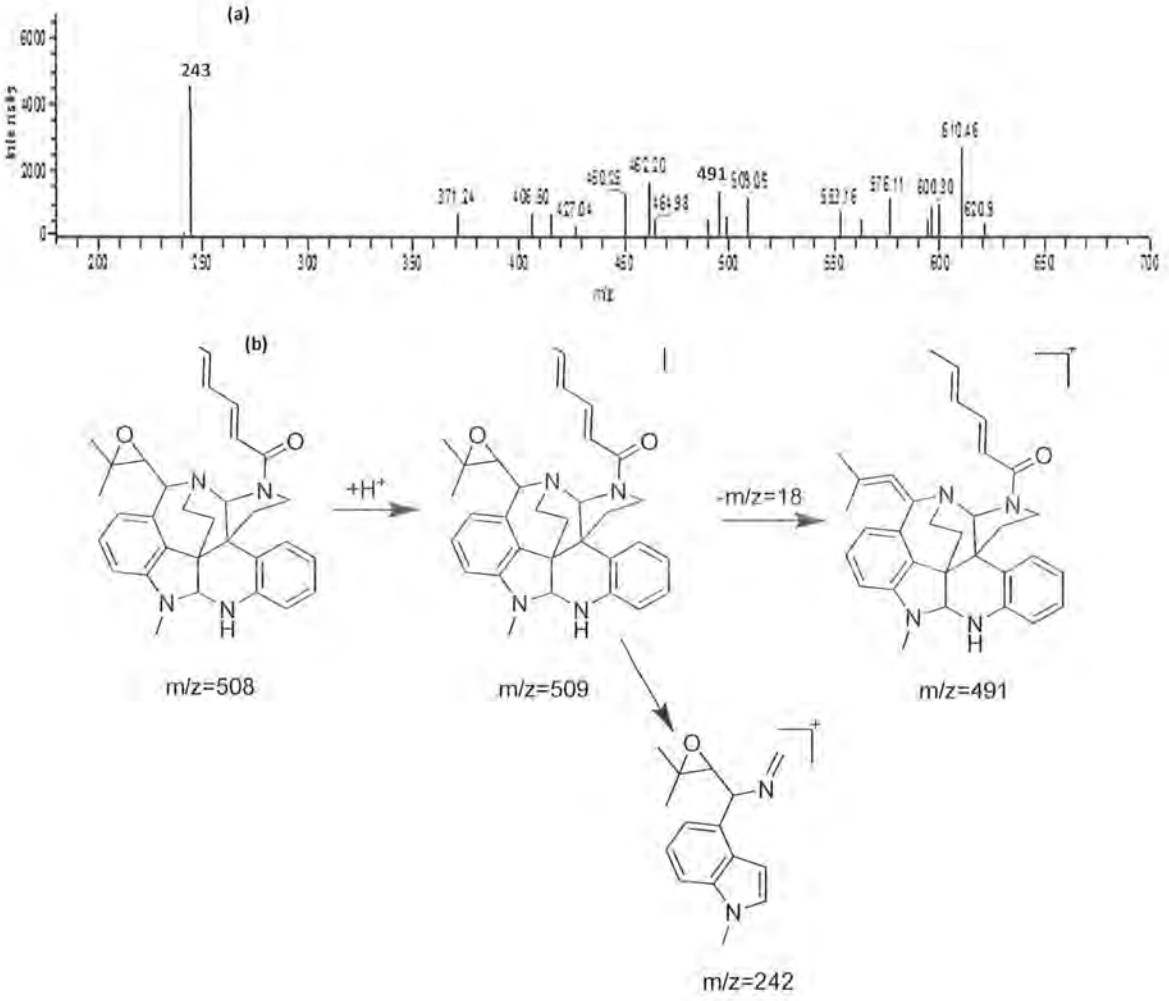


Fig 5.48: Fragmentation pattern of Communosine g (b) (Fraction B-c) [(showing an intense molecular ion peak of m/z 509 and the fragments with m/z 491 and 242 when subjected to LCMSMS) (a)] predicting its structure.

5.1.4.4 Pyrrocidine b

Fraction C-a 1-4 was found to contain an intense peak of m/z 477 which when subjected to LCMSMS showed the fragments with m/z 478 and 354 which corresponded to the structure of Pyrrocidine b (Fig. 5.49).

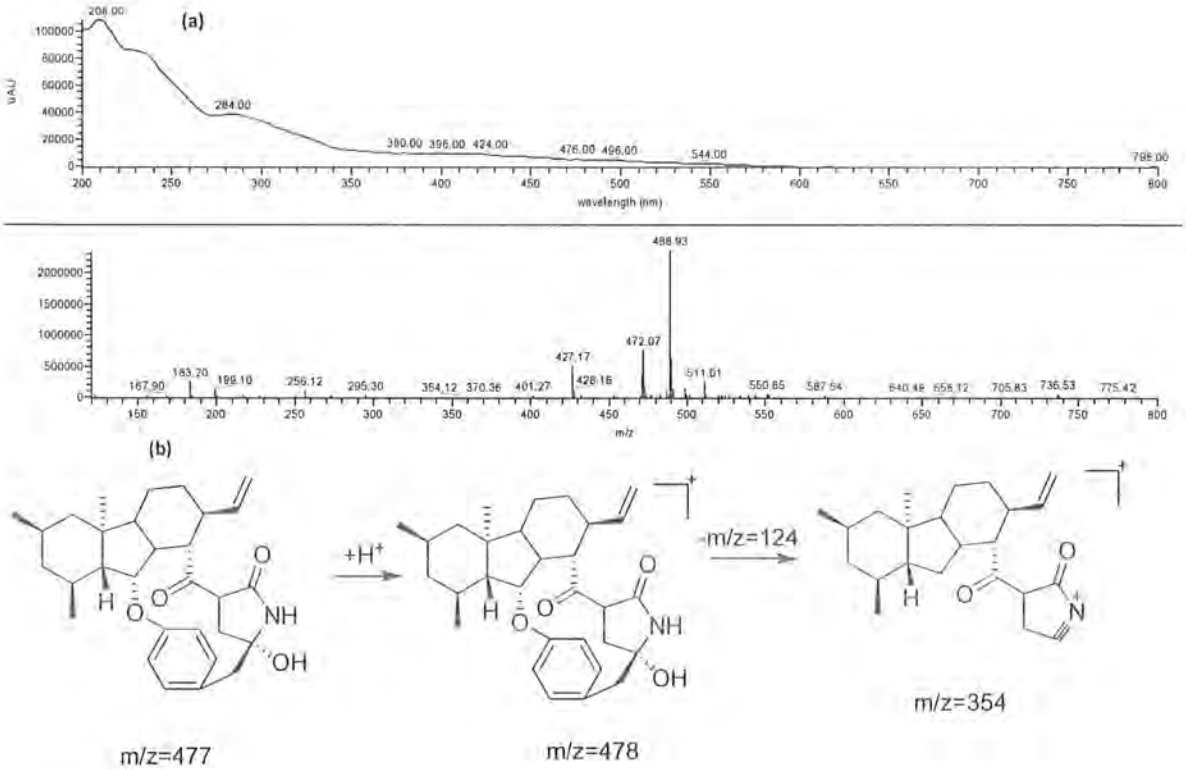


Fig 5.49: Fragmentation pattern of Pyrrocidine b (b) (Fraction C-a 1-4) [(showing an intense peak of m/z 477 and the fragments m/z 478 and 354 in LCMS)(a)] predicting its structure.

5.1.4.5 Nivalenol

Fractions C.a 11-15 were combined and found to contain two intense peaks. One peak of m/z 311 in LCMSMS was detected and its logical fragmentation confirmed it to be compound Nivalenol (Fig. 5.50).

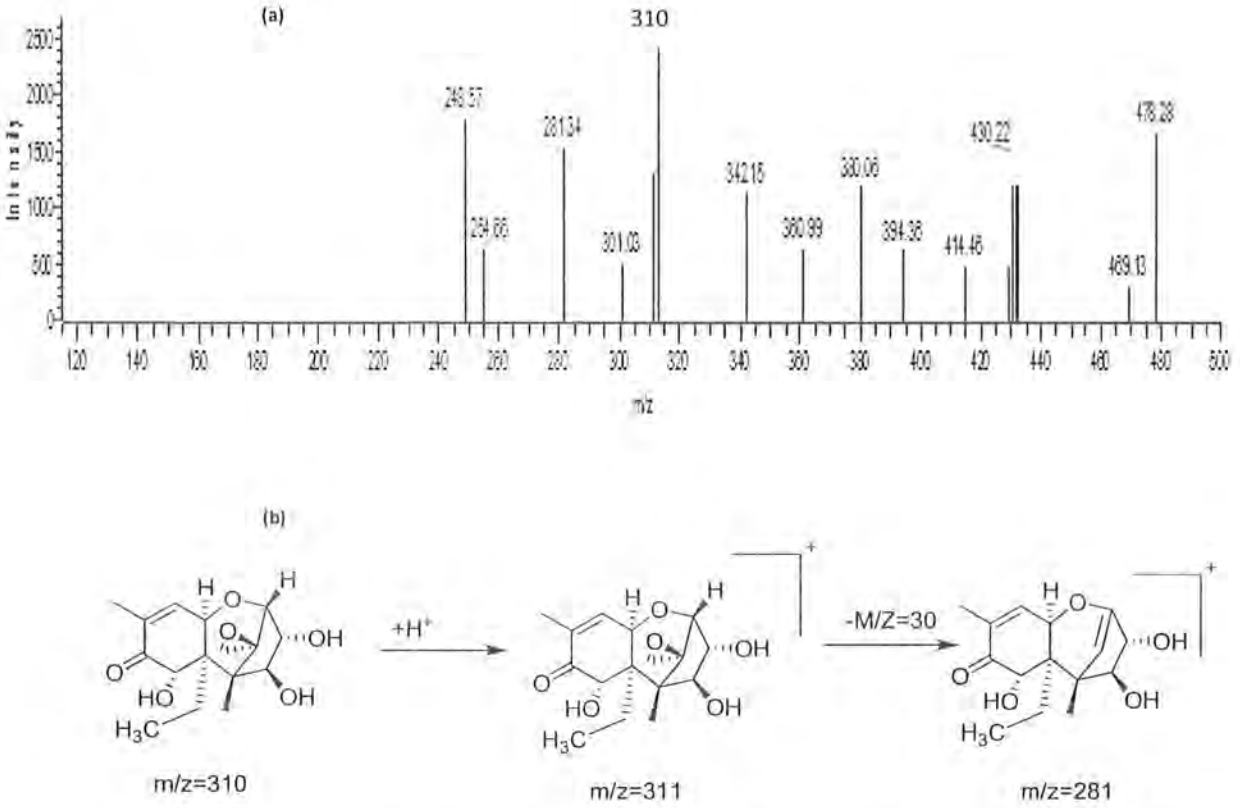
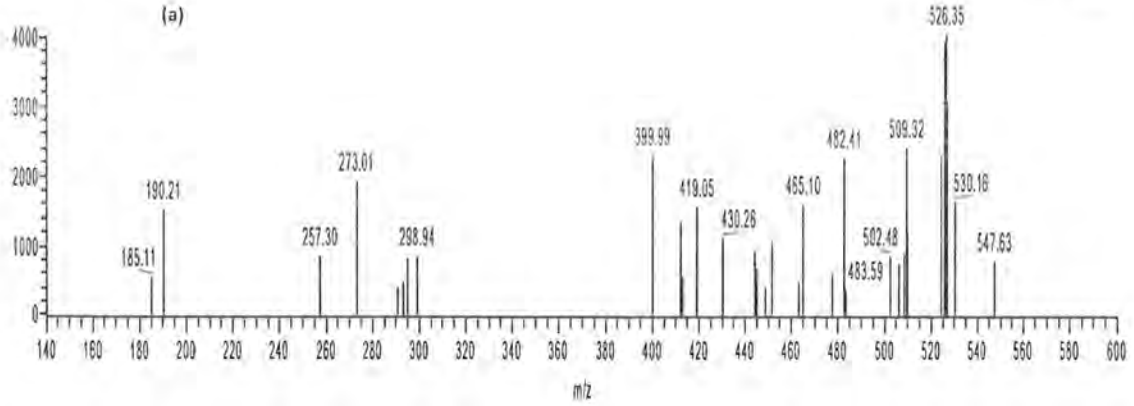


Fig 5.50: Fragmentation pattern of Nivalenol (C.a11-15) (Fraction C.a 11-15) [(Showing an intense peak of m/z 311 the fragments with m/z 281 in LCMSMS) (a)] predicting its structure.

5.1.4.6 Teritrem

The second intense peak of molecular ion in fractions C.a 11-15 was of m/z 526 which when subjected to LCMSMS showed the fragments with m/z 482, 465, 430, 400 and 190 corresponding to the structure of Teritrem (Fig. 5.51).



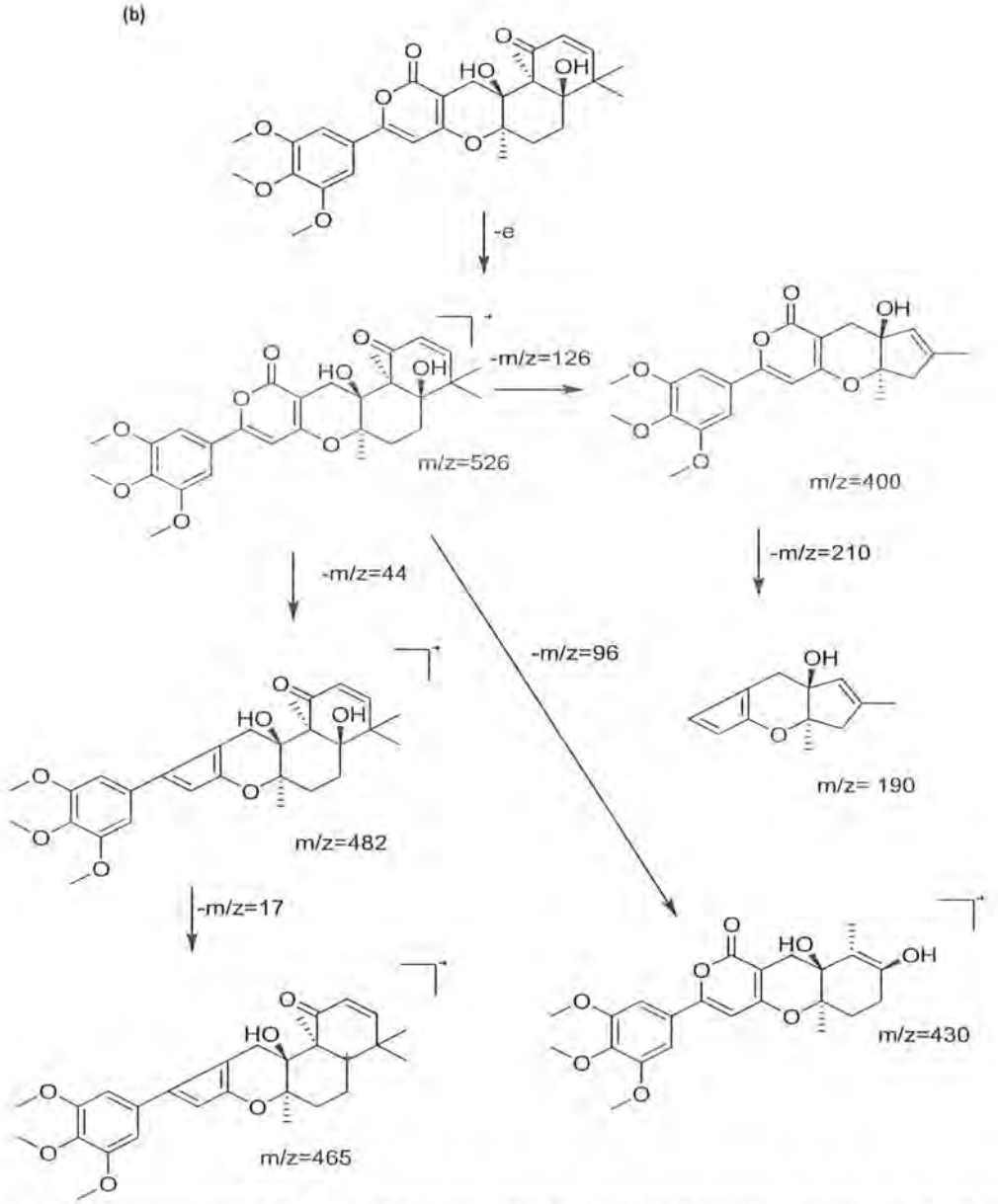
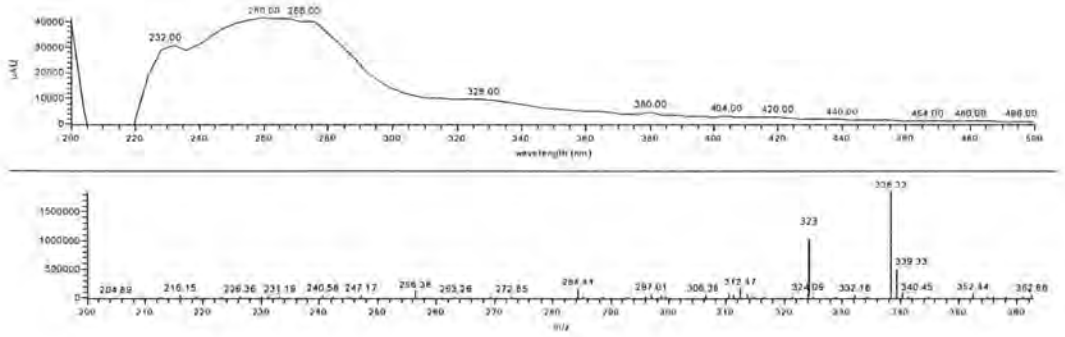


Fig 5.51: Fragmentation pattern of Teritrem (b) (C.11-15) [(showing an intense peak of m/z 526 and the major fragments with m/z 482, 465, 430, 400 and 190 in LCMSMS) (a)] predicting its structure.

5.1.4.7 Sterigmatocystin

Fraction C.a11-15 contained another important compound Sterigmatocystin having m/z 324.



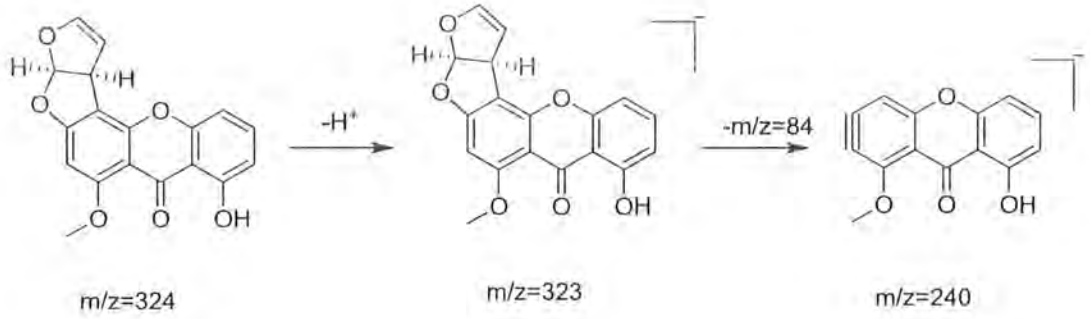


Fig. 5.52 Fragmentation pattern of Sterimatocystin [showing presence of molecular ion peak m/z 324 and fragment 240 m/z] corresponding to its structure

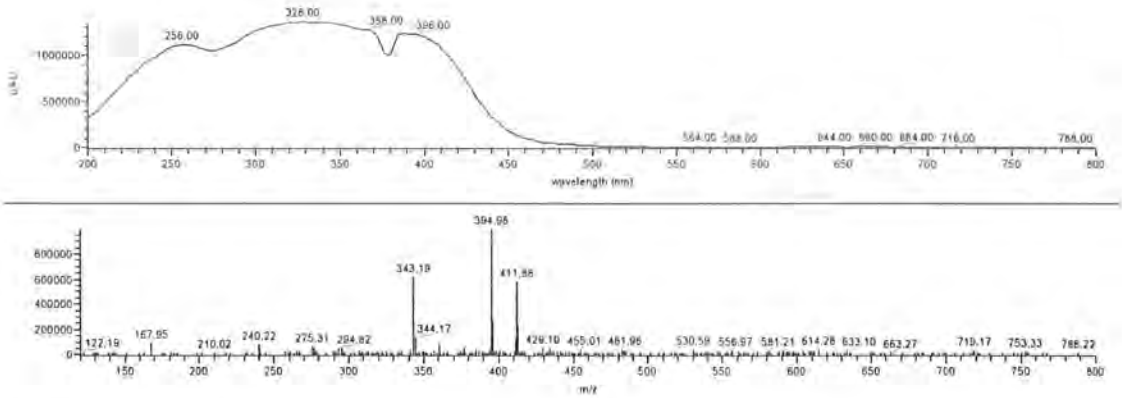


Fig 5.53: Fraction B.a-c obtained after processing of methanolic pigmented filtrate of *Aspergillus fumigatus* contained peaks of m/z 343, 394 and 411 in LCMS (Unknown peak).

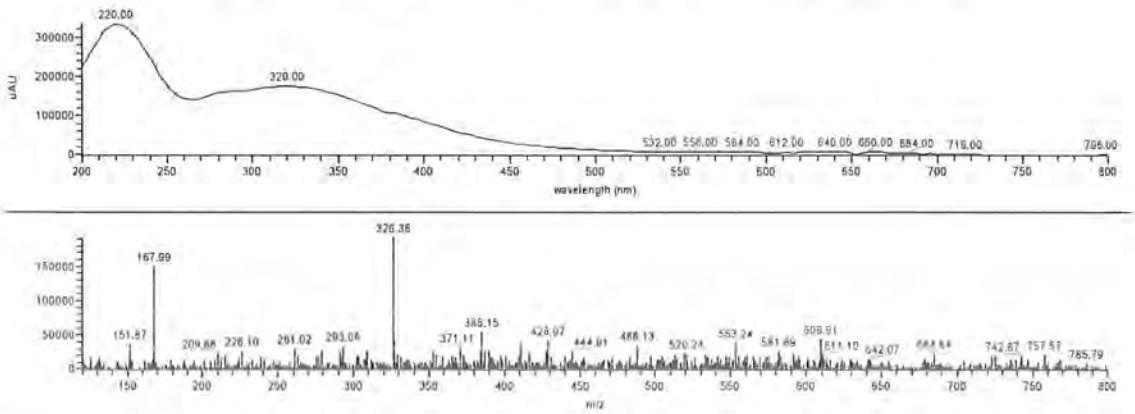


Fig 5.54: Fraction B.d obtained after processing of methanolic pigmented filtrate of *Aspergillus fumigatus* contained peak of m/z 326 in LCMS (Unknown compound).

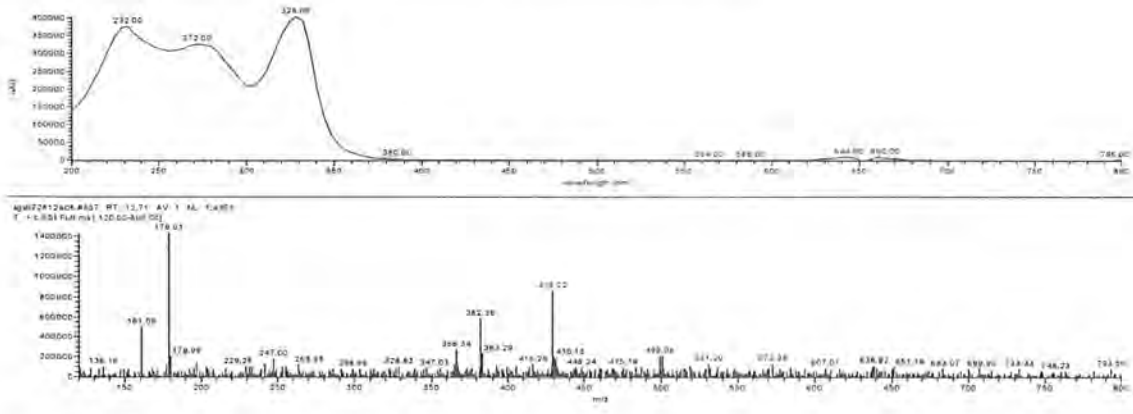


Fig 5.55: Fraction D.a obtained after processing of methanolic pigmented filtrate of *Aspergillus fumigatus* contained a peak of m/z 429 in LCMS (Unknown compound).

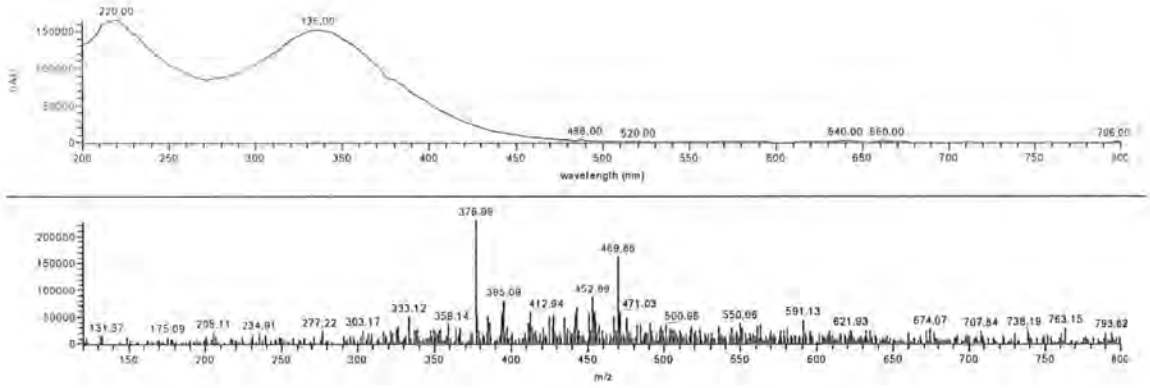


Fig 5.56: Fraction D.b obtained after processing of methanolic pigmented filtrate of *Aspergillus fumigatus* contained peaks of m/z 376 in LCMS (Unknown compound).

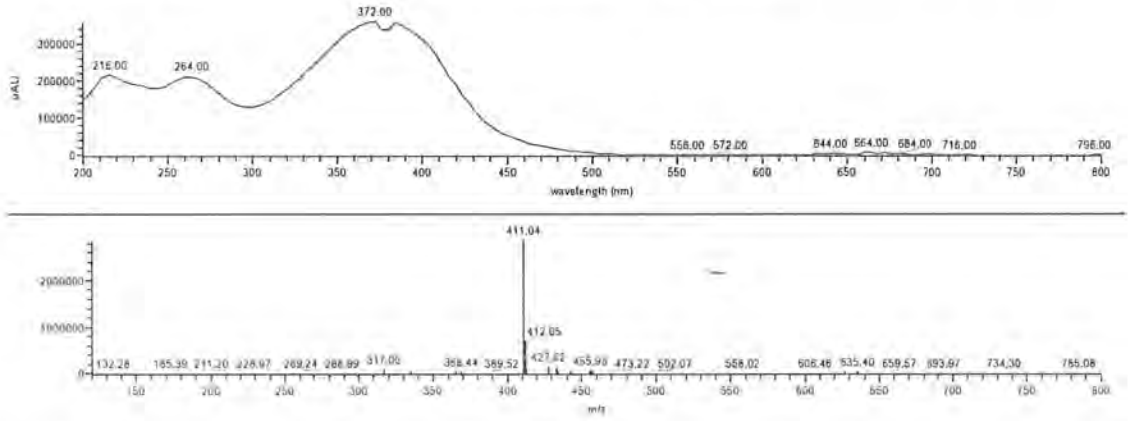


Fig 5.57: Fraction D.c obtained after processing of methanolic pigmented filtrate of *Aspergillus fumigatus* contained peaks of m/z 411 in LCMS (Unknown peak).

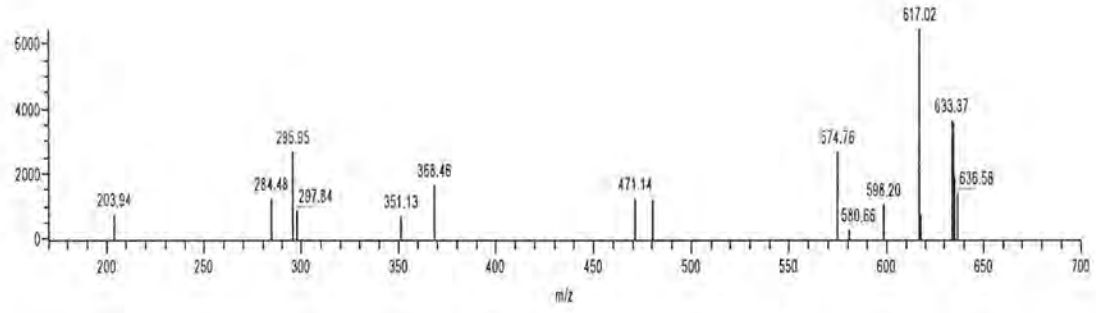


Fig 5.58: Fraction D.d obtained after processing of methanolic pigmented filtrate of *Aspergillus fumigatus* contained peak of m/z 635 in LCMSMS (Unknown compound).

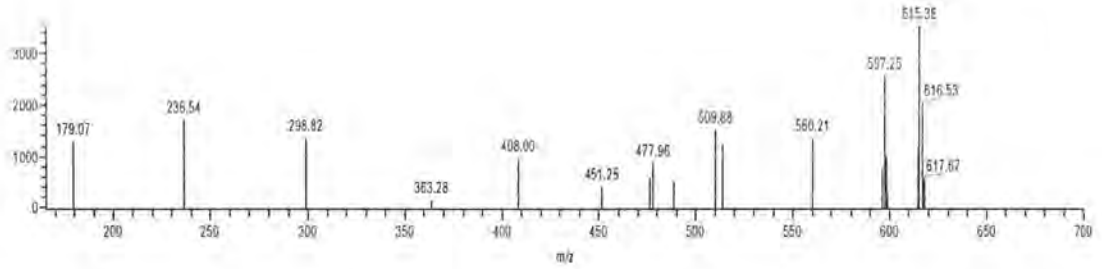


Fig 5.59: Fraction D.d obtained after processing of methanolic pigmented filtrate of *Aspergillus fumigatus* contained peak of m/z 616 in LCMSMS (Unknown compound).

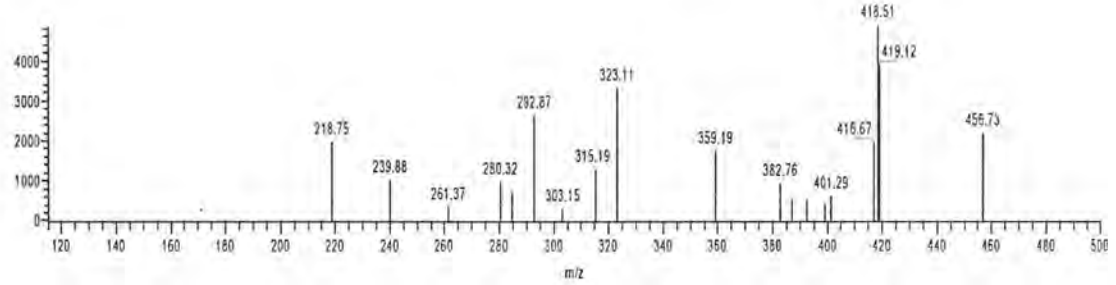


Fig 5.60: Fraction D.e obtained after processing of methanolic pigmented filtrate of *Aspergillus fumigatus* contained peaks of m/z 418 in LCMSMS (Unknown compound).

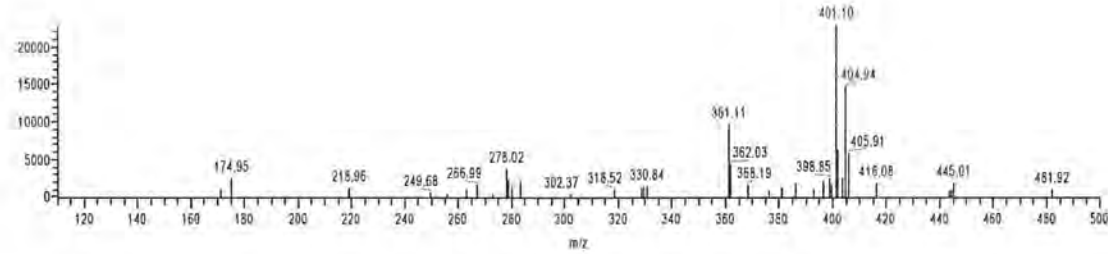


Fig 5.61: Fraction D.e obtained after processing of methanolic pigmented filtrate of *Aspergillus fumigatus* contained peaks of m/z 405 in LCMSMS (Unknown compound).

5.4 Mammalian Cell line cytotoxicity assays of *Aspergillus fumigatus* fractions

The cytotoxicity against HSCT6 (43.5±84) was significantly lower and differed significantly from all the other four cell lines (Fig. 5.62) (Table 5.6; A18, A19, A21, A24 & A25). Overall, Crude pigmented filtrate showed least cytotoxicity against all the five cell lines followed by Pyrrocidine (9.0±10)> Communosine (10±0.35) (Non-significantly differed from PPR Monascorubramine, Fusarubin and Teritrem). In case of KA3IT, maximum cytotoxicity was shown by Pyrrocidine (1.0± 0.84) (non significantly differed from Fusarubin, PPR Monascorubramine, Teritrem and

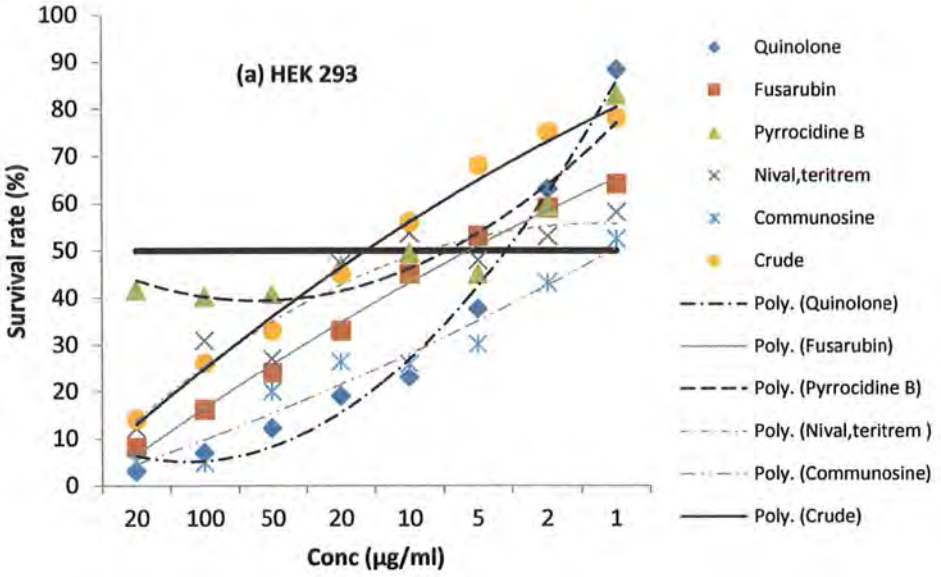
Communosine). In MDCK, Least cytotoxicity was shown by crude filtrate followed by PPR Monascorubramine (20± 0.88)> Communosine g (4.0± 0.45) (differed non significantly from Pyrrocidine, Fusarubin and Teritrem). In HSCT6, least cytotoxicity was again shown by crude pigmented filtrate (200± 0.76) followed by Fusarubin (20±0.89) (non significantly differed from Pyrrocidine) which differed significantly from Communosine (10.0±0.35) (differed non significantly from PPR Monascorubramine). Whereas maximum cytotoxicity (1.0± 1.2) was shown by Teritrem containing fraction. In HEK cell line, least cytotoxicity was shown again by crude filtrate (10± 0.09) (non significantly differed from Communosine) followed by Fusarubin (5.0±0.53) >Quinolone (2.0± 0.72) (non significantly differed from Pyrrocidine and Teritrem). In NIH3T3, least cytotoxicity was shown by crude (20± 0.47) (Non significantly differed from Communosine and Pyrrocidine) followed by PPR Monascorubramine (2.0± 0.59) (Non significantly differed from Fusarubin and Teritrem).The statistical analysis of the IC_{50} values and the graphical representation of IC_{50} values of different fractions are shown in Table 5.6 and Figure 5.62.

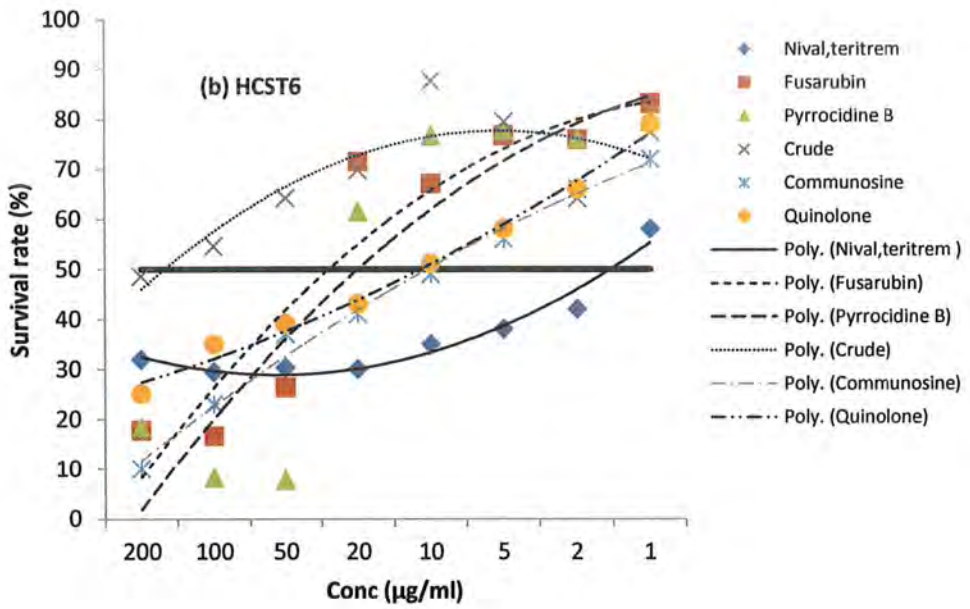
Table 5.6: Cytotoxic effect (IC_{50}) of different compounds and major fractions of culture filtrate of *Aspergillus fumigatus* SG against different normal and cancer cell lines (LSD) (Average values sharing a common letter do not differ significantly, other differ significantly ($p < 0.05$))

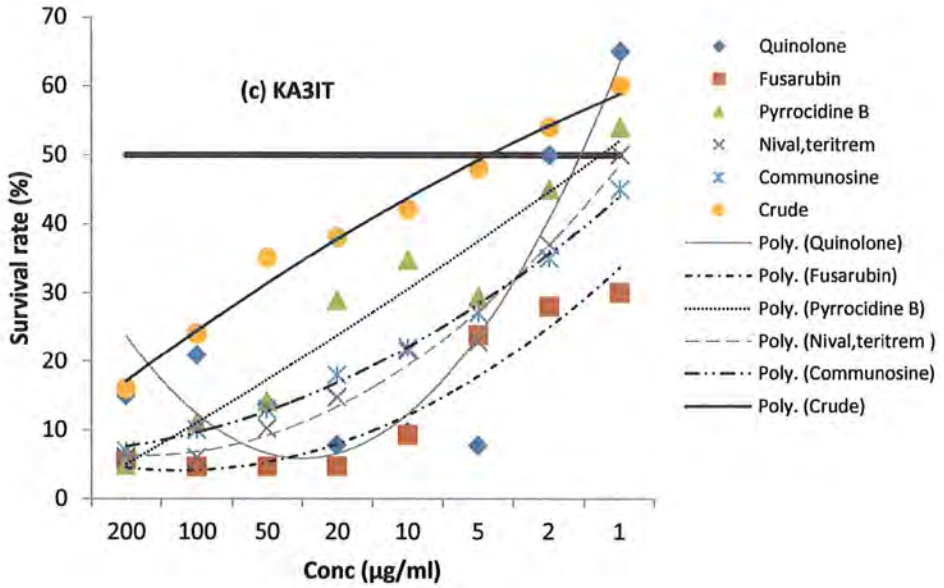
Fungal Filtrate		Mammalian cell lines											
Main fractions	Sub-fractions	KA31T (µg/ml)		MDCK (µg/ml)		HSCT6 (µg/ml)		HEK (µg/ml)		NIH3T3 (µg/ml)		Combine effect	
		Ave.±SD (n = 3)	R	Ave.±SD (n = 3)	R	Ave.±SD (n = 3)	R	Ave.±SD (n = 3)	R	Ave.±SD (n = 3)	R	Ave. (n = 5)	R
B													
C													
	Monascorubramine	1.0± 1.63	B	20± 0.88	B	10± 0.44	C	2.0± 0.72	C	2.0± 0.59	B	7.0±8.1	B
	Fusarubin	1.0±0.89	B	2.0±0.54	C	20±0.89	B	5.0±0.53	B	2.0± 0.89	B	6.0±7.9	B
	Pyrrocidine B/Tryptoquialanine	1.0± 0.84	B	2.0± 0.17	C	20± 0.89	B	2.0± 0.82	C	20± 0.72	A	9.0±10	B
	Communosine	1.5+ 0.89	B	4.0± 0.45	C	10.0± 0.35	C	10± 0.88	A	20± 0.76	A	9.0±8.2	B
	Teritrem/Nivalenol	1.0± 1.5	B	1.0± 0.99	C	1.0± 1.2	D	1.0± 1.5	C	1.0± 0.48	B	1.0±4.0	B
Crude culture filtrate		2.8± 0.44	A	59±0.89	A	200± 0.76	A	10± 0.09	A	20± 0.47	A	58±84.5	A
	Ave. (n = 13)	1.38±0.72		14.9±22.8		43.5±84		3.5±3.5		10.8±10.1			
	R	B		B		A		B		B			

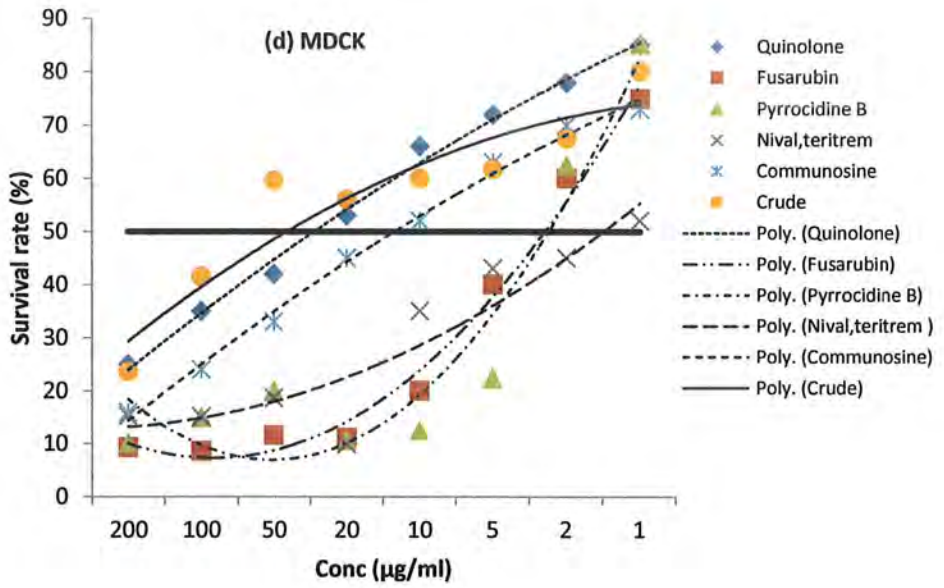
Foot notes: Vertically ranking associated with IC_{50} of different fractions with values against different cell lines. Horizontally ranking depict average of IC_{50} values from all cell lines. IC_{50} was calculated by formula= Treated cell conc (OD)/untreated cell conc. (OD)*100.

LSD = Least Square Difference; R = Rank order; SD = Standard deviation; Ave = Average; NIH3T3 = cells mouse embryonic fibroblasts; HSCT6 cells = rat hepatic stellate cell line; HEK293 = cell line derived from human embryonic kidney cells grown in tissue culture; MDCK Line = Madin-Darby Canine Kidney Epithelial Cells; KA3IT = virally transformed cancerous cell line.


(a) HEK 293
Survival rate (%)
Conc (µg/ml)
100
90
80
70
60
50
40
30
20
10
0
20 100 50 20 10 5 2 1
Quinolone
Fusarubin
Pyrrocidine B
Nival,teritrem
Communosine
Crude
Poly. (Quinolone)
Poly. (Fusarubin)
Poly. (Pyrrocidine B)
Poly. (Nival,teritrem)
Poly. (Communosine)
Poly. (Crude)


(b) HCST6
Survival rate (%)
Conc (µg/ml)
100
90
80
70
60
50
40
30
20
10
0
200 100 50 20 10 5 2 1
Nival,teritrem
Fusarubin
Pyrrocidine B
Crude
Communosine
Quinolone
Poly. (Nival,teritrem)
Poly. (Fusarubin)
Poly. (Pyrrocidine B)
Poly. (Crude)
Poly. (Communosine)
Poly. (Quinolone)


(c) KA3IT
Survival rate (%)
70
60
50
40
30
20
10
0
200 100 50 20 10 5 2 1
Conc (µg/ml)
Quinolone
Fusarubin
Pyrrocidine B
Nival,teritrem
Communosine
Crude
Poly. (Quinolone)
Poly. (Fusarubin)
Poly. (Pyrrocidine B)
Poly. (Nival,teritrem)
Poly. (Communosine)
Poly. (Crude)


(d) MDCK
Survival rate (%)
90
80
70
60
50
40
30
20
10
0
200 100 50 20 10 5 2 1
Conc (µg/ml)
Quinolone
Fusarubin
Pyrrocidine B
Nival,teritrem
Communosine
Crude
Poly. (Quinolone)
Poly. (Fusarubin)
Poly. (Pyrrocidine B)
Poly. (Nival,teritrem)
Poly. (Communosine)
Poly. (Crude)

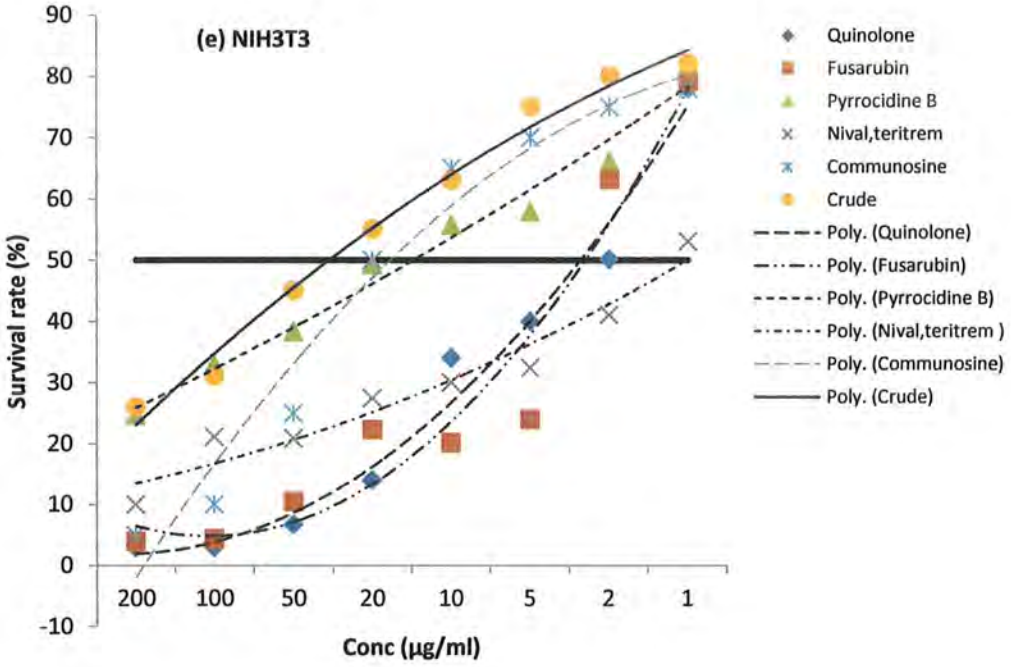


Fig. 5.62: Cytotoxicity (IC_{50}) of different fractions of *Aspergillus fumigatus* containing important colored and bioactive compounds against five different cell lines i.e., HEK293 (a; Table A18), HSCT6 (b; Table A19), KA3IT (c; Table A21), MDCK (d; Table 24), NIH3T3 (e; Table 25)

5.5 Pure orange compound of *Chaetomium strumarium*

The orange colored compounds labeled as S2 was obtained after the Sephadex LH20 column of *Chaetomium Strumarium*. The fraction E.d 31-32 containing orange spots were combined and crystallized in Methanol and Ethyl acetate and the crystals were subjected to LCMS and Proton NMR for structural elucidation. This orange compound was dissolved in DMSO and its LCMS showed the presence of M^+ 539 m/z and a major fragment of 167 m/z (Fig. 5.63). The proton NMR of compound was obtained by dissolving the crystals in DMSO-d6. In Proton NMR, The peaks at 2.51 and 3.34 ppm were due to DMSO. The signals for 6 protons in the aromatic region fall between 6.62-7.30 ppm. The signals for two aromatic protons designated as a,a' lies at 6.62 ppm as singlet. This shielding results due to vicinity of two electron donating hydroxyl groups. The signal at 7.14 ppm is for b,b' Hs and appear as singlet with integration for 2 protons. The signal of c,c' protons appear at 7.28 ppm as singlet. They are most deshielded because of their neighbouring groups as compared to other protons located close to donating groups. The broad signal from 5.1-5.6 ppm is due to OH protons. The signals at 2.33 ppm for 6 protons is due to methyl protons.The proton NMR also confirmed the structure to be 2,2',4,4',5,5'-hexahydroxy-7,7'-dimethyl-1,1'-bianthracene-9,9',10,10'-tetrone (Fig. 5.64).

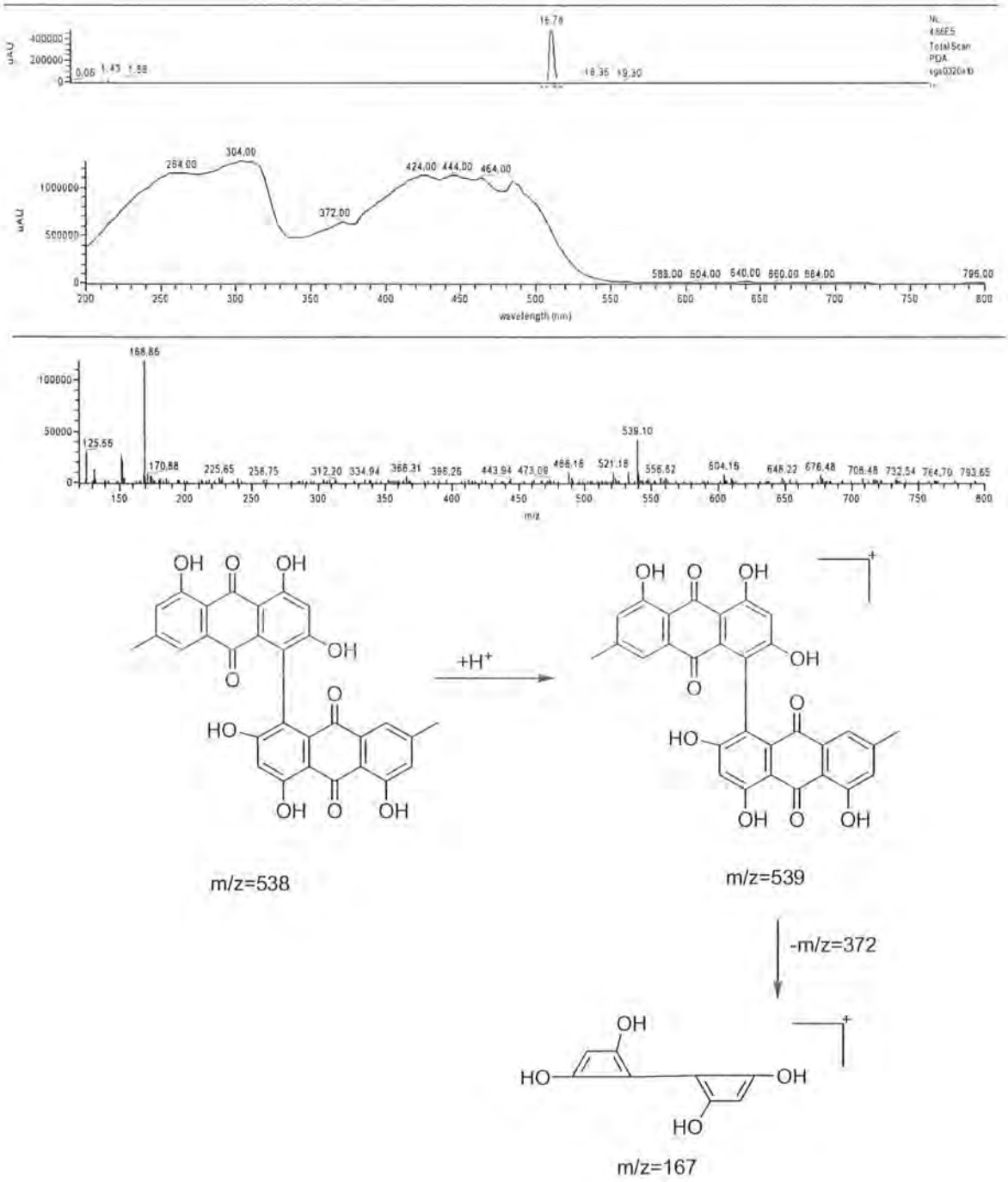
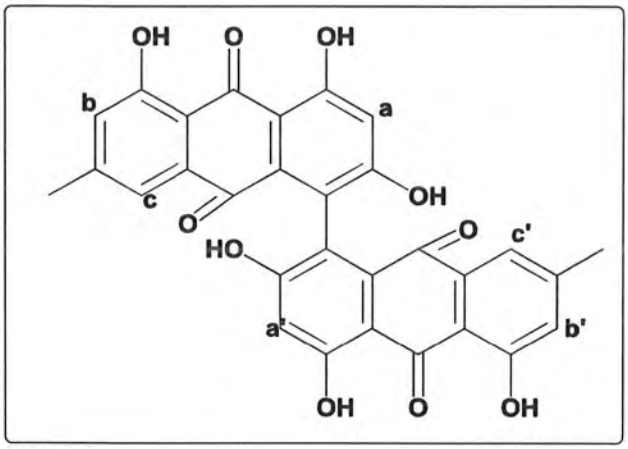


Fig 5.63: Fraction E.d was found to contain an intense peak of m/z 539 which when subjected to LCMS showed the major fragment m/z 167 which correspond to the structure of Anthraquinone

Anthraquinone



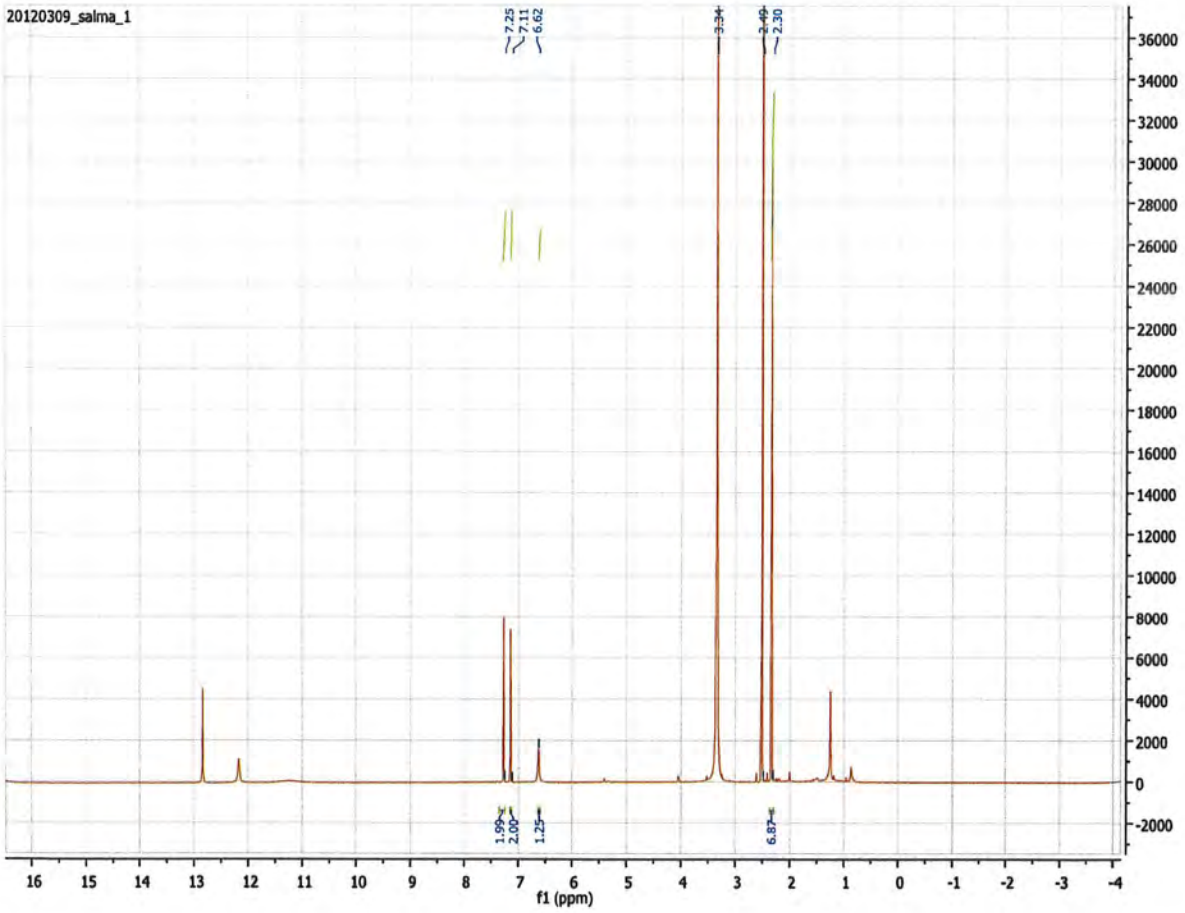


Fig 5.64: Proton NMR of orange crystals obtained from *Chaetomium strumarium* culture filtrate

5.6 Xrd of Pure Yellow Crystal of *Penicillium verruculosum*

Fraction B-c was processed through LH20 and 2 fractions containing yellow spots were crystallized by dissolving in chloroform at room temperature and kept for slow evaporation in a vial closed with two layers of tissue paper. Clear yellow prismatic crystals were observed after full evaporation. The obtained pure yellow crystals were subjected to 3D XRD.

Table

Crystal data and structure refinement parameters for phenazine-1-carboxylic acid (**1**)..... (Shanmugaiah et al., 2010).

Chemical formula	C_{13} H_8 N_2 O_2
Chemical formula weight	224.21
Crystal system	monoclinic
Space group	?

Unit cell dimentions

a	3.944(2)	Cell measurement temperature	296(2)
b	19.141(14)	Density	1.493
c	13.337(8)	F(000)	464
α (Å)	90	Crystal size	(0.350×0.140×0.120)
β (Å)	97.93(3)	Radiation wavelength	0.71073
γ (Å)	90	Radiation type	MoKα
Volume	997.3(11)		
Z	4		

The molecular structure of phenazine-1-carboxylic acid (**1**) is presented in Table above. The compound was crystallized in a monoclinic system. The unit cell packing of (**1**) shows that the molecule exists as single asymmetric unit. The bond length O2-C1 (1.31(2) is greater than that of O1-C1 (1.22(2)) confirming the single bond character of the bond. The torsion angles O1-C1-C2-C3 (-2(3)), O2-C1-C2-C3 (-178.5(16)), O1-C1-C2-C7 (-177(2)) and O2-C1-C2-C7 (6(3)) show that the

carboxylate moiety is lying out of plane from that of phenazine moiety. The packing of the molecules was further stabilized through vander Waal's interactions. The molecular dimensions are normal (Fig 5.65 and 5.66).

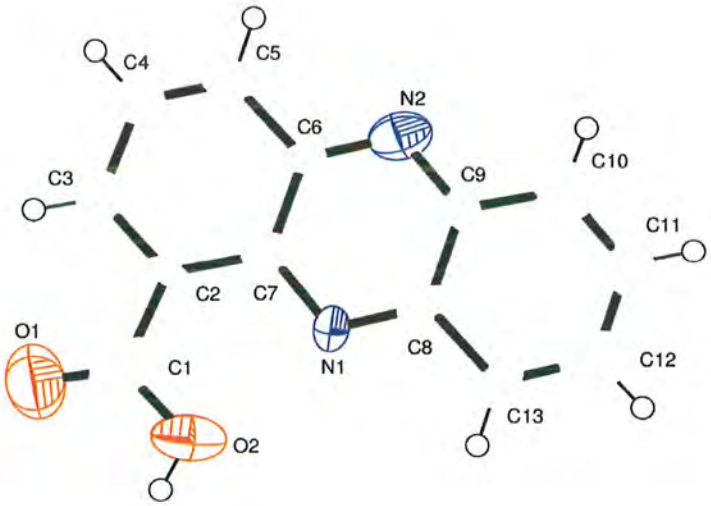


Fig 5.65: ORTEP drawing of compound 2 with displacement ellipsoids drawn at the 50% probability level and atomic numbering scheme. H-atoms are shown as small spheres of arbitrary radius.

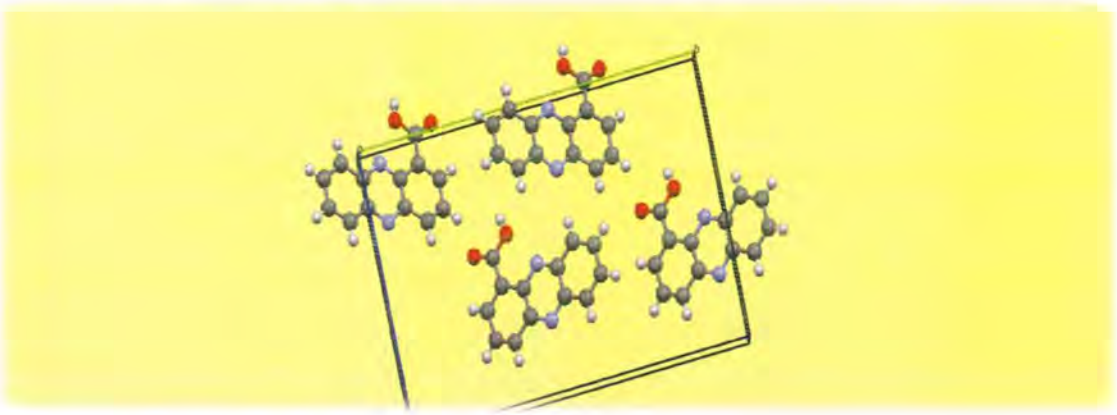


Fig 5.66: Unit cell packing of (1), which shows that the molecule exists as a monomer.

The three fungi produced extracellular pigments that seemed to be industrially important with an additional advantage of pharmaceutical importance. Further, the

composition of the pigmented filtrate was explored by LCMS and LCMSMS to see that the apparent natural pigment is combination of how many colors and what other bioactive compounds might be present contributing to the biological activities. These fungi proved to be a potential source of natural pigments in addition to many bioactive compounds and unknown compounds. The presence of pure compound Phenazine 1 carboxylic acid which is reported for the first time from any fungi gives an important insight in to the fact that these fungi can be having some unique behavior that needs to be further explored. The other bright orange compound 'Anthraquinone' can be overlooked in future as a better source of colorant.

The toxicity of colorants is largely an unexplored domain. Considering in view this fact, different cytotoxicity assays using mammalian cell lines were carried out in order to determine IC_{50} values of the various fractions of the three fungi containing important compounds. Moreover, a pair of normal cell line and cancer cell line proved the anticancer potential of the pigmented filtrate and various fractions of the fungi.

DISCUSSION

Discussion

Colorants have various applications in food, pharmaceutical and dyeing industry. Considering harmful effects of synthetic colorants i.e., carcinogens and non-biodegradable, several microbial pigments/colored metabolites have also been investigated. They proved to be useful as colorants with an additional advantage of their possibility to be used as drugs e.g., antimicrobial (Anthraquinones), antioxidant and anticancer drugs (Phenolics, Carotenoids, flavonoids, Melanins). The drawbacks associated with natural sources of colorants are dependence on the supply of raw materials, seasonal variations and ethical restrictions in killing the plants and animals (Mapari et al., 2005). As a result, a trend towards procurement of drugs and colorants from fungi especially ascomycetes was developed. Several fungal species are rich in stable colorants such as anthraquinone carboxylic acids and pre-anthraquinones (Raisanen et al., 2001) with most important broad bioactive class being azophilones/ polyketides having applications in food and pharmaceutical industries (Chen et al., 2013).

Ascomycetes are preferred group of fungi for pigments production due to ease of growing them in controlled environments and diverse array of both intracellular and extracellular colored metabolites. The potential of this group of fungi (Ascomycete) need to be explored further with reference to enhanced pigment production at optimum conditions before scaling up and their screening at initial level for their use at appropriate industrial levels. Considering this aspect, three different fungi belonging to asmycota including one endophyte *Chaetomium strumarium* (Fig. 4.3) and other two soil isolates *Penicillium verruculosum* (Fig. 4.1) and *Aspergillus funigatus* (Fig. 4.2) isolated from Kala pani forest Pakistan were explored in details for pigments production, their identification and characterization with respect to their stability, bioactivity and enhanced production.

These fungal species were studied for the first time for pigments production whereas their other associated species in the same genera were studied previously (Engstrom et al., 1982; Suhr et al., 2002; Dufossé, 2006; Méndez- Zavala et al., 2007; Hernández-Rivera et al., 2008). Typically, pigment producing ability of fungal species *Chaetomium strumarium* (Fig. 4.3), an endophyte of *Taxus baccata* has been reported for the first time in this research work. Other two fungi were isolated from Kala Pani

soil (Khyber pakhtun khawa, Pakistan) an area not studied with respect to diversity in microbial flora and its biotechnological potentials.

Generally, fungi play different roles in their environment by producing pigments as secondary metabolites taking selective advantages for their survival within their ecological niche by competitive antagonism (Rose 3rd ed). Besides, they withstand harsh environmental condition through their secondary metabolites (Valmaseda et al., 1989; Nosanchuk and Casadeval, 2003). The production of water soluble exogenous red pigments from species *Penicillium verruculosum* (Fig. 4.3; 4.18) is being reported for the first time under noncompetitive (antagonism) environment (Fig. 4.18) as previously there has been one report on extracellular red pigment production from *Penicillium verruculosum* L9 upon interaction with *Trichoderma viridae* L6 both in solid and liquid media (Nasuno and Asai, 1962). *Penicillium* genera have been reported previously to produce homologues of *Monascus* pigments which have similar chromophore i.e., polyketides (Espinoza-Hernández et al., 2004; Mapari et al., 2009). Moreover, the study on *Aspergillus fumigatus* (Fig. 4.2; 4.18) with respect to extracellular natural pigments is being reported for the first time. These fungi being ascomycete are selected for future evaluation as natural colorants and in biomedical industry as they could easily be produced in high yields because of the availability of cultivation technology and potential use of bio-engineering tools.

Secondary metabolites biosynthesis has been well integrated with different environmental cues, including the carbon and nitrogen source, ambient temperature, light and pH (Bennet and Ciegler, 1983; Berry, 1988). Initially, different operational and nutritional parameters were optimized with respect to enhanced pigment production by three different fungi. Five different media used (Table 4.1) for pigments production were also previously been used and PDB proved to be the best culture medium (Frisvad and Thrane, 2004; Boonyapranai, et al., 2008; Mendez et al., 2011) and this observation significantly coincided with present findings (Table 4.1). The major difference between PDB and other media was that it contained potato starch and the others did not.

The potato starch might have been potentially effecting enhanced production of pigments. Similarly, starch as a substrate for pigments production (Table 4.1) has been used previously as it was more easily assimilated than other lignocellulosic

substrates (Wheat bran, Sugar beet pulp) (Rosenblitt et al., 2000; Joshi et al., 2003; Prado et al., 2004; Santa et al., 2005). Contrarily, some studies showed that complex carbon sources like starch reduce both growth and pigment production due to poor oxygen mass transfer resulting from increased viscosity of starch solutions (Lee et al., 1994; 1995).

Moreover, complex carbon source like rice has been preferred for pigments production especially in solid state culture media (Lin, 1973; Teng and feldheim, 1998). In the present findings (Table 4.1), Czapek dox media was the least productive media for pigments yielding however, this media when used with carbon source like xylose led to increased pigment production previously (Mendez et al., 2011). The reason for comparatively low pigments production in Czapek dox media in present study can be due to the sucrose and simple inorganic nitrogen source i.e., sodium nitrate (Table 4.1) (Fig. 4.16; 4.14). Overall, each fungus displayed a specific behavior with respect to utilization of carbon source present in culture media for growth and pigments production. The use of malt extract by fungi for effective pigments production was reported previously similar to the present findings (Table 4.1) (Mendez et al., 2011). The reason for pigments production in this media can be amino acids that might play role in release of pigments into the media by making them water soluble.

Among the most important variables affecting biotechnological processes, pH and temperature are environmental conditions with a strong effect on the biosynthesis of metabolites such as pigments. Thus, it is very important to control them in industrial bioprocesses. Generally, fungi prefer slightly acidic pH (5.0–6.0), however, the metabolism and associated physiology of different fungi vary at different pH with reference to production of specific metabolites (Bae et al., 2000; Cho et al., 2002). The results in current study depicted the same trend. The fungi were able to produce pigments from acidic (5.5) to neutral (7) pH with 5.5 pH being the most optimum (Fig. 4.12).

Responses to varying pH (s) are transmitted through Cys2 His2 zinc-finger global transcription factors that mediate through PacC proteins (Tilburn et al., 1995; Martin, 2000) signaling. pH effects function of cellular growth, production of primary and secondary metabolites, fermentation and the oxidation processes of the cell (Mendez

et al., 2011). Moreover, cell membrane function, cell morphology and structure, the solubility of salts, the ionic state of substrates, the uptake of various nutrients and product biosynthesis were also affected by pH of the media (Kim et al., 2003). The pH range for pigments production in the present investigation (Fig. 4.12) falls within the pH range already reported i.e. 4-7, whereas, it varies in range of 2.5-8 for growth of fungi among different species (Yongsmith et al., 1993).

The pH profile of the media depends on type and concentration of nitrogen sources (Wong and Bau, 1977; Pirt, 1985; Matinkova and Patakova, 1999). In *Monascus* sp. the initial pH proved to have a significant effect on pigments production and by then it can be adjusted to a suitable level depending upon nitrogen sources used in fermentation. Perversely, the yellow pigment production was reported at lower pH 3-4 in *Monascus* (Yongsmith et al., 1993) and at pH 5.5, the pigments production/coloration shifted towards orange and reddish with predominantly red pigment being produced in basic pH (Mak et al., 1990). However, in present study (Fig. 4.12), different pigments (red, yellow and purple) were observed in pH range of 5-7. Similarly, other studies report pigments production at pH range of 5.5-9 (Sharma et al., 2005; Boonyapranai et al., 2008; Liu et al., 2008). Nonetheless, optimum pigment production has also been reported at basic pH range 8-10 by different fungi (Boonyapranai, et al., 2008; Mendez et al., 2011). It suggested that pigments produced from different fungi were having different basic structures and they were also showing transformation in structures and associated colors at minor change in pH due to addition or deletion of function groups (auxochrome groups) and cations/anions ratios of the aqueous medium (Kim et al., 2003).

The pigments produced and extracted from the three fungi were also checked for their stability at various pH (Fig. 4.20 and 4.21). The pigments of *Chaetomium strumarium* (Fig. 4.20b; 4.21b) and *Aspergillus fumigatus* (Fig. 4.20c; 4.21c) were stable at neutral and basic pH whereas, the pigments concentration decreased drastically at acidic pH (3). Nevertheless, pigments of *Penicillium verruculosum* (Fig. 4.20a; 4.21a) were stable at wide pH range of pH 3-9 which is in accordance with previous reports (Dangles and Brouillard, 1992; Inami et al., 1996; Wongjewboot and Kongruang, 2011). In submerged cultures, the pigments are pH dependant and are more sensitive to heat and variations in pH whereas the reason for the wide pH tolerance range

shown by *Penicillium verruculosum* (3-9) in present study (Fig. 4.20a; 4.21a) might be due to the pigmented extract obtained from solid media (Mak et al., 1990; Yongsmith et al., 1993; Hajjaj et al., 1997; Hamdi et al., 1997; Hajjaj et al., 2000).

The trend shown by *Chaetomium* sp (Fig. 4.20b; 4.21b) and *Aspergillus* sp (Fig. 4.20c; 4.21c) in the present study i.e., instability at acidic pH was shown previously by *Monascus* (Wongjewboot and Kongruang, 2011). Also this kind of behavior has been shown previously by plant pigments and the results (Table 4.2) showed the presence of different phytochemicals. It might be due to the change in the maximum absorbance (λmax) at varying wavelengths (λmax) at varying pH associated with change in the color intensities revealing a possible hyperchromic effect and bathochromic shift (Δλmax), resulting from a copigmentation reaction. The visible absorption maxima suffer a bathochromic shift at low pH. Hypochromic shifts were observed at higher pH then 6 up to 9. At higher pH, hypochromic shifts with time are related to chemical changes in the molecule (Inami et al., 1996) due to interactions with the buffer used. The similarity in behavior of plant and fungal pigment at various pH suggested some structural similarity or nature between their metabolites/pigments.

Temperature affects the rate of biochemical reaction thus growth and metabolites (pigments) production in fungi (Pirt, 1975). Mostly the optimum temperature for pigment production has been reported under mesophilic temperatures ranging from 25-30°C which is in accordance with the present findings (Fig. 4.13) (Shepherd, 1977; Lin, 1991; Desai et al., 2003; Liu et al., 2008; Mendez et al., 2011). Similarly, optimum temperature for pigments production by *Monascus* was 30°C in previous study (Padmavathi and Prabhudessai, 2013) which is in accordance with optimum temperature for pigment production by *Aspergillus fumigatus* (Fig. 4.13). Whereas optimum temperature for pigment production in *Fusarium* sp was previously reported to be 28°C (Pradeep and Pradeep, 2013) which is near the optimum temperature for pigments production by *Penicillium verruculosum* and *Chaetomium strumarium* in present case (Fig. 4.13). However, in some cases a high temperature (37°C) promoted growth and pigments production in some fungi (Carvalho et al., 2005).

The pigments produced by the three fungi were checked for their stability at varying temperatures (-20-100°C). The pigments were mostly stable at 100°C≥ with red

pigments of *Penicillium verruculosum* being most stable at even 100°C (Fig. 4.22 and 4.23). The trend of pigments instability at higher temperatures range 100°C≥ was shown previously by *Monascus* (Wongjewboot and Kongruang, 2011). The reason for instability at higher temperatures can be due to decrease in copigment bond intensity and its hyperchromic shift. Copigment complexes are exothermic and are particularly sensitive to temperature (Dangles and Brouillard, 1992).

The type and concentration of carbon source is known to affect the growth and in turn secondary metabolism by fungi. The best carbon source for pigment production by the three fungi was glucose (Fig. 4.14) followed by fructose> sucrose>lactose>glycerol. Previously, similar predictions were being made showing glucose as most suitable carbon source for pigments production (Lin 1973; Lauro, 1991; Lin and Demain, 1991; Subhasree et al., 2011). The role of carbon source is very important as they are involved in the biosynthetic pathways of polyketides which occurs in a stepwise manner from simple 2, 3, or 4-carbon building blocks acting as starters. The common starters are acetyl- CoA and its activated derivatives malonyl-CoA which are formed by the carbon source like glucose. Additional degrees of complexity in polyketides arise from the use of different starter units and chain elongation units as well as the generation of new stereoisomers (World of Polyketides, 2007). Several studies (Tudzyunski et al., 1999; Ehrlich et al., 2003) indicated that responses to environmental signals are transmitted through Cys2 His2 zinc-finger global transcription factors that mediate through CreaA proteins for carbon (Dowzer and Kelly, 1989).

It was seen that with increase in complexity of carbon source there is a decrease in pigments production. In nature, source of carbon is CO_2 that living organism utilize to make different carbohydrates including monosaccharides (glucose and fructose), disaccharides (lactose) and polysaccharides (starch). The monosaccharides are readily available source of carbon and thus energy whereas, starch act as energy reserves and broken down into its monomers during stress conditions to be utilized by living organisms. Being heterotrphic organism, fungi can utilize a variety of carbon sources. The reason for maximum pigments production when glucose is used as a substrate (Fig. 4.14) is that it is readily available source of carbon interfered with the biosynthesis of many secondary metabolites (Liu et al., 2008; Pradeep and Pradeep,

2013). It might be due to this reason that fungi utilize it readily and enters into the secondary metabolism phase for its survival producing extracellular pigments as secondary metabolites. Glucose also provides the carbon for making various cell structures, organic chemicals and metabolites (Broder and Koehler, 1980; Martin and Edward, 1990; Lin and Demain, 1991).

On the other hand some studies suggested other complex carbon sources like disaccharides and ethanol as preferred carbon source for pigments production. The reason can be that these complex sugars slows down the growth of fungi creating stress thus halting primary metabolism leading to production of pigments as secondary metabolites (Santerre et al., 1995; Juzlova et al., 1996). Whereas lactose, fructose and xylose are known to be inferior for growth and pigment production in *Monascus* sp. (Lin 1973; Lin and Demain, 1991) which differs from the present findings (Fig. 4.14) where fructose is a better source of carbon following glucose. But the differences in nutritional requirements can be due to the fact that different fungi and their different species have their own genetic makeup that might be responsible for their different behavior in different operational conditions. It has been reported that if ecological conditions and physiology is varied (Del Hoyo et al., 2011), then a combination of different polyketides may be produced by the availability of different types of starting units.

Optimum glucose concentration was found to be 20-40 g/l in the present study (Fig. 4.15). Optimum glucose concentration is also very necessary for pigment production as increase in glucose concentration up to a certain level leads to increase in pigments concentration and afterwards the pigment concentration decreases due to crabs tree effect which inhibits respiratory enzyme and increases ethanol production (De Deken, 1966; Han and Mudget, 1992; Lee et al., 1994; Pastrana et al., 1995; Boonyapranai, et al., 2008). The need of 40g/l of glucose by endophytic fungus can be due to the reason that this fungus lives in mutualistic relation with plant and can access easily the carbohydrates from their host as compared to other fungi living in stress conditions adapted to low glucose requirements. So, glucose concentration is an important factor for growth and pigment production. Results supporting the present findings (Fig. 4.15) are observed in *Monascus* sp. where carbon source concentration greater than 20g/l was used in order to avoid crabs tree effect that is supposed to occur at

concentrations less than 20g/l. Other studies also report the pigment production from fungi at 2-20g/l of glucose concentration (Liu et al., 2008; Pradeep and Pradeep, 2013).

Organic nitrogen source i.e., yeast extract and peptone favored pigments production as compared to inorganic nitrogen source (ammonium nitrate and sodium nitrate) with yeast extract as the best nitrogen source in boosting pigments production (Fig. 4.16). Nitrogen comprises about 10 % of weight of fungi (Pirt, 1975). Moreover, the type of nitrogen source effects growth, sporulation and type of pigments production by fungi. Nevertheless, the nitrogen also plays an important role at the level of metabolic pathway as pathway specific regulatory proteins selectively activate the nitrogen catabolic structural genes encoding enzymes involved in a particular pathway. These proteins act only in combination with globally acting proteins for activation of majority of target genes. Responses to environmental signals are transmitted through Cys2 His2 zinc-finger global transcription factors that mediate through AreA proteins for nitrogen (Kudla et al., 1990).

The enhanced pigment production by fungi (Fig. 4.16) in presence of yeast extract might be due to the reason that it can be a source of amino acids, vitamins, coenzymes and growth factors as previously reported (Pradeep et al., 2013). Similar to present findings (Fig. 4.16), in *Monascus* sp., monosodium glutamate, peptone and some amino acids increased both growth, red and yellow pigment production (Lin, 1991; Chen and Johns, 1993; Juzlova et al., 1994; 1996; Cho et al., 2002; Gunasekaran and Poorniammal, 2008) but contrary to the present case (Fig. 4.16), yeast extract decreased both growth and pigments. Similarly, the use of inorganic nitrogen source also supported pigment production and growth in *Monascus* sp except sodium nitrate which reduces growth and pigment production similar to present findings (Fig. 4.16). Whereas, use of ammonium salt supported less pigment production specifically red pigment production is decreased in presence of it due to acidification of media which promotes orange pigments. Acidification weakens the reaction of orange pigments i.e., Rubropunctatine and Monascorubrine with amino acids in the media and their consequent transformation in to red pigment (Juzlova et al., 1996; Martinkova and Patinkova, 1999) supporting the decrease in red, yellow and magenta pigment

production in present case with inorganic nitrogen source i.e., ammonium nitrate (Fig. 4.16).

However there is another contradictory report in which various kinds of amino acids containing inorganic nitrogen sources are essential for secondary metabolite biosynthesis (Carels and Shepherd, 1977; Jung et al., 2003). When using rice for angkak production, no more nitrogen source is needed to be added as it already have proteins 5-8 % as nitrogen source (Carvalho et al., 2003) while in PDB, despite the fact that it contains 4% proteins as nitrogen source, addition of nitrogen source like yeast extract in present case increased pigment production (Fig. 4.16) which is in accordance with previous report where PDB was modified by addition of yeast extract and enhancement in pigments was observed (Boonyapranai et al., 2008). Another relevant report mentioned maximum pigments production using peptone as being the second best nitrogen source (Fig. 4.16) used (Liu et al., 2008; Pradeep and Pradeep, 2013).

The pigments production was comparatively higher on solid state and stationary liquid cultures whereas no pigment was observed in shaking condition in present study (Fig. 4.1-4.3; 4.18). The use of static conditions in flask culture experiments in the present case resulted in release of water soluble pigments in to the media after formation of thick mat of cellular mass. It is suggested that this thick mat of mycelia might be providing solid support mimicking natural solid habitat of fungi thus, resulting in release of pigments. Moreover, only fungi have the ability to grow in solid state cultures with minimum water content as fungal mycelia have the ability to penetrate inside solid substrate thus increasing its accessibility to nutrients and more pigment production while prohibiting feedback inhibition (Mitchel and Lonsane, 1992; Prabhakar et al., 2005; Couto and Sanroan, 2006; Singhania et al, 2009). Another reason might be that the fungi went in stress condition in absence of oxygen and produced the extracellular pigment for its survival.

Previously, it has been reported that despite advantages of submerged culture i.e., ease of operation and controlled conditions, solid state fermentation is preferred in some cases due to increased pigment productivity (Evans and Wang, 1984; Chen and johns, 1994; Lee et al, 2001; Pandey, 2003; Carvalho et al., 2005; Holker and lenz, 2005).

The reason for low productivity in submerged cultures might be due to product inhibition during fermentation as mostly hydrophobic pigment remains inside mycelia halting further pigment production by feedback inhibition. Moreover, pigments might oxidize due to aeration in shaking conditions (Fig. 4.18) but on the other hand, pigment production is reported to increase in *Monascus* by aeration (Turner, 1971). Similarly, previously both excessive and limited oxygen supply (aeration) has negative impact on growth and pigment production of fungi by formation of metabolites like ethanol and various metabolites L maltose, succinate and dicarboxylic acid.

Phytotoxicity (Raddish seeds assay) (Table 4.5) and cytotoxicity (Brine shrimp assay) (Table 4.6) were carried out with the pigments produced by the three fungi in order to see that whether these pigments were toxic or not. All the three fungi studied showed phytotoxic and cytotoxic activities that were dose and time dependent with some variations in different fungi. Very low phytotoxicity was observed in all the fungi as shown by % seed germination and inhibition (10-20%) (Table 4.5). This showed that these natural colorants are not harmful to plants. The slightly decreased seed germination might be attributed to phytotoxins produced by the fungi which could change plant metabolic activities, causing chlorosis and affecting seed germination (Baker and Tatum, 1998).

The cytotoxicity of the three fungi in present study was directly related to pigment concentration and time. After 48 hours and at maximum pigment concentration (1000ppm), *Aspergillus fumigatus* and *Chaetomium strumarium* showed high cytotoxicity (73%) whereas *Penicillium verruculosum* showed moderate cytotoxicity (53%). But low to moderate cytotoxicity (13-50%) was observed at lower concentrations after 24 hours. Previously, compounds isolated from *Chaetomium globosum,* an endophyte of *Ginko biloba* were active against brine shrimp (Artemia salina) and *Mucor miehei* (Qin et al., 2009) corresponding to presence of some bioactive and cytotoxic compounds present in the pigmented filtrate of the three fungi (Table 4.6). The water soluble fungal pigment, anthraquinones derivatives have also been reported to have antiprotozoal and cytotoxic activities (Nelson and Marasas, 1983; Okamura et al., 1993) which also corresponds to the present study where extracellular pigments are also water soluble and have cytotoxic activities (Table 4.6).

Their possibility to be explored as food colorant can made in view of their low phytotoxic (% seed germination) and cytotoxic activities with their levels determined through IC_{50} by nonlinear regression (Table 4.5 and 4.6). Considering in view this fact, the dosage of the natural obtained from these three fungi can be carefully selected and can be applied in future. However, these aspects of toxicities require more investigation, with the help of animal models (Baker and Tatum, 1998; Mawthols et al., 2005).

The slight to strong presence of alkaloids, anthraquinones, flavonoids, glycosides and terpenoids (Table. 4.2) in all these three fungi is in accordance with previous reports (Langfelder et al., 1998; Kanokmedhakul et al., 2002; Lucas et al., 2007; Nicoletti et al., 2007; Ge et al., 2009). The presence of different phytochemicals in genera *Penicillium* and endophytes (Table 4.2) have been evident by the presence of phenolic compounds, steroids, cardiac glycosides, tannins, alkaloids and flavonoids in an endophytic *Penicillium* sp (Koolen et al., 2002; Devi et al., 2012).

The phenols and flavonoids, which are the pigmented class of plants have also been isolated in all these fungi and interestingly in endophyte (Fig. 4.24 and 4.25). It might be due to transfer of biosynthetic gene between fungus and plant as evident from high antioxidant activity shown by the *Taxus* plants and now its endophyte *Chaetomium strumarium* in present study. Moreover, the stability behavior (Fig. 4.20 and 4.22) of the fungal pigments towards variations in pH and temperature further affirms some similarity between the fungal and plant pigments.

The flavonoids are plant natural products with potentially useful pharmacological and nutraceutical activities. These natural products usually exist in plants as glycosides (He et al., 2008). Previously, study on endophytic fungi producing orange pigment isolated from *Ginkgo Biloba* revealed presence of flavanoids (Liu et al., 2008) which is coinciding with the present study showing presence of flavonoids in both endophytic and non endophytic fungi (Table 4.2) (Fig. 4.24). The presence of flavonoids in these fungal groups (Fig. 4.24) is supported by the production of a benzylated flavonoid by *Penicillium griseoroseum* (Da Silva et al., 2010) and ability of *Aspergillus niger* to produce flavonoid Glycoside Hydrolase (Liu et al., 2012).

Previously, endophytic fungi *Chaetomium globosum* isolated from medicinal plants *Curcuma wenyujin* and *Ginko biloba biloba* (Ding et al., 2006; Qin et al., 2009; Wang et al., 2012; Li et al., 2013) were reported to produce bioactive metabolites corresponding to bioactive potential of endophytic *Chaetomium* in present study (Table 4.2). Moreover, another relevant report in which *Chaetomium* sp is reviewed as a rich source of chaetoglobosins, azaphilones, xanthones, anthraquinones, alkaloids, terpenoids, and steroids further corresponds to the results obtained (Table 4.2) (Fig. 4.24 and 4.25) (Zhang et al., 2006; 2012).

Presence of phenols in *Penicillium verruculosum* (Fig. 4.24) is further supported by the previously reported phenolic compounds in *Penicillium brevicompactum* for chemotaxonomic characterization (Andersen, 1991). Similarly, the presence of phenols in genera *Aspergillus* supported the phenol production by *Aspergillus fumigatus* in current investigations (Fig. 4.25) (Hassall and Lawrence, 1964). Nevertheless, the presence of azaphilones and phenolic glycosides from *Chaetomium elatum* is coherent with the presence of these compounds in the present study in *Chaetomium strumarium* (Fig. 4.25) (Chen et al., 2012). Bioactive anthraquinones and alkaloids have been previously reported from genera *Penicilium*, *Chaetomium* and *Aspergillus* (Bu Lock and Smith, 1968; Bennett et al., 1981; Bachmann, 1992; Huang et al., 1995; Kanokmedhakul et al., 2002; Scherlach and Hertweck, 2006). similar to the findings (Table 4.2) where different species of these fungi are able to produce these compounds. The presence of alkaloids and polyketides in endophytic *Penicillium* sp (Ferreira Koolen et al., 2012; El-Neketi et al., 2013; Lai et al., 2013) supports the presence of these compound classes in endophytes similar to present findings (Table 4.2). Similarly, alkaloids from *Penicillium fellutanum* (Kozlovsky et al, 2000), *Aspergillus* sp. (Scherlach & Hertweck, 2006), *Penicillium aurantiogriseum* (Vinokurova et al., 2004), *Chaetomium globosum* (Ding et al., 2006; Li et al., 2013) and airborne fungus *Aspergillus fumigatus* (Coyle and Panaccione, 2005) are evident of the presence of these compounds classes in soil isolates other than endophytic fungi corresponding to the currentt report (Table 4.2).

Presence of glycosides (Table 4.2) in these fungal groups is evidenced by numerous studies i.e., Glycoside hydrolase production by an anaerobic rumen fungus *Caecomyces communis* (Bata and Gerbi, 1997) and improvement of phenyl ethanoid

glycosides production by a fungal elicitor in cell suspension culture of *Cistanche deserticola* has been reported (Lu & Mei, 2003). The evidence of presence of coumarins in endophytic fungi (Table 4.2) is evident from their presence in endophytic fungus *Pestalotiopsis* sp. of Chinese mangrove plant *Rhizophora mucronata* (Xu et al., 2009; Ye et al., 2013).

The pigmented filtrate obtained from the three fungi showed good antibacterial and antifungal activities (Table 4.3 and 4.4) with maximum activity shown by *Chaetomium strumarium* and *Aspergillus fumigatus* against *B.spizizeni* (23-24mm) whereas *Penicillium verruculosum* extract was least active against this bacteria (7±6.9). Moreover, *Chaetomium strumarium* was least active against *S. typhimorium* (6.6±6.1) (Table 4.3). Overall, PDA and SDA media were significantly effective against all the bacteria (9-24mm) as well as fungi i.e., upto 30mm zone of inhibition against *A. niger* while Czapek dox media showing least pigments was least effective (Table 4.3 and 4.4). The maximum antimicrobial activity shown by pigmented filtrate of fungi in PDA and SDA (Table 4.3 and 4.4) gives an important insight into the fact that antimicrobial activity is directly related to intensity of the pigments released in to culture media and these microbial pigments could be used in both perspectives i.e., medicinal and as bio-control agents (Lawrey, 1986, Huneck, 1999; Gauslaa, 2005; Sipiczki, 2006).

The antimicrobial activity can be correlated to qualitative presence of generalized group of compounds (Table 4.2) with emphasis on quantitative presence of phenols and flavanoids which was highest in *Penicillium verruculosum* followed by *Chaetomium strumarium* and then *Aspergillus fumigatus* (Fig. 4.24 and 4.25). Flavonoids (Kosalec et al., 2012) and anthraquinones isolated from various species of fungus and lichens showed anti-bacterial activity against several Gram positive and Gram-negative bacteria (Yagi et al., 1993). Similarly evidence contributing to antimicrobial activities of these fungi is presence of terpenoids and phenols (Table 4.2) used previously as bactericidal, fungicidal with applications in various industrial sectors i.e., medicinal, cosmetic and food industries (Properzi et al., 2013).

Antimicrobial alkaloid produced previously by *Penicillium* sp. an endophytic fungus isolated from *Mauritia flexuosa* (Ferreira Koolen et al., 2012) showed that the strong

presence of this class of compound in *Penicillium verruculosum* and moderate presence in the other two fungi might be contributing to the antimicrobial activity used in the present study (Table 4.2; 4.3; 4.4). In *Chaetomium strumarium*, pigments might protect the plant from invasion of pathogens and at the same time giving endophyte a selective advantage in present case. Previously, the finding of polyketides in forest soils, where they were exposed to harsh environmental conditions with other competing organisms, has led to the suggestion that those polyketides with antagonistic properties may structure the microbial communities in the soil (Kellner & Zak, 2009) that is in accordance with the presence of polyketide pigments in the two soil isolates i.e., *Penicillium verruculosum* (Fig. 5.2; 5.3; 5.4; 5.5) and *Aspergillus fumigatus* (Fig. 5.46). Similarly, in case of *Chaetomium strumarium*, not living in soil, polyketide pigments (Fig. 5.28) might allow them to survive in discrete ecological niches by reacting to environmental variables such as light, drought or protecting themselves from predators and parasites as previously reported (Huneck, 1999).

Some specific compounds of each fungus detected in LCMS spectra might play a strong role in their biological activities. In *Chaetomium strumarium* fractions, Dihydromaltophytin (Antifungal agent) (Fig. 5.26) and Cochliodinol (Antibacterial agent) (Fig. 5.25) detected in LCMS can be one of the strong reasons for its antimicrobial activities. A pure compound isolated from this fungus being an anthraquinone might also contribute to antibacterial activities (Fig. 5.63 and 5.64). Similarly, in *Penicillium verruculosum*, bioactive *Monascus* pigments (5.1-5.4), bioactive compounds Citrinadin (Fig. 5.7) and Demethyl Calcimycin (Fig. 5.8), the pure compound phenazine 1 carboxylic acid (Fig. 5.65; 5.66) might contribute to antimicrobial activities (Saha et al., 2008). Similarly, the presence of Fusarubin (Fig. 5.47) and Pyrrocidine B (Fig. 5.49) being antibiotic in nature might be a cause of antimicrobial activities shown by *Aspergillus fumigatus*.

The antioxidant activities shown by the three fungi (Fig. 4.26 and 4.27) seemed to be directly related to total flavonoids (Fig. 4.24) and phenolic content (Fig. 4.25) in the present findings. The highest activity was shown by *Penicillium verruculosum* having highest phenols and flavonoids contents (Fig. 4.24 and 4.25) (Table 4.2) followed by *Chaetomium strumarium* and then *Aspergillus fumigatus*. It is evident in a previous

report where flavanoids and phenolics (colored class of compounds) derived from fungi possessed antioxidant and free radical scavenging activities (Dhale and Vijay Raj, 2009). Reactive oxidant species (ROSs) play an important role in degenerative condition such as aging cancer, neuron degenerative disorders, atherosclerosis and inflammations (Aruoma, 1998). These free radicals occur in the body during an imbalance between ROSs (Reactive Oxygen Species) and antioxidants. Hence, the dietary intake of antioxidants is necessary and important to balance the antioxidant status that would reduce the pathological conditions induced by free radicals (Essawi & Srour, 2000).

The antioxidant activity of phenolic compounds depends on the structure, in particular the number and positions of the hydroxyl groups and the nature of substitutions on the aromatic rings. The antioxidant activity increases with increase in degree of hydroxylation and substitution of hydroxyl group at 3 and 5 positions with methoxyl group reduces its activity (Rice Evans et al., 1996). Glycosides attached to flavonoids can significantly increase their antioxidant activity but in case of phenolic acids, they are sufficient alone to cause antioxidant action.

Antioxidant properties of phenolic compounds from *Aspergillus* sp. was previously reported (Palumbo et al., 2007). Moreover, terpenoids and phenols (Table 4.2) present in pigmented filtrate of the three fungi (Table 4.2) have also been previously reported to have antioxidant activities (Properzi et al., 2007). Nevertheless, presence of *Monascus* pigments in all of the three fungi in present study contributing to antioxidant activity resulting in ultimate anticancer activities can be evidenced by a previous report where *Monascus* pigments and their azophilone derivatives from ascomycetes were reported to have antitumour properties (Hosoe et al., 1999; Jongrungruangchok et al., 2004; Fang et al., 2006; Li et al., 2010; Zheng et al., 2010). Antioxidant activity, reducing power and free radical scavenging activity of monascal pigments in rice was previously reported (shcherba et al., 2000).

Antioxidant activity of the pigments of *Aspergillus* genera was previously reported (Zaika and Smith, 1975) with melanins playing a major role (Sheherba et al., 2000; Youngchim et al., 2004) whereas, in present case (Fig. 4.26 and 4.27), *Monascus*

pigments and antibiotic pigment Fusarubin in *Aspergillus fumigatus* might be playing the same role. Different pigments like carotenoids are also known to have a protective action against photooxidation (Berkelmans & van Oppen, 2006) but recently, it was hypothesized that polyketides play a role in protection against oxidative stress in fungi (Luo et al., 2009; Reverberi et al., 2010). Similarly, the pigments and other bioactive compounds detected in LCMS of the three fungi in present case also belong to polyketide class of compounds thus affirming their contribution in antioxidant activity. Previously endophytic *Chaetomium* globosum is known to produce antioxidant compound Flavipin (Ye et al., 2013) supporting the presence of antioxidants in endophytic *Chaetomium* genera coherent with the antioxidant activities shown by endophytic *Chametomium strumarium* (Fig. 4.26 and 4.27). Similarly, anthraquinones from *Chaetomium* sp are previously reported to have anticancer activities corresponding to antioxidant activity shown by *Chaetomium strumarium* can be due to strong presence of anthraquinones (Table 4.2) and a pure anthraquinone compound isolated (Fig. 5.63 and 5.64) while anthraquinones were also strong to moderately present in other two soil isolates i.e., *Penicillium verruculosum* and *Aspergillus fumigatus* (Table 4.2) (Chen et al., 2011).

Fungi are known to produce various classes of bioactive and colored compound classes i.e., polyketides and carotenoids. LCMS profiling of the three extracellular pigment producing fungi was done in order to explore the possibility of presence of previously known or novel compounds in addition to various colored and bioactive compounds. The exogenous release of *Monascus* pigments into media by *Penicillium verruculosum* SG, *Chaetomium strumarium* and *Aspergillus fumigatus* which used to be otherwise cell bound and hydrophobic in nature might be due to their aminophilic moieties that reacted with amino group-containing compounds in the culture medium resulting in formation of water-soluble pigments called azophilones (Lin et al., 1992; Hajjaj et al., 1997).

In the present study, different fungal isolates produced polyketide class of compounds. Moreover, they produced *Monascus* pigments i.e., Monascin (Fig. 5.2), Monascorubrin (Fig. 5.3) and glutamyl Monascorubrin (Fig. 5.4) in *Penicillium verruculosum,* Monascorubramine (Fig. 5.28) in *Chaemtomum strumarium,* while *Aspergillus fumigatus* showed the production of PPR Monascorubramine (Fig. 5.46).

These fungi proved to be the potential candidate of *Monascus* pigments belonging to azophilone group. The reason for multiple extracellular pigments formation in these fungi might be due to the use of complex nitrogen as yeast extract in the culture medium. Yeast extract has been previously reported for the production of Rubropunctatin and/or Monascorubrine in the media possibly by Schiff base formation type of reaction mechanism (Lin et al., 1992; Hajjaj et al., 1997).

Previously, various *Monascus* pigments have been reported in different species of *Penicillium*. For example, *P. marneffei* (Mapari et al., 2005), *Penicillium purpurogenum, Penicillium aculeatum, Penicillium pinophilum* have been reported to produce *Monascus* pigments; Monascorubramine, and Monascin (Buchi et al., 1965; Frisvad, 1989; van Reenen-Hoekstra et al., 1990). The presence of reddish orange pigment, Monascorubrine in *Penicillium verruculosum* SG (Fig. 5.3) has future implications in dyeing and printing due to its stable nature apart from its application in food industry. Moreover, there are many previous reports of this pigment on anti-inflammatory (Yasukawa et al., 1994; Hsu et al., 2012) and antibiotic activity (Martinkova and Vesely, 1995). The yellow pigments, Monascin (Fig. 5.2) showed immunosuppressive activity (Martinkova, 1999). Monascin from red mold was known as a novel antidiabetic, antiobesity and antioxidative stress agent in rats and *Caenorhabditis elegans* (Shi et al., 2012; Lee et al., 2013). Another *Monascus* pigment N-glutamyl Monascorubrin (Fig. 5.4) had been found to be produced by *Penicillium verruculosum* SG. It has been previously reported to be produced by *Monascus ruber* as an extracellular water soluble pigment (Lin et al. 1992; Hajjaj et al. 1997). The red pigments Monascorubramine displayed stronger cytotoxicity and antimitotic effects, which is probably due to the higher reactivity of the dihydropyridine ring was also found in LCMS spectra of the *Chaetomium strumarium* (Fig. 5.27 and 5.28) (Knecht and Humpf, 2006). Similarly the reddish purple pigment PPV Monascorubramine (Fig. 5.46) detected in *A. fumigatus* was previously reported from *Penicillium* sp (Ogihara et al., 2001).

There were a number of some specific compounds produced by each fungal isolate in the current study. For example in *Penicillium verruculosum*, A yellow colored compound Orevactaene previously reported from *Epicoccum nigrum* had been seen in LCMS spectra in one of the fractions of *Penicillium verruculosum* SG (Fig. 5.7). This

compound has been reported to be a binding inhibitor of HIV-1 Rev protein to the Rev response element (RRE) with an IC_{50} value of 3.6 μM (Shu et al., 1997). Calcimycin (Fig. 5.9) is also found in this fungus which is a rare divalent cation specific ionophore antibiotic that has many biochemical and pharmaceutical applications. This compound was previously reported from *Streptomyces chartreusis* (Wu et al., 2011). A novel pentacyclic spiroindolinone alkaloid, citrinadin A, was previously isolated from the fungus *Penicillium citrinum* (Tsuda et al., 2004). It was also indicated in LCMS chromatogram of *Penicillium verruulosum* SG (Fig. 5.8). Pyripyropenes A±R which is acyl-CoA:cholesterol acyltransferase inhibitors is *Aspergillus fumigatus* metabolites biosynthesized from terpene and polyketide called meroterpenoids (Kwon et al., 2002) having antiangeogenic properties. This compound has also been indicated in LCMS chromatogram of *Penicillium verruculosum* SG (Fig. 5.6). Scirpentriol was detected in LCMS spectra of *Penicillium verruculosum* (Fig. 5.11) whose derivatives were previously known to have as antitumor agents (US patent) and as an anti larvicidal (Grove & Hosken, 1975).

A pure crystalline compound, phenzaine 1 carboxylic acid is purified and reported for the first time in *Penicillium verruculosum* SG (Fig. 5.65 and 5.66). Phenazines are N-containing heterocyclic pigmented compounds produced by the species of the bacterial genera and also by the actinomycetes genus, *Streptomyces* (Budzikiewicz, 1993; Li et al., 2007). Phenazine 1 carboxylic acid is a broad range antibiotic and is also used in pesticide M18 (Biocontrol agent against fungal plant pathogens) with high efficiency, low toxicity and good environmental compatibility (Smirnov et al., 1997; Chin-A-Woeng et al. 2001; Hu et al., 2005; Kumar et al., 2005; Smirnov et al., 1997 ;Chin-A-Woeng et al. 2001). The XRD of the phenazine 1 carboxylic acid has been previously reported from florescent *Pseudomonas aurogínosa* (Shanmugaiah et al., 2010).

In case of endophytic *Chaetomium strumarium*, a long period of co-evolution might have resulted in adaptation to its special microenvironments gradually by genetic variation, uptaking some plant DNA segments into their own genomes and vice versa. This could have led to certain endophytes to biosynthesize some "phytochemicals" originated from their host plants (Table 4.2) (Stierle et al., 1993; Zhang et al., 2006).

Alizarin is an anthraquinone and has been known in plant defense mechanism (Eichinger et al., 1999). It was depicted in LCMS chromatogram of endophytic *Chaetomium strumarium* (Fig. 5.29) showing some transfer of biosynthetic gene from plant to fungi. Some plants as well as fungi can synthesize anthraquinones in a different route, specifically, from acetyl-CoA via the polyketide pathway (Eichinger et al., 1999). Previously, bioactive secondary metabolites from the endophytic fungus *Chaetomium sp.* have been isolated from *Salvia officinalis* growing in Morocco (Debbab et al., 2009). The extracellular purple compound i.e., Cochliodinol, an antibiotic metabolite of *Chaetomium* spp. reported previously by Brewer et al., (1970) was also found in *Chaetomium strumarium* (Fig. 5.24 and 5.25). Trytoquialanine A and B (Fig. 5.30 and 5.31) belonging to the group of indole alkaloids were found in LCMS spectra of *Chaetomium strumarium.* They were previously found in *Aspergillus fumigatus* and *Penicillium* sp. and are quinazoline-containing tryptoquivaline bioactive compounds (Clardy et al., 1975; Yamazaki et al., 1979; Gao et al., 2011; Ariza et al., 2002; Yu et al., 2007).

A very important compound Dihydromaltophytin also known as heat stable antifungal factor (HSAF) detected in LCMSMS spectra of *Chaetomium strumarium* (Fig. 5.26). It was previously isolated from *Lysobacter enzymogenes* strain C3, a bacterial biological control agent of fungal diseases (Yu et al., 2007). HSAF shows a novel mode of antifungal action by disrupting the biosynthesis of a distinct group of sphingolipids. It was synthesized by hybrid PKS-NRPS that catalyzed the biosynthesis of the unique macrolactam system that is found in many biologically active natural products isolated from marine organisms (Yu et al., 2007). The BK-channel antagonist Paxilline also detected in LCMS spectra of *Aspergillus fumigatus* (Fig. 5.32) was previously known to possess significant anticonvulsant activity (Sheehan et al., 2009). An anthraquinone orange compound's crystals were purified (Fig. 5.63 and 5.64) from *Chaetomium strumarium* which can be a potential candidate as a natural colorant. Similarly, the presence of the bioactive group of anthraquinone class was previously found in *Chaetomium* genera having anticancer and antimycobacterial activity (Kanokmedhakul et al., 2002; Wijeratne et al., 2006).

The metabolites specific to *Aspergillus fumigatus* were also observed. *Aspergillus fumigatus* is previously known to have antifungal, antifeedant and toxic activities (Li

et al., 2012). *Aspergillus fumigatus* is shown to produce Naphthoquinone pigment (Fig. 5.47) i.e., fusarubin having antibiotic activities whereas it was previously reported from *Fusarium* genus and *Nectria haematococca* (Ammar et al., 1979; Parisiot et al., 1991). Pyrrocidine B which was having antibiotic nature found to be produced by *Aspergillus fumigatus* (Fig. 5.44) and was previously known to be produced by filamentous fungi and *Acremonium zeae* (He et al., 2002; Wicklow and Poling, 2009). *Aspergillus fumigatus* also showed the production of Nivalenol (NIV) (Fig. 5.50) which is known previously to belong to the B-type trichothecene and was produced by *Fusarium* species (Sugita Konishi and Kimura, 2013). The teritrem which is a neuroactive fungal metabolite primarily indole-derived compound produced by *Aspergillus fumigatus* (Fig. 5.51) was reported previously from *Aspergillus terreus* (Peng et al., 1985) having significant insecticidal activity (Dowd et al., 1988). The reason might be the interactions with glutaminergic (Norris et al., 1980) or GABAnergic (Norris et al., 1980; Gant et al., 1987; Yao et al., 1989) receptors occurring in insects and are likely to be target sites for these compounds. Sterigmatocystin (ST) is a yellow polyketide (Versilovskis & De Saeger, 2010) produced by filamentous fungi was also seen to be produced by *Aspergillus fumigatus* (Fig. 5.52) (Rank et al., 2011).

Pigments from basidiomycetes and ascomycetes have been reported to have antitumour properties (Hosoe et al., 1999; Fang et al., 2006). Cytotoxicity of *Monascus* pigments and their azophilone derivatives to human cancer cells showed their anticancer and bioactive nature (Jongrungruangchok et al., 2004; Li et al., 2010; Zheng et al., 2010). Anthone–anthraquinone heterodimers are a series of fungal extracellular color metabolites showing cytotoxic activities against human cancer cell lines (Chen et al., 2013). Numerous reports proved that various fungi showed selective cytotoxicity against human cancer cell lines at a concentration non toxic to normal cells (Li et al., 2010).

The crude pigmented filtrate (Mixture) of *Penicillium verruculosum* used in this study (Table 5.2) have IC_{50} values for cancerous cell line almost equal to that for normal cell lines but after fractionation through column chromatography, the different fractions containing colored and bioactive compounds selectively triggered cancer cell death giving comparatively less harm to normal cells (Table 5.2). The reason is

that the significant decrease of the ratio of cells in the ana and telophase to cells in the pro-meta and metaphase proved a stop of the mitosis at the meta- to anaphase control point. The compounds caused mitotic arrest and the formation of structural damages like c-mitosis through interaction with the mitotic spindle. These effects point to an aneuploidy inducing potential, which has been linked to cancer formation. So, fractions active only against cancer cells were interfering with cell division by effecting spindle fibers or any other mitotic apparatus (Oshimura and Barret, 1986; Miller and Adler, 1989; Pihan and Doxsey, 1999). This cytotoxic effect might have been compensated due to fast re-growth and rehabilitation of normal cells (Langhorne 1997). Previously, various *Penicillium* sp. i.e., extracts of *P. terrestre* tested by Chen et al., (2007), exhibited cytotoxic effects (HL-60, MOLT-4, BEL-7402, and A-549 cell lines). Many toxicological data were available on the red Arpink Red TM from *Penicillium oxalicum* pigment: acute oral toxicity and micronucleus test in mice, anti-tumour effectiveness and AMES test (*Salmonella typhimurium* reverse mutation assay) (Patent 2002; Sardaryan et al., 2004).

Monascorubrine being azophilone have been previously reported to inhibit tumor promotion by 12-O-tetradecanoylphorbol-13-acetate in two-stage during carcinogenesis in mice (Yasukawa et al., 1994). This idea supports the high anticancer activity showed by Monascorubrin (5µg/ml) against KA3IT cell line (Table 5.2) as its anti-inflammatory activity can be one of the reasons to inhibit tumor progression (Yasukawa et al., 1994). Monascin containing fraction also showed cytotoxicity to cancer cell lines in present case. The cytotoxicity study for the yellow and orange pigments Monascin (Blanc et al., 1994), Ankaflavin (Hajjaj et al., 2000), Rubropunctatin, and Monascorubrin when observing embryotoxicity using chicken embryos showed differences in their effects depending on the length of the side chain. However, only the C_5H_{11} side chain homologs Rubropunctatin and Monascin (Blanc et al., 1994) were teratogenic, which was attributed to the higher hydrophility (Martinkova, 1999). Previously, Monascin was known to have no effect in human cancer cell lines Hep G2 and A549 (Blanc et al., 1994; Hajjaj et al., 2000). So the cytotoxicity shown by the Monascin containing fraction might be due to some other anticancer compound in this fraction or due to the difference of cell line.

Brown algal compounds were previously known to have anticancer activities against KA3IT cell line (Ayyad et al., 2003). Cucurbitacins-type triterpene with potent activity on mouse embryonic fibroblast from *Cucumis prophetarum* was studied by Ayyad et al., (2011). Two compounds were found having IC_{50} values lower in cancerous cell line (KA3IT) than normal cell line (NIH3T3) and it is in-line with our findings. The potent cytotoxic activities of different fractions and colored bands (Table 5.2) toward NIH3T3 and KA31T highlighted *Penicillium verruculosum* SG as an important organism in finding novel anticancer drugs. Orevactaene showed IC_{50} value of 5 µg/ml equaling to Monascorubrine and moderate IC_{50} value was also observed for Pyripyropene (Table 5.2) against KA3IT as compared to high IC_{50} value for normal cell line i.e., NIH3T3 showing them as promising source of anticancerous compounds. Numerous studies against cancer cell line showed that some bioactive compounds have anticancer activity at a concentration at which they were not toxic to normal cells (Li et al., 2010).

Cytotoxic azophilone alkaloids and various other antitumour compounds were previously isolated from *Chaetomium* sp. with activities against human cancer cell lines (Wang et al., 2012; Li et al., 2013). Anthraquinones from *Chaetomium* sp. were also reported to have anticancer activities (Wijeratne et al., 2006). The crude pigmented filtrate in the present study (Table 5.4) showed least cytotoxicity revealing the potential of colored filtrate to be used in various industries. Cochliodinol containing fraction in *Chaetomium strumarium* (Table 5.4) showed the highest activity against cancer cell line KA3IT at a concentration lower then IC_{50} for normal cell line NIH3T3, followed by Alizarin and Dihydromaltophytin. All the three mentioned compounds were already reported as bioactive compounds showing their antimicrobial potential but Alizarin is also known to have antioxidant capability that might also contribute to anticancer activity.

The low cytotoxicity to cancer cell line KA3IT by Monascorubramine containing fraction whereas this compound was previously known to have good anticancer activity might be due to the fact that there must be some compound in the fraction that counter the effect of Monascorubramine and have low anticancer activity (Table 5.4) as different length of the hexanoyl or octanoyl side chain did not seem to have an influence on the toxicity in case of Monascorubramine. A pure orange anthraquinone

(Fig. 5.63 and 5.64) compound was isolated and purified from this fungus and reported for the first time from *Chaetomium* genera. Previously, anthraquinones from endophytic fungus *Chaetomium globosum* demonstrated anticancer activity (Wijeratne et al., 2006) and were found to exhibit strong cytotoxicity against a panel of seven human solid tumor cell lines by disrupting the cell cycle leading to the accumulation of cells in either G2/M or S phase, and induce classic signs of apoptosis and another antimycobacterial compound have been isolated from *Chaetomium* sp. previously (Kanokmedhakul et al., 2002).

The pigmented filtrate of *Aspergillus fumigatus* (Table 5.6) contained very good anticancer activities in all the fractions containing important compounds except only one fraction containing teritrem which showed IC_{50} similar in both normal and cancer cell lines. It was the only fungus in which even the crude pigmented filtrate showed good anticancer activity. *Aspergillus fumigatus* is a potential candidate for the production of anticancer compounds (Kusari et al., 2009; Sallam et al., 2012). The antitumor activity of the *Aspergillus* genus pyranone derivatives is previously reported with cytotoxicity of 7µg/ml for cancerous cell line and no toxicity to normal cell line (Elaasser et al., 2011). The IC_{50} values obtained against cancer cell line (Table 5.6) gives potential insight in to the fact that much effective anticancer drugs can be obtained from this fungus as it has much lower IC_{50} values.

PPR Monascorubramine and Fusarubin containing fraction showed IC_{50} value for cancerous cell lines lower than normal cells but the difference was not much. PPR Monascorubramine and Fusarubin were reported to be bioactive pigmented compounds. They might have contributed to some anticancer activity shown by the fractions containing them but their cytotoxicity studies are not done yet up to best of our knowlwdge. Whereas, in case of crude extract, Pyrrocidine b and Communosin g containing fractions there was a high difference between IC_{50} for cancer and normal cell lines with lower IC_{50} values for cancerous cells (Table 5.6). Pyrrocidine b was previously reported to have antibiotic nature whereas Communosine g was not having any prominent bioactivity up till now. So Pyrrocidine B might have contributed in the anticancer activity. Previously, cytotoxicity studies have not been carried out with these compounds.

Some of the fractions proved to be much toxic to normal cell lines too in addition to cancer cell lines. One of the fractions B-d in *Penicillium verruculosum* showed high cytotoxicity to normal cell lines as compared to cancerous cell lines and it contained compounds Verrucine A and Scirpentriol (Table 5.2). It is in accordance with results of Blanc et al., (1994) where he reported presence of such compounds when he modified yeast extract sucrose medium (YES) by adding 160g/l of sucrose and 40 g/l of yeast extract i.e., C:N = 4:1. The high toxicity of Scirpentriol containing fraction might be due to the hydroxyl group at position 3 and decrease in number of acyl groups like other tricothecences was not the pattern followed in Scirpentriol toxicity (Richardson and Hamilton, 1990). In *Chaetomium strumarium*, Tryptoquialanine/ Nivalenol containing fraction also showed good anticancer activities with similar IC_{50} against normal and cancer lines. Whereas in *Aspergillus fumigatus*, Teritrem and Pxilline containing fractions were more cytotoxic to normal cell lines. These compounds were not studied previously with respect to their anticancer activities.

CONCLUSIONS

FUTURE PROSPECTS

Conclusions and Future Prospects

1. **Impact of the study;** Fungal isolates proved to be an important source of various known, novel secondary metabolites and pigments with considerably importance in areas of; pharmaceuticals as antimicrobial and anti-cancer drugs; food, beverages industries as nutritional and coloring supplements in addition to dyeing and printing industries as ecological safe coloring agents. Extracellular water soluble pigments of different hues in the species of *Penicillium, Aspergillus and Chaetomium* have been explored in the present study. The present study also brought out the future cell factories of filamentous fungi that produced both polyketide pigments and many bioactive compounds naturally without the use of genetic manipulation techniques. Also, the presence of these important fungal groups from such less studied places focuses light on future exploration of fungal diversity from competitive environments i.e., soil and plants as they can be a good source of such compounds of immense importance in sectors of food, dyeing and pharmaceuticals.

2. **Effect of nutritional and physiochemical factors in pigment production:** Different physiochemical and nutritional factors played an important role in increasing the pigments producing abilities of fungi. Fungi produced colored metabolites both in liquid and solid state fermentation but solid state was more preferred because of efficient pigments yield. Moreover, pigment production was effected by varying pH, temperature, carbon and nitrogen source and their combined ratios. Maximum pigment yield was attained at pH 5.5, under mesophilic temperatures with glucose and yeast extract as being source of C and N respectively. A systematic evaluation of different factors effecting pigments production and/or a better understanding of the factors inducing pigments should be further studied in future. Moreover, refinement of bioprocess procedures and associated conditions is needed in terms of strategy of fungal cultivation and application for efficient yield, scale up and development of continuous operations (Reactors).

3. **Stability of pigments;** The pigmented extracts of the three fungi showed stability in basic pH and neutral range with red color produced by *Penicillium verruculosum* to be stable at even acidic range. Whereas, the pigments were able to withstand temperatures of almost ≤100°C. This showed a significant structural integrity of the pigments of fungi at varying environmental conditions further depicting their efficacy in commercial products. The *Penicillium verruculosum* pigments were least cytotoxic (Brine shrimp) and phytotoxic with moderate to high toxicity in other two fungi. The pH and temperature stability in addition to the less toxic nature of the natural dyes increased their possibility to be used in food, pharmaceutical and dyeing industry. Moreover, their antioxidant potential can decrease the possibility of cancer. Further investigations with respect to applications should be carried in order to establish their compliance with the industrial level applications.

4. **Phytochemicals in fungi;** Initial qualitative and quantitative screening of fungi showed the presence of important group of compounds in the three fungi i.e., anthraquinones, phenols, flavonoids, terpenes, coumarins and alkaloids. The presence of phenols and flavonoids in the fungi gives an important insight in to the fact that whether some fungal pigments are of plant origin. Moreover the behavior of *Chaetomium strumarium* and *Aspergillus fumigatus* pigments at acidic pH also corresponds to the hypothesis of presence of plant pigments in fungi. A detailed chromatography with emphasis on un known pigments could reveal some novel pigments in fungi like presence of Phenazine 1 carboxylic acid was produced in case of *Penicillium verruculosum*, which was previously a pigment of bacteria.

5. **Secondary metabolites profiling of fungi;** Fungal metabolites profiling using tools suchas HPLC-DAD-MS having a priori knowledge of fungal extrolites gave an important insight in-to variety of compounds present in the three fungi. Monascus pigments were present in all the three fungi. *Penicillium verruculosum* contained more number of important compounds. The most important being Orevactaene, Pyripyropene and Citrinadin in addition to a bioactive yellow compound Phenazine 1 carboxylic acid. The second most important fungi with respect to known compounds was *Chaetomium*

strumarium containing Dihydromaltophytin as most potent compound with another pure orange compound of anthraquinone nature. The third fungi *Aspergillus fumigatus* produced an important bioactive pigment Fusarubin and an antibiotic Pyrrocidine B as important compounds. Furthermore, these important compounds can be purified for future research, applications and their production should be scale up. It is a balanced approach towards choice of possibly safe polyketide natural colorant and bioactive compounds producing fungal cell factories. The structure of the compounds detected in LCMS should be further reconfirmed by purification and analysis by various techniques like NMR for complete structural elucidation.

6. **Cytotoxic studies;** Cytotoxicity studies of colored fractions and crude extracts revealed them non toxic to four normal mammalian cell lines (MDCK, HEK293, HSCT6 and NIH3T3). However, cytotoxicity of the different fractions of the crude color filtrates of the fungi revealed them bioactivity against cancer cell line (KA3IT) showing their anticancer potential. Dose related response should be investigated by using animal models in order to design safe natural colorants for food and pharmaceutical industries. Moreover, future studies on various cancer cell lines and with purified compounds should be carried out in order to explore further the pharmacological potential of fungal compounds.

7. **Unexplored compounds;** Various unknown compounds detected in LCMS have also demonstrated that these filamentous fungi contained some novel compounds which were not previously reported. These compounds should be purified and identified in order to precisely characterize their bioactive potential and other applications in commercial sectors.

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APPENDIX

Appendix

Table A1: Pigments concentration at varying temperatures (15-37) by three pigment producing fungi

Temperature (°C)	*Penicillium verruculosum*	*Chaetomium strumarium*	*Aspergillus fumigatus*
15	12±0.50	14.6±0.34	6.5±0.3
25	17±0.26	15.3±0.30	7.0±0.57
30	10±0.61	8.5±0.230	9.5±0.11
37	7.5±0.28	5.30±0.55	4.0±0.50

Table A2: Pigments concentration (AU) at varying pH (3-9) by three pigment producing fungi

pH	*Penicillium verruculosum*	*Chaetomium strumarium*	*Aspergillus fumigatus*
3	2.10±0.21	3.40±0.56	0.97±0.54
5.5	25±0.440	19.5±0.44	12.1±0.11
7	17.4±0.31	15.4±0.33	8.4±0.320
9	0.50±0.24	0.95±0.65	0.51±0.58

Table A3: Pigments concentration at varying carbon sources by three pigment producing fungi

Carbon source (20g/l)	*Penicillium verruculosum*	*Chaetomium strumarium*	*Aspergillus fumigatus*
Sucrose	20±0.66	12.5±0.98	9.0±0.66
Fructose	21±0.89	17±0.52	10±0.45
Lactose	12±0.87	9.0±0.34	7.5±0.67
Glycerol	10±0.54	6.0±0.47	6.0±0.78
Glucose	25±0.76	19.5±0.44	12±0.66

Table A4: Pigments concentration at varying nitrogen sources by three pigment producing fungi

Nitrogen source	*Penicillium verruculosum*	*Chaetomium strumarium*	*Aspergillus fumigatus*
Peptone	26±0.11	21.0±0.45	16.0±0.49
Yeast	29±0.32	25.5±0.66	17.5±0.21
NaNO3	9.0±0.33	11.0±0.64	8.50±0.86
NH4NO3	14±0.43	19.0±0.78	15.0±0.54

Table A5: Pigments concentration at varying glucose concentration by three pigment producing fungi

Glucose conc (g/l)	*Penicillium verruculosum*	*Chaetomium strumarium*	*Aspergillus fumigatus*
40	19±0.990	20.0±0.70	9.20±0.59
20	27.0±0.70	16.0±0.54	12.4±0.40
10	20.5±0.65	13.0±0.30	10.6±0.50
5	17.0±0.63	12.5±0.21	7.30±0.60
2	10,0±0.33	8.50±0.56	5.10±0.33
1	6.0±0.200	6.50±0.49	2.60±0.32
0.5	1.25±0.11	0.65±0.59	0.95±0.16
0	0.02±0.24	0.19±0.90	0.01±0.19

Table A6: Pigments concentration at varying C: N ratio by three pigment producing fungi

C:N ratio	*Penicillium verruculosum*	*Chaetomium strumarium*	*Aspergillus fumigatus*
20:01	2.02±0.22	17.0±0.75	15.0±0.33
10:01	22.5±0.33	30.0±0.66	17.5±0.23
8:01	24.5±0.89	28.5±0.43	20.0±0.33
5:01	25.5±0.56	24.0±0.21	17.5±0.89
4:01	35.0±0.11	12.5±0.21	9.50±0.77

Table A7: UV vis absorbance of the *Chaetomium strumarium* after treatment at different pH for 6 hrs.

Chaetomium strumarium			
Time (hrs)	pH 9	pH 7	pH 3
0	290±0.22	30.0±0.57	15.0±0.31
2	28.5±0.45	29.5±0.69	12.5±0.55
4	27.5±0.56	29.0±0.99	10.0±0.44
6	27.0±0.55	28.0±0.88	10.0±0.17

Table A8: UV vis absorbance of the *Aspergillus fumigatus* after treatment at different pH for 6 hrs

Aspergillus fumigatus			
Time (hrs)	pH 9	pH 7	pH 3
0	20.±0.97	20.0±0.76	10.0±0.76
2	20±0.35	20.0±0.73	7.05±0.22
4	20±0.21	19.5±0.16	7.50±0.19
6	19±0.78	19.0±0.19	5.00±0.22

Table A9: UV vis absorbance of the *Penicillium verruculosum* after treatment at different pH for 6 hrs

Penicillium verruculosum			
Time (hrs)	pH 9	pH 7	pH 3
0	35.0±0.99	35.0±0.33	32.5±0.99
2	34.5±0.55	34.5±0.21	31.0±0.32
4	34.0±0.47	34.0±0.22	29.5±0.99
6	32.5±0.47	33.5±0.11	28.5±0.21

Table A10: UV/Vis spectroscopy of the pigmented filtrates of *Penicillium verruculosum* after treatment at different temperatures for 6 hrs

Penicillium verruculosum							
Time (hrs)	_ 20 °C	4°C	15° C	37° C	50 °C	75° C	100°C
0	35±0.21	35.0±0.35	35.0±0.99	35.0±0.99	35.0±0.56	35.0±0.54	35.0±0.11
2	33±0.22	34.0±0.38	35.0±0.88	34.5±0.21	33.5±0.0	32.5±0.55	33.0±0.66
4	30±0.44	32.5±0.98	33.5±0.64	32.5±0.46	31.5±0.0	30.0±0.66	29.5±0.77
6	28±0.55	29.5±0.67	32.0±0.33	30.0±0.65	29.5±0.01	29.5±0.21	29.0±0.50

Table A11: UV/Vis spectroscopy of the pigmented filtrates of *Chaetomium strumarium* after treatment at different temperatures for 6 hrs

Chaetomium strumarium							
Time (hrs)	_ 20 °C	4°C	15° C	37° C	50 °C	75° C	100°C
0	30.0±0.99	30.0±0.55	30.0±0.34	30±0.45	30±0.77	30±0.12	30±0.20
2	29.0±0.88	29.5±0.43	29.0±0.14	28±0.63	27±0.87	26±0.84	25.5±0.5
4	28.0±0.77	29.5±0.32	29.0±0.00	27±0.68	26±0.98	25±0.16	25±0.55
6	27.0±0.54	28.5±0.21	28.0±0.16	27±0.76	25±0.36	24.6±0.11	25±0.40

Table A12: UV/Vis spectroscopy of the pigmented filtrates of *Aspergillus fumigatus* after treatment at different temperatures for 6 hrs

Aspergillus fumigatus							
Time (hrs)	_ 20 °C	4°C	15° C	37° C	50 °C	75° C	100°C
0	20±0.20	20±0.14	20±0.870	20±0.210	20±0.13	20±0.21	20±0.31
2	19±0.33	20±1.10	19.5±0.22	19.5±0.33	18±0.33	17.5±0.1	16.5±0.4
4	18±0.87	18.5±0.23	18±0.990	17.5±0.55	17±1.10	16.5±0.2	16.5±0.3
6	17±0.77	17.5±0.55	18±0.770	16±0.330	15±0.40	14±0.42	15.5±0.3

Table A13: Total antioxidant activity (%) of the three fungi

Conc	*Chaetomium strumarium*	*Aspergillus fumigatus*	*Penicillium verruculosum*
0.	0.00±0.00	00.00±0.98	0.00±0.00
25.	67.32±0.76	48.75±0.98	70.45±0.99
50.	67.56±0.12	67.32±0.14	73.90±1.1
100.	70.45±2.89	70.45±0.66	74.90±1.6
200.	71.45±0.98	74.90±0.86	89.51±2.1
250.	89.51±3.76	80.00±2.10	92.00±2.6
300.	91.00±2.32	81.00±2.80	95.24±2.4

Table A14: DPPH free radical scavenging activity (%) of the three fungi

Conc	*Penicillium verruculosum*	*Aspergillus fumigatus*	*Chaetomium strumarium*
0.	0.00±0.00	0.0±0.00	0.0±0.00
25.	69.2±1.20	38.77±2.1	33.8±1.2
50.	73.78±2.1	58.97±1.2	67.0±0.99
100.	75.09±2.3	69.8±0.99	69.8±0.88
200.	91.0±0.99	80.0±0.66	89.5±0.54
250.	94.00±3.2	84.2±3.10	92.2±1.9
300.	99.10±3.1	91.0±2.10	95.5±1.87

Table A15: Total ABTS scavenging activity (%) of the three pigment producing fungi

Conc	*Penicillium verruculosum*	*Aspergillus fumigatus*	*Chaetomium strumarium*
0.	0.00±0.00	0.00±0.00	0.00±0.0
25.	13.1±0.99	16.6±0.54	12.5±1.6
50.	33.1±0.54	29.5±0.98	23.3±3.2
100.	58.41±1.2	36.8±1.20	42.8±1.4
200.	65.1±0.76	59.1±1.70	68.6±0.77
250.	70.4±0.54	61.3±2.30	73.3±0.65
300.	81.9±1.2	78.2±1.20	80.1±1.2

Table A16: Total reducing power (%) of the three pigment producing fungi

Conc	*Aspergillus fumigatus*	*Penicillium verruculosum*	*Chaetomium strumarium*
0.	0.00±0.00	0.00±0.00	0.00±0.0
25.	15.1±0.16	26.6±0.18	16.6±2.7
50.	22.4±0.78	27.3±0.78	25.4±0.43
100.	30.2±2.90	35.8±1.2	32.2±0.13
200.	34.6±0.12	39.1±0.96	36.5±0.56
250.	39.1±0.96	43.4±0.17	41.2±3.78
300.	40.2±3.40	45.2±1.2	42.2±0.27

Table A17: Cytotoxicity assays against Mammalian cell line (HEK) by the various fractions containing important compounds

Conc	Monascorubr amine	Dihydomaltop hytin	Trypto, pax	Alizarin	Crude	Cochliodinol
200	5.9±0.70	34.0±1.40	5.6±0.900	6.92±0.90	8±0.20	10±0.10
100	5.2±0.98	37.9±1.50	5.9±0.550	16.37±0.88	17±0.23	26±0.30
50	21.1±0.55	63.5±1.60	17.5±±0.98	39.94±0.44	33±0.54	41±0.44
20	52.7±1.10	56.4±1.30	41.9±0.40	48.09±1.20	42±0.98	55±0.55
10	59.4±1.20	52.2±0.98	46.5±1.30	54.01±1.40	51±0.99	62±0.21
5	62.4±2.10	52.7±0.99	43.6±0.76	53.0±1.66	56±0.54	70±0.44
2	79.6±1.50	55.7±0.56	78.4±0.99	60.23±1.80	69±0.44	75±0.77
1	95.5±0.99	79.3±0.44	75.0±0.65	70.58±3.20	81±0.21	82±0.21

Table A18: Cytotoxicity assays against Mammalian cell line (HEK) by the various fractions containing important compounds

Conc	Quinolone	Fusarubin	Pyrrocidine B	Nival,teritrem	Communosine	Crude
20	3.0±0.00	8±0.66	41.5±0.99	12.1±0.87	5.1±0.67	14.0±0.1
100	7.0±0.99	16±0.43	40.1±0.99	30.8±0.99	4.8±0.61	26±0.2
50	12.2±0.6	24±0.21	40.7±0.45	27.0±0.76	20±0.26	33±0.22
20	19±0.34	33±0.55	46.7±0.32	47.1±0.35	26.3±0.6	45±0.34
10	23±0.21	45±0.21	49.4±0.99	53.5±0.66	25.8±0.23	56±0.45
5	37.6±0.3	53±0.11	45.0±0.12	47.8±0.47	30.12±1.2	68±0.88
2	63.0±0.4	59±0.55	59.1±0.18	53.0±0.25	43.12±1.4	75±0.76
1	88.3±0.9	64±0.77	82.3±0.88	58.0±0.37	52.43±2.1	78±0.77

Table A19: Cytotoxicity assays against Mammalian cell line (HSCT6) by the various fractions containing important compounds

Conc	Nival,teritrem	Fusarubin	Pyrrocidine B	Crude	Communosine	Quinolone
200	31.8±0.59	17.7±0.29	18.17±0.57	48.5±1.9	10±0.99	25±0.19
100	29.4±1.10	16.5±0.14	8.20±0.81	54.5±0.8	23±0.55	35±0.18
50	30.2±0.90	26.2±0.43	7.74±0.86	64.1±0.76	37±0.32	39±0.15
20	30.0±0.98	71.4±0.48	61.4±0.19	69.7±0.71	41±0.21	43±0.22
10	35.0±1.1	67.0±0.43	76.6±0.76	87.5±0.43	49±0.11	51±0.29
5	38.0±1.50	76.6±0.76	77.7±0.81	79.4±0.48	56±0.20	58±0.33
2	42.0±0.99	75.8±0.95	76.0±0.52	64.0±3.3	66±0.19	66.0±1.3
1	58.0±0.24	83.1±0.14	79.5±0.19	77.2±0.81	72±0.54	79.0±2.1

Table A20: Cytotoxicity assays against Mammalian cell line (HSCT6) by the various fractions containing important compounds

Conc	Cochliodinol	Monascorubramine	Dihydomaltophytin	Alizarin	Trypto, Pax	Crude
200	23.0±0.44	18.0±0.32	20.0±2.70	2.0±0.00	29±0.66	10±0.22
100	25.0±0.51	22.0±0.11	26.0±3.30	5.98±0.92	33±0.11	17±0.90
50	29.0±0.61	50.2±0.69	33.0±3.00	16.7±0.47	40±0.12	22±0.91
20	48.0±0.73	65.0±0.88	49.1±0.99	50.0±0.11	45±0.33	45±0.90
10	65.0±0.79	86.0±0.94	65.0±0.66	65.0±0.1	54±0.35	69±1.90
5	78.0±0.00	86.0±0.43	74.0±0.22	70.0±1.1	73±0.37	87±2.80
2	79.2±0.69	88.0±2.60	82.8±0.92	72.0±2.3	81±0.49	90±1.90
1	83.0±2.20	93.0±1.20	96.0±0.75	78.0±2.4	84±0.50	92±1.60

Table A21: Cytotoxicity assays against Mammalian cell line (KA3IT) by the various fractions containing important compounds

Conc	Quinolone	Fusarubin	Pyrrocidine B	Nival,teritrem	Communosine	Crude
200	15±0.110	5.6±0.00	4.9±0.40	6.03±0.11	7±0.710	16.0±0.21
100	20.9±0.190	4.6±0.33	11.06±0.5	5.96±0.67	10±0.73	24.0±0.98
50	13.7±0.330	4.6±0.33	14.1±1.10	10.2±0.19	13±0.69	35.0±0.76
20	7.76±0.670	4.6±0.67	28.9±0.98	14.7±0.88	18±0.55	38.0±0.66
10	9.66±0.670	9.2±0.67	34.76±0.19	21.5±0.66	22±0.44	42.0±0.37
5	7.7±0.110	23.8±0.11	29.4±0.85	22.8±0.83	27±0.91	48.0±2.1
2	50.0±0.13	28.0±0.16	45±0.120	37.0±0.92	35±0.81	54.0±1.4
1	65.0±1.80	30.0±0.22	54±0.400	50.0±0.93	45±0.51	60.0±0.88

Table A22: Cytotoxicity assays against Mammalian cell line (KA3IT) by the various fractions containing important compounds

Conc	Cochliodinol	Monascorubramine	Dihydomaltophytin	Trypto, pax	Alizarin	Crude
200	6.19±0.78	6.66±0.67	7.56±0.27	6.98±0.92	5±0.88	7±0.43
100	5.4±0.650	15.6±0.45	6.47±0.88	5.98±0.92	8±0.77	10±0.4
50	6.2±0.180	50.2±0.69	5.94±0.76	16.7±0.71	19±0.55	15±0.48
20	11.6±0.67	81±0.88	49.1±0.65	66.82±0.53	50±0.33	42±0.77
10	76±0.88	86.0±0.88	73.7±0.49	80±0.88	65±0.88	66±0.79
5	78.0±0.54	86.07±0.43	69±0.88	88.49±0.02	73±0.81	87±0.70
2	79.2±0.69	90±0.77	82.8±0.92	84.88±0.35	81±0.62	90±1.1
1	87±0.88	93.0±0.43	96±0.88	90±0.88	84±0.31	92±2.5

Table A23: Cytotoxicity assays against Mammalian cell line (MDCK) by the various fractions containing important compounds

Conc	Monascorubramine	Dihydomaltophytin	Trypto, pax	Alizarin	Crude	Cochliodinol
200	7.78±0.14	19.3±0.94	7.5±0.04	8.9±0.76	6±0.88	7±0.88
100	15.4±0.98	30.45±0.98	47.3±0.73	20.6±0.45	13±0.99	13±0.00
50	26.3±0.55	73.64±0.06	18.4±0.76	45.9±0.96	38±0.77	23±0.61
20	61.9±0.57	81.6±0.06	65.4±0.76	93±0.88	89±0.66	70±0.71
10	75.8±0.53	90±0.88	79.5±0.02	96.7±0.88	92±0.22	79±0.78
5	88.4±0.59	92.7±0.88	94.7±0.49	91.01±0.61	94±0.43	82±0.90
2	90±0.88	91.6±0.67	96±0.88	94.9±0.57	95±0.54	90±0.75
1	94±0.89	96±0.88	98±0.99	94.5±0.02	98±0.67	94±0.89

Table A24: Cytotoxicity assays against Mammalian cell line (MDCK) by the various fractions containing important compounds

Conc	Quinolone	Fusarubin	Pyrrocidine B	Nival,teritrem	Communosine	Crude
200	10±0.88	9.20±0.78	23.6±0.02	15.2±0.22	16±0.74	25±0.91
100	15±0.81	8.5±0.01	41.4±0.87	15.0±0.39	24±0.60	35±0.88
50	19.9±0.05	11.5±0.24	59.5±0.99	18.6±0.45	33±0.50	42±0.71
20	10.5±0.43	11.1±0.18	55.9±0.97	9.8±0.55	45±0.20	53±0.69
10	12.3±0.89	20±0.80	59.9±0.05	13.2±0.38	52±0.30	66±0.81
5	22.2±0.52	40±0.89	61.6±0.14	20±0.78	63±0.77	72±0.09
2	62.3±0.89	60±0.85	67.4±0.26	28±0.80	70±0.79	78±0.90
1	85.1±0.32	75±0.76	80±0.88	51±0.70	73±0.91	85±0.87

Table A25: Cytotoxicity assays against Mammalian cell line (NIH3T3) by the various fractions containing important compounds

Conc	Quinolone	Fusarubin	Pyrrocidine B	Nival,teritrem	Communosine	Crude
200	3±0.98	3.88±0.94	24.68±1.2	9.92±0.55	5±0.66	26±0.79
100	3±0.91	4.28±0.42	32.92±0.90	21.12±0.91	10±0.76	31±0.54
50	6.72±0.93	10.4±0.94	38.32±0.78	20.8±0.73	25±0.92	45±0.90
20	14±0.76	22.24±0.99	49.28±0.96	27.4±0.99	50±0.21	55±0.72
10	34±0.72	20.12±0.71	55.64±0.77	29.96±0.71	65±0.99	63±0.49
5	40±0.79	23.92±0.72	57.88±0.92	32.36±0.92	70±0.43	75±0.91
2	50.12±0.43	63.16±0.43	66.04±2.1	41±0.46	75±0.79	80±0.00
1	78±0.41	79.12±0.55	81.88±2.3	53±0.61	78±0.43	82±2.1

Table A26: Cytotoxicity assays against Mammalian cell line (NIH3T3) by the various fractions containing important compounds

Conc	Monascorubr amine	Dihydomaltop hytin	Crude	Alizarin	Trypto, pax	Cochliodinol
200	9.70±0.77	13.05882	11.6±0.20	24.96078	27±0.38	23±0.50
100	29.39±0.79	37.9±0.21	24.3±0.77	31.82±0.7	38±0.87	33±0.87
50	25.86±0.72	67.76±0.77	48.09±0.21	51.7±0.74	46±0.45	47±0.20
20	48.8±0.20	68.13725	61.84314	75.7±0.77	60±0.45	66±0.20
10	69±0.23	79.23±0.71	72.96±0.74	79.27451	73±0.20	79±0.50
5	79±0.50	83.5±0.77	82.52941	84±0.20	84±0.50	88±0.45
2	84±0.87	87±0.27	90±0.87	88±0.87	88±0.87	90±0.45
1	92±0.50	90±0.29	93±0.50	90±0.20	90±0.20	92±0.20

Table A27: Cytotoxicity assays against Mammalian cell line (KA3IT) by the various fractions containing important compounds

Conc	B	E	F
200	8.91±0.45	5.45±0.70	4.79±0.45
100	22.9±0.70	5.8±0.55	9.11±0.45
50	57.7±0.55	10.2±0.70	20±0.70
20	58±0.55	36.8±0.55	40±0.55
10	66±0.70	64.7±0.45	60±0.45
5	70±0.30	94.2±0.45	73.6±0.49
2	79±0.30	79.3±0.30	81.7±0.70

Table A28: Cytotoxicity assays against Mammalian cell line (KA3IT) by the various fractions containing important compounds

Conc	Monascin	Analogue monascorubrin	Monascorubrin
200	4.9±0.81	5.20±0.81	3.91±0.55
100	7.973199	4.37±0.55	3.16±0.81
50	11.6±0.81	20.7±0.81	3.0±0.30
20	45±0.55	50±0.30	10.5±0.55
10	93.9±0.55	70.5±0.81	40.37±0.81
5	94.15±0.55	80.1±0.30	72.6±0.30
2	95.5±0.81	95.0±0.55	73.6±0.45
1	99.3±0.55	92.8±0.30	76.2±0.45

Table A29: Cytotoxicity assays against Mammalian cell line (KA3IT) by the various fractions containing important compounds

Conc	Glutamyl monascorubrin	Scirpentriol, verrucine	Orevactaene
200	4.9±0.45	5.20±0.70	3.9±0.55
100	6±0.81	4.37±0.89	3.1±0.81
50	10±0.55	35±0.81	3.04±0.70
20	38±0.30	50±0.45	10.5±0.45
10	65±0.55	70.5±0.55	40.3±0.81
5	94.1±0.70	80.1±0.55	72.6±0.70
2	95.5±0.70	95.0±0.30	73.0±0.45
1	99.3±0.70	92.8±0.30	76.2±0.70

Table A30: Cytotoxicity assays against Mammalian cell line (KA3IT) by the various fractions containing important compounds

Conc	Calcimycin	Citrinadin	Pyripyropene
200	4.9±0.55	5.2±0.55	3.9±0.55
100	7.9±0.70	4.3±0.70	3.1±0.70
50	15±0.30	20. ±0.7	10±0.45
20	38±0.81	50±0.70	25±0.81
10	68±0.30	60±0.81	50±0.30
5	94.0±0.5	73±0.30	72.6±0.70
2	95.5±0.5	81±0.45	73.6±0.45
1	99.3±0.4	89±0.45	76.2±0.45

Table A31: Cytotoxicity assays against Mammalian cell line (MDCK) by the various fractions containing important compounds

Conc	Monascin	Monascorubrin	Analogue monascorubrin
200	8.58±0.30	5.35±0.87	5.0±0.810
100	7.60±0.26	7.16±0.35	17.1±0.00
50	55.0±0.81	45.0±0.30	60.0±0.30
20	70.0±0.84	64.0±0.26	65.0±0.26
10	76.0±0.84	78.0±0.81	75.0±1.20
5	76.45±0.30	71.6±0.30	85.0±0.30
2	83.5±0.77	93.9±±0.55	91.4±0.30
1	92.5±0.82	96.1±0.89	96.3±0.81

Table A32: Cytotoxicity assays against Mammalian cell line (MDCK) by the various fractions containing important compounds

Conc	Glutamyl monascorubrin	Orevactaene	Scirpentriol, verrucine
200	8.50±0.81	5.35±0.81	5.0±0.30
100	7.60±0.70	7.10±0.30	17.1±0.81
50	47.0±0.81	54.0±0.81	25.0±0.70
20	60.0±0.70	69.0±0.30	35.0±0.35
10	76.05±0.81	78.0±0.70	45.0±0.30
5	76.45±0.30	71.3±0.81	60.0±0.70
2	83.5±0.35	93.9±0.35	75.0±0.35

Table A33: Cytotoxicity assays against Mammalian cell line (MDCK) by the various fractions containing important compounds

Conc	Pyripyropene	Calcimycin	Citrinadin
200	8.5±0.35	5.35±0.81	5±0.45
100	7.6±0.81	7.16±0.81	17.1±0.3
50	50±0.30	45±0.81	60±0.30
20	56±0.81	65±0.81	67±0.35
10	70±0.30	78±0.30	75±0.81
5	76.4±0.3	71.3±0.3	84±0.81
2	83.5±0.81	93.9±0.4	91±0.81
1	92.5±0.81	96.1±0.4	90±0.35

Table A34: Cytotoxicity assays against Mammalian cell line (HSCT6) by the various fractions containing important compounds

Conc	Monascin	Monascorubrin	Analogue monascorubrin
200	6.0±0.45	4.3±0.81	9.40±0.81
100	4.35±0.81	3.3±0.70	3.50±0.45
50	46±0.55	50±0.81	60±0.81
20	67±0.59	77.5±0.70	81.95±0.45
10	87.0±0.45	93.9±0.45	84.7±0.54
5	77.75±0.54	87.5±0.70	91.5±0.70
2	88.9±0.55	98.0±0.45	82.5±0.70
1	94.2±0.55	89.46±0.81	88.76±0.45

Table A35: Cytotoxicity assays against Mammalian cell line (HSCT6) by the various fractions containing important compounds

Conc	Pyripyropene	Calcimycin	Citrinadin
200	6.0±0.59	4.3±0.79	9.40±0.79
100	40±0.70	10±0.45	3.5±0.81
50	60±0.45	30±0.70	45±0.45
20	80±0.70	72±0.81	65±0.81
10	87.0±0.45	82±0.30	76±0.59
5	77.7±0.79	90±0.39	84±0.30
2	88.9±0.59	98.0±0.59	90±0.70
1	94.2±0.70	89.4±0.79	91±0.30

Table A36: Cytotoxicity assays against Mammalian cell line (HSCT6) by the various fractions containing important compounds

	Glutamyl monascorubrin	Orevactaene	Scirpentriol, Verrucine
200	6.0±0.810	4.30±0.59	9.4±0.45
100	15.0±0.59	10.0±0.45	3.5±0.30
50	60.0±0.66	55.0±0.77	15±0.79
20	74.0±0.45	68.0±0.59	35±0.59
10	87.0±0.30	77.0±0.79	50±0.45
5	77.7±0.45	87.5±0.30	65±0.30
2	88.0±0.77	98.0±0.53	70±0.79
1	94.2±0.48	89.4±0.45	80±0.45

Table A40: Cytotoxicity assays against Mammalian cell line (NIH3T3) by the various fractions containing important compounds

Conc	Monascorubrin	Analogue of monascorubrin	Monascin
200	10±0.30	19±0.30	9±0.77
100	37±0.77	25±0.77	30±0.30
50	40±0.45	45±0.45	60±0.59
20	65±0.79	61±0.77	53±0.79
10	70±0.59	70±0.30	60±0.45
5	75±0.77	72±0.79	68±0.59
2	81±0.79	70±0.59	85±0.30
1	90±0.30	75±0.45	87±0.79

Table A41: Cytotoxicity assays against Mammalian cell line (NIH3T3) by the various fractions containing important compounds

Conc	Calcimycin	Citrinadin	Pyripyropene
200	10±0.79	19±0.45	9.0±0.30
100	37±0.77	25±0.30	30±0.59
50	50±0.30	60±0.59	53±0.66
20	60±0.59	70±0.45	60±0.79
10	65±0.45	75±0.59	70±0.77
5	75±0.77	79±0.79	78±0.30
2	81±0.59	83±0.77	85±0.66
1	90±0.30	93±0.66	87±0.69

Table A42: Cytotoxicity assays against Mammalian cell line (NIH3T3) by the various fractions containing important compounds

Conc	Orevactaene	Scirpentriol,verrucine	Glutamyl Monascorubrin
200	10±0.66	3±0.66	9±0.77
100	37±0.22	10±0.77	30±0.23
50	40±0.77	30±0.45	53±0.66
20	53±0.30	40±0.11	60±0.32
10	65±0.45	45±0.77	70±0.45
5	75±0.44	55±0.66	80±0.65
2	81±0.55	63±0.99	85±0.54
1	90±0.66	75±0.87	87±0.45

Table A43: Cytotoxicity assays against Mammalian cell line (NIH3T3) by the various fractions containing important compounds

Conc	B	E	F
200	10±0.30	19±0.55	9.0±0.21
100	35±0.84	30±0.65	30±0.30
50	55±0.55	52±0.99	60±0.99
20	65±0.87	61±0.30	70±0.55
10	70±0.99	80±0.87	74±0.87
5	75±0.77	85±0.12	80±0.30
2	81±0.55	85±0.55	85±0.87
1	90±0.22	91±0.76	87±0.89