SECONDARY METABOLITES

Plants, humans and animals are highly susceptible to various diseases. These diseases are either airborne, waterborne or contiguous; in general terms they are microorganisms borne. Similar to higher organisms, these microbes are also susceptible to certain chemicals. Therefore, the problem of pathogenic microorganisms can be solved through the use of such chemicals directly, which inhibit their growth and metabolism. However, use of these chemicals is lethal, as it may kill the useful organisms present in the host body. One way to solve these problems is to find substance that directly inhibits the metabolic pathway of these pathogenic microbes without harming useful microflora required by the host for its metabolism. Several such synthetic chemicals/drugs are available in the market, which are able to control these pathogenic organisms. However, these chemicals are also reported harmful to host body. Alternative option to these problems is to isolate secondary metabolites produced by certain group of microbes which are highly effective against the pathogenic organisms. Use of such bioactive natural products started since the second world war with the invention of penicillin by Alexander

Fleming (1929). Since that time, several such secondary metabolites are isolated and are marketed with various brands by different pharmaceutical companies. In the recent years, extensive studies are initiated to overcome the problems of side effects of these synthetic drugs. In this connection, in the present study attempt has been made to explore the possibility of any such biological molecule from different fungal isolates.

4.1 Natural products:

The usual definition of natural products in the widest sense emphasizes that, "they are chemical compounds isolated from diverse living things". These compounds may be derived by primary or rather secondary metabolism of living organisms. Nature produces an amazing variety and number of products. About 100,000 secondary metabolites of molecular weight less than 2500 Da have been characterized, mainly from plants (Roessner and Scott, 1996) and some 50,000 are from microorganisms (Fenical and Jensen, 1993; Berdy, 1995).

Soils are complex ecosystems, consisting a consortium of different microorganisms. Thus, it is not surprising that its inhabitants have evolved chemical defenses against each other for their survival and competition for food and space. These defense mechanisms are nothing but the synthesis of unique chemical substances referred as secondary metabolites, which are important part of the natural products. Most of the compounds extracted from microorganisms usually exhibit some kinds of biological activities, known as the bioactive secondary microbial metabolites (Berdy, 2005). The exact definition of secondary metabolites for long time is the most disputed and most obscure field in the whole area of microbiology. Most characteristic features are their incredible array of unique chemical structures, with their very frequent occurrence and versatile bioactivities (Demain and Fang, 2000).

Advantages of natural products

- Natural products offer unmatched chemical diversity with structural complexity and biological potency (Verdine, 1996).
- Natural products have been selected by nature for specific biological interactions. They have evolved to bind to proteins and have drug like properties (Nisbet and Moore, 1997).
- Research on natural products has led to the discovery of novel mechanisms of action, for example, the discovery of the role of guggulsterone (Urizar *et al.*, 2002).
- Natural products are powerful biochemical tools, serving as "pathfinders" for molecular biology and chemistry and in the investigation of cellular functions (Hung *et al.*, 1996).
- Natural products can guide the design of synthetic compounds (Breinbauer *et al.*, 2002).

The secondary metabolites isolated from microbes possessing either antimicrobial (antibacterial, antifungal, antiprotozoal), antitumor and/or antiviral activities, are called as antibiotics. Antibiotics can also be defined as secondary metabolites which regulate growth processes, replications, and/or attribute some kind of responding actions (regulating, inhibiting, stimulating) to the (life cycle of) prokaryotic or eukaryotic cells at the biochemical level in minimal concentration (Berdy, 2005).

The practical importance of antibiotics and other secondary metabolites is tremendous. They are widely used in veterinary, agriculture, scientific research and in countless other areas. In general, natural products including the microbial metabolites may be practically utilized in three different ways: i) Applying the natural/fermentation product directly in the medicine, agriculture, or in any other fields. ii) Using as starting material for subsequent chemical or microbiological

modification (derivatization) and iii) Using as lead compounds for chemical synthesis of new analogs or as templates in the Rational Drug Design (RDD) studies. Now a days, most commonly used technique is the chemical synthesis of new analogs from naturally derived products. They are termed as semi synthetic products.

4.1.1 Semi synthetic products

The molecules produced by microbes that are subsequently modified by a chemist to enhance their antimicrobial properties. These natural products are chemically modified in order to:

- improve the effieciency of the natural product.
- reduce its side effects.
- circumvent developing resistance by the targeted bacteria/microbes.
- expand the range of bacteria/microbes that can be treated with it.
- to improve bioavailability of product.
- easy dosage form (can be given orally, parental form).
- as diluent, glident, lubricant, binder in sustained release and novel drug delivery system.
- manufacturing routes are known and are not complex.
- usage of different precursors used to make one drug.

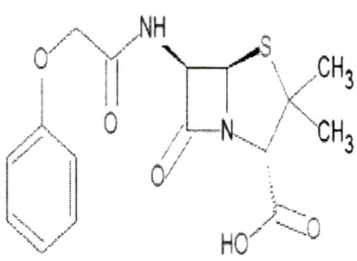
Precursor for production of all these semi synthetics are natural products. As a source of natural products, fungi are diverse and valuable resources for the discovery of novel compounds. The chemical potential of fungi is enormous and new approaches are devised to access the genetic and chemical diversity efficiently to develop new medicines/drugs. Most fungi produce plethora of bioactive natural products. Fungal metabolites display broad range of activities against pathogenic microbes, exemplified best by antibiotic penicllin (Dirk and Keller, 2007). The number of fungal species in the world is estimated to be at least 1.5 million (Hawksworth, 1991) thus, fungi are

the most probably one of the major sources for new and useful metabolites. According to Dreyfuss and Chapela (1994), approximately 4,000 secondary metabolites of fungal origin have been described to possess biological activities.

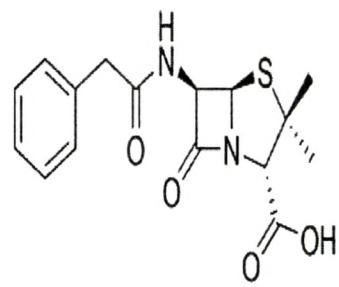
4.2 Antibiotics:

Antibiotics include a chemically heterogenous group of small organic molecules that are of microbial in origin. At low concentrations, they are deleterious to growth and metabolic activities of other microorganisms (Thomashow and Weller, 1995). Currently, it is the most important field of research in microbial biotechnology due to their highly significant effects on bacterial and fungal infections (Wainwright, 1994). They represent the greatest contribution for drug therapy and health care of increasing population of the world. It also provides effective control of many microbial pathogens that have been causing death of humans and animals (Robbers *et al.*, 1996).

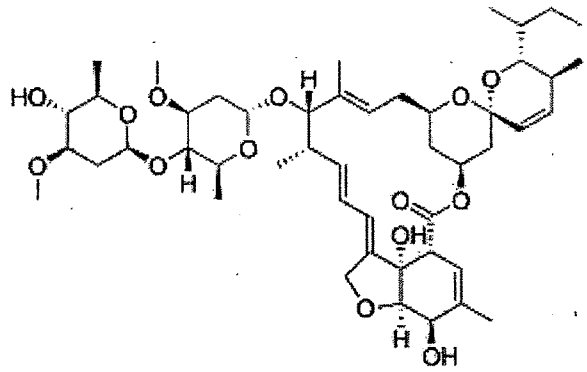
Use of fungal drugs started with the discovery of penicillin by Alexander Fleming (1929) from the fungus *Penicillium notatum.* It was reported in the British Medical Literature in 1929 (Flannigan and Miller, 1994).



Penicllin V



Penicllin G



Avermectin

Figure 23: Structure of Penicillin V, G and Avermectin.

The antifungal agent 'griseofulvin' from *Penicillium griseofulvum* (Rehm, 1980) and the cholestrol biosynthesis inhibitor 'lovastatin' from *Aspergillus terreus* (Alberts *et al.*, 1980) are some of the important fungal metabolites. Isolation of Avermectins from *Streptomyces avermilis* MA-4680 also led to significant increase in isolation and characteristion of metabolites from microbes (Babu *et al.*, 1989; Miller and Savard, 1989).

Near about 40% of compounds used in market as therapeutic agents are from fungal origin. When compared with total drugs available in the market and number of drugs obtained from fungi, it can be said that we are using only about 5% of the total fungi known to science (Hawksworth, 1991). Therefore, looking to such a vast biodiversity of fungi, several species are yet to be explored for it's usefulness to the mankind. Fungal genera such as *Aspergillus, Penicillium, Trichoderma, Acremonium, Fusarium,* etc. have been studied earlier by several workers for metabolites production and they are known for their ability to synthesize diverse chemical structures (Alberts *et al.*, 1980, Rehm, 1980). However, secondary metabolites produced from fungi vary in production, function and specificity to a particular fungus (Keller *et al.*, 2002). Therefore, process of new drug discovery is driven by the

desire to identify a structurally novel compound that possesses novel and potentially useful biological activity (Dreyfuss and Chapela, 1994).

4.2.1 Characteristics of antibiotics

Antibiotics may have a cidal (killing) effect or a static (inhibitory) effect on a range of microbes. The range of bacteria or other microorganisms that is affected by a certain antibiotic is expressed as its spectrum of action. The spectrum range of each antibiotics may vary. Depending on their spectrum of action, antibiotics may be distinguished into: broad spectrum, narrow spectrum and limited spectrum. Antibiotics that are effective against prokaryotes that kill or inhibit a wide range of gram positive and gram negative bacteria are said to be broad spectrum antibiotics. Antibiotics effective against gram positive or gram negative bacteria, are narrow spectrum. If it is effective against a single organism or disease, they are referred to as limited spectrum.

General characteristics of antibiotics

- It should have a wide spectrum of activity with the ability to destroy or inhibit many different species of pathogenic organisms.
- It should be nontoxic to the host and without undesirable side effects.
- It should be nonallergenic to the host.
- It should not eliminate the normal flora of the host.
- It should be able to reach the part of the human body where the infection is occurring.
- It should be inexpensive and easy to produce.
- It should be chemically stable (have a long shelf life).
- Microbial resistance is uncommon and unlikely to develop.

4.2.2 Mode of action

Generally, antibiotics have been classified according to their mode of inhibition. However, conventional antibiotics either inhibit cell wall synthesis or they may inhibit DNA, RNA and protein synthesis (Rang and Dale, 2007). With the advent of technology and development of modern medicines, a new forms of semisynthetic peptides have emerged for synthesizing the antibiotics. These newly developed antibiotics are antimicrobial peptides having more ways to inhibit parasites (Sahfer, 2006). Besides conventional antibiotics, it can inhibit enzyme synthesis, membrane disruption and autolysin. Below is the schematic representation of coventional as well as antimicrobial peptides modes of inhibition.

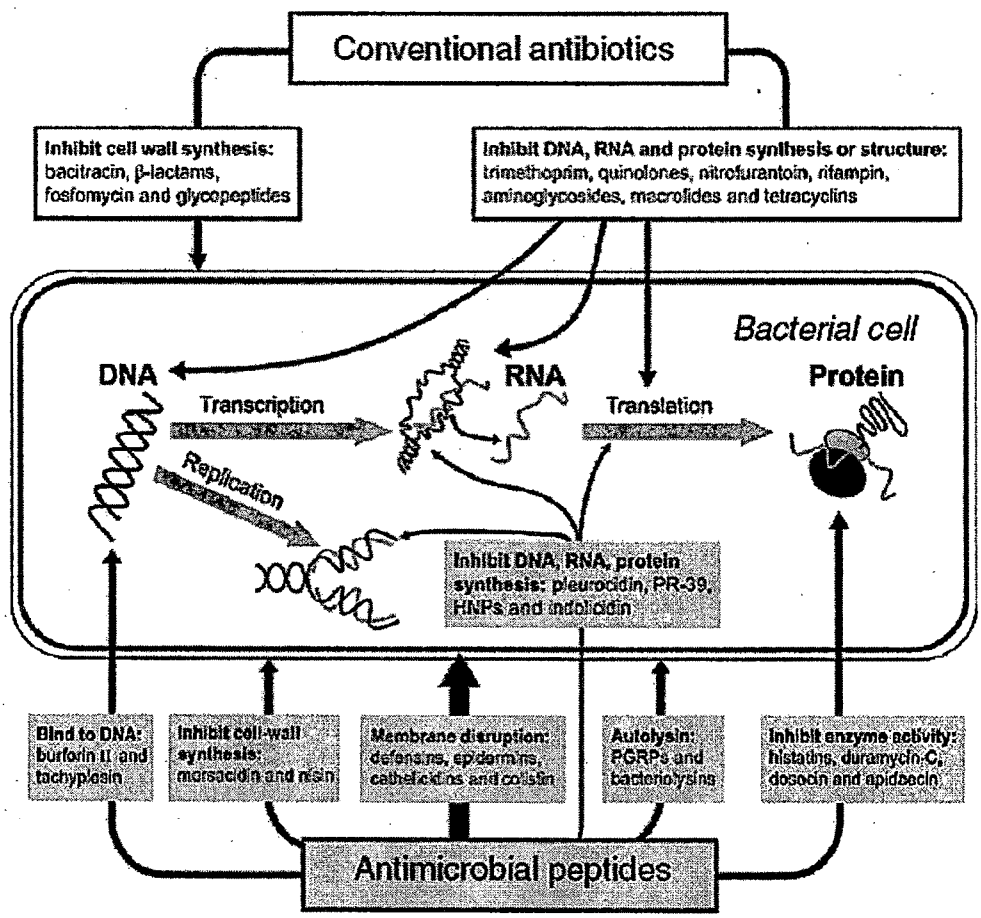


Figure 24: Mode of inhibtion of conventional and antimicrobial peptides.

4.3 Current production of antibiotics and secondary metabolites from different fungi:

Last three decades are characterised by the novel discoveries of microorganisms that are capable of producing different novel antibiotics and in the development of semisynthetic drugs (Hawksworth, 1991). More than 70,000 derivatives are produced, from which approximately 70% are being used in clinical medicine. These discoveries contributed to widening of practical applications in oncology, parasitology and plant protection (Hawksworth, 1991). A number of higly potential drugs based upon fungal metabolites have been developed and marketed by various companies.

Fungi have been existing on planet earth at least from one thousand million years. Since that time, they have exploited and evolved secondary metabolism for the production of bioactive compounds for their survival and competition with other microbes (Heckman *et al.*, 2001). It occupies the largest part of the total microbial protoplasm because of the large diameter and the extensive network of fungal filaments. Recently, dozens of new natural bioactive compounds have been characterised from mushrooms by their biological and chemical investigation. Novel terpenoids, phenolics and nitrogen containing compounds have also been isolated and characterised from Basidiomycetes and Ascomycetes group of fungi (Ji kai liu, 2007). From the systematic view point, the most prolific producers of pharmaceutically relevant molecules are the Ascomycetes. By their evolutionary counterpart, the Basdiomycetes are equally rich and often unique in their secondary metabolism, however they cannot be compared for significance in terms of clinically used compounds (Dirk and Keller, 2007).

Fungi produce a wide range of secondary metabolites with high therapeutic value such as antibiotics, cytotoxic substances, insecticides, promoters and inhibitors for growth, attractor and various repellent (Demain, 1999). Even if the natural function of secondary metabolites often is unknown, it is assumed that they play an important

role in chemical defence and communication (Krohn, 1996). Fungal products are critical in human health care. In addition to penicillins and cephalosporins, there are other success stories of new compounds such as cyclosporin-A, which reduces risk of rejection in organ transplantation, and statins for the control of cholesterol levels (Tripathi, 2008). The annual sales of amoxicillin (a penicillin) in the USA alone amounts to around US $500 millions, and statins are being taken by some 30 million people worldwide and generating US $25 billions annually for the producing companies. The full range of fungal compounds beneficial to health are staggering and expanding day by day (Pelaez, 2005).

4.3.1 Important metabolites from wood rotting fungi

A novel benzofuran lactone, named concentricolide (Liu, 2005), was isolated along with four known compounds (friedelin, cytochalasin *L*-696,474, armillaramide and russulamide) from the fruiting bodies of the xylariaceous ascomycete *Daldinia concentrica*. Two more new aromatic steroids were isolated from the fruiting bodies of *D. concentrica* (Ye *et al.*, 2005), whereas another important compound Grifolin, a natural active substance was isolated from the fruiting bodies of *Albatrellus confluens* (Qing *et al.*, 2004). It is said that Grifolin strongly inhibits the tumor cells lines CNE1, HeLa, MCF7, SW480, K562, Raji, and B95-8. Other groups of fungi which produce many important metabolites are included in group *Xylaria*. Unique secondary metabolites have been found in this genus are: cytochalasins, globoscin, lactones, maldoxin, sesquiterpenoids, xylaramide, xylarin, and xyloketals (Wang *et al.*, 2005). Detailed chemical investigation from the fungus *Xylaria euglossa* has been performed and a new nitrogen containing compound, xylactam has been reported by Xing *et al.*, (2005). Not only the fungal metabolites are used in pharmaceuticals but fungal pigments (new butenolide type fungal pigment "pulverolide" isolated from the fresh fruiting bodies of *Pulveroboletus ravenelii*) are also having therapeutical values in

pharmaceutical industries (*Zhang et al.*, 2006). These are only few metabolites which are isolated from the wood rotting fungi and much more researches have already been reported in the literature (Baldwin *et al.*, 1964; Ayer and Browne, 1981; Wang *et al.*, 2005).

Most fungi studied to date have been isolated either from soil, wood or marine condition and all of them are proven to have a very high creativity index, i.e. new and interesting secondary metabolites. Genera such as *Aspergillus, Penicillium, Acremonium, Fusarium*, all typical isolates, are known for their ability to synthesize diverse chemical structures (Flannigan and Miller, 1994). Dreyfuss (1986), however described a problem which is often encountered in screening of fungal isolates for their secondary metabolite content. Increasingly, known metabolites are rediscovered making screening programmes less efficient. This may be due to the use of well established protocols and same fungal species for isolation of metabolites. Other alternative to investigate more fungal species for new secondary metabolites may be isolation of them from other ecological niches (Dreyfuss, 1986). This may prove a more direct approach towards to get a novel fungal bioactive metabolite. Some relatively unexplored fungal groups derived from such ecosystems are; e.g. freshwater fungi, marine fungi and endophytic fungi (Dreyfuss and Chapella, 1994).

Another important factor responsible for the search of new bioactive metabolites from the new source is development of antibiotic resistance which is continuously increasing against known microbial species. This problem developed an urgent need for finding an alternative to use natural sources for production of bioactive compounds which has direct action against parasitic growth. Development of new technologies like microbial engineering, strain improvement, fermentation technologies resulted into more production of metabolites from same known species. For example, gene cloning technique increased pencillin acylase production upto

twenty fold with less toxic end products (Yang *et al.*, 1987). Moreover, features such as low cost and high productivity of the secondary metabolites have attracted many research efforts in both molecular-genetic techniques and bioprocess improvements (Finkelstein and Ball, 1992; Banerjee *et al.*, 2003).

Eventhough, improvement of science and technology are finding a way to fight against diseases, use of synthetic antibiotics has certain limitations due to their side effects and change in the infection cycle of parasite. Therefore, production of these drugs demand the improvement in pharmacokinetics properties (Sprecher and Hanssen, 1985). Similarly, there is an urgent need of suitable antibiotics in many fields of human medicine or in non medicinal areas like plant diseases and as food preservatives. It is therefore very much essential to find out a new source of more effective and less toxic antibiotics, which can inhibit parasites, causing damage to hosts. In this context, present investigatoin was carried out to screen some wood rot fungi for the production of secondary metabolites for antibacterial activity. From the isolated cultures, *C. asperatum* a member of Ascomycetes gave positive results for antibacterial activity. Members of Ascomycetes are reported to be active producers of antimicrobial compounds, which have high therapeutic values (Quang *et al.*, 2002). Therefore, one of the objective of the present study was to screen these wood rot fungi for the production of antimicrobial compound (if any?).

MATERIAL AND METHODS

4.4 Isolates used for screening of antibacterial antibiotics:

Near about 30 fruiting bodies of different wood rot fungi were collected from dead as well as infected living trees growing in different forests (*viz.* Girnar forest-Junagadh, Satpuda forest of Dediapada and Panchmahal, Pavagadh and Shivrajpur) of Gujarat state. Among all the strains screened, *Chrysosporium asperatum* Carmichael gave positive results for antibacterial activity, thus it is used further for screening of antibacterial antibiotics. Details of the isolation and identification of fungal isolates are described in previous chapters. Pure cultures were established by serial transfer and the cultures were maintained on 3% malt extract agar in refrigerated condition.

4.4.1 Bacterial strains and chemicals

Bacterial strains: *Salmonella* sp., *Shigella* sp., *E. coli*, *Klebsiella* sp., *Staphylococcus aureus* and *B. subtilis* were used in the present study. All these strains were procured from Microbial Technology Laboratory of Department of Biochemistry, The Maharaja Sayajirao University of Baroda. All the bacterial strains were maintained on nutrient agar slants under refrigerated conditions. Before screening for antibacterial activity, all the strains were activated in nutrient broth. Nutrient agar and Nutrient broth were procured from Himedia (India). All the experiments for screening of antibacterial antibiotics were performed using double distilled water.

4.4.2 Fungal growth and extraction of crude extract:

4.4.2.1 In shaking condition

Spores of *Chrysosporium asperatum* from mature slants were inoculated in 3% malt broth for 14 days. Three such flasks containing 100 ml of media were incubated in shaking condition. The resultant fermented broths were treated with 100 ml of

methanol, chloroform and ethyl acetate followed by filtration with cheesecloth to remove the mycelial biomass. The organic extract was separated and dried.

4.4.2.2 In static condition

Under static condition, *C. asperatum* has been inoculated on 3% malt extract agar. For static condition, cultures were grown for 35-40 days. After the 35–40 days of incubation, media containing *C. asperatum* showed brownish exudates, which was collected in eppendorf centrifuge tube and stored in refrigerated condition for further analysis. Media was homogenized after treating it with methanol and kept in shaking condition for two days for efficient mixing. This homogenate was centrifuged and filtered with cheesecloth. Filtered homogenate was kept in oven for drying.

4.5 Antibacterial activity:

Antibacterial activities of methanol, chloroform and ethyl acetate extracts were analyzed by agar diffusion method (Anonymous, 1996). For the preparation of nutrient agar, initially base nutrient agar plate was prepared and on the top of which top nutrient agar was poured. As shown in Figure 25, a well of 6mm diameter was made on the growth media using cup borer and 25 µl of filtrate was inoculated in these wells. For control, one of these wells was either filled with methanol, chloroform or ethyl acetate to verify its effect on bacterial growth. Bacterial strains were activated in nutrient broth and the cultures were maintained in environmental incubator at 37 °C. These activated bacterial strains were inoculated on the top of nutrient agar. Petri plates were then incubated in environmental incubator at 37 °C for 24-48 hours for observations.

4.6 Effect of incubation time and media on production of antibiotics:

Media composition plays an important role in growth of organism and production of the metabolites. Therefore, different media were also examined for the production of

metabolites. Along with growth media, effect of incubation period plays a crucial role in production of metabolites. Thus, fungal isolates were cultivated on media for different incubation periods to extract secondary metabolites.

4.7 Analysis of exudate collected:

Exudate, which was collected from static culture of *C. asperatum*, was checked for its antibacterial activity. Nature of the exudate was checked by Thin Layer Chromatography (TLC), for which readymade TLC plates were procured from Merck India Pvt. Ltd. Extraction of the compound present in exudates was carried out by using different combinations of solvent mixture. Compounds separated by TLC plate were observed under UV light for fluorescence. Active fraction was scraped out from TLC and maximum absorbance of that compound was noted down.

Bands seperated on TLC plate were scrapped out for the checking of biological activity against bacterial species. Scrapped bands were dissolved in ethanol and inoculated in petri plates containing different bacterial strains. Zone of bacterial growth inhibition was recorded and photographs were taken by Sony digital camera DSC T10 with 8 megapixel.

RESULTS AND DISCUSSION

4.8 Preliminary screening of antibacterial activity in shaking condition:

Fungi are well known to show antibacterial, antifungal, larvicidal, molluscicidal, antioxidant and free radical scavenging activities (Keller *et al.*, 2002). Though, *Trichoderma sp.* has been well established for producing antifungal antibiotics (Ridout *et al.*, 1986; Whilhite *et al.*, 1994), but it shows negative results for production of antibacterial antibiotics in the present investigation. The ethyl acetate fraction of *C. asperatum* showed antibacterial activity against both gram positive and gram negative bacteria when cultured on a rotary shaker (Figure 25). Krohn (1996) also reported antibacterial activity against *E. coli* and *B. megaterium* using bioactive compound isolated from *Phomopsis sp.*

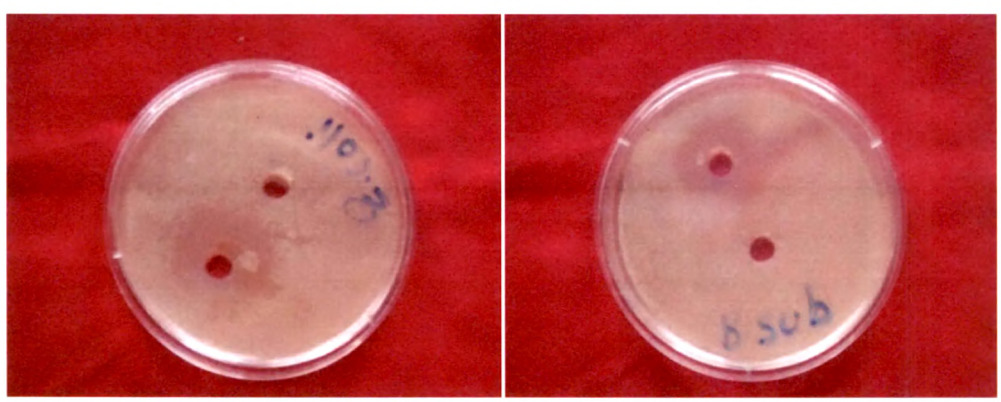


Figure 25: Plate showing antibacterial activity of ehtylacetate fraction of *Chrysosporium asperatum* against *E. coli* and *B. subtilis.*

4.8.1 Effect of parameters studied in shaking flasks condition

For efficient production of antibacterial compound from *C. asperatum,* following two parameters were taken into consideration. The first parameter was optimisation of incubation period for production of antibacterial compound and the second one was optimisation of media for production of antibacterial compound. Cultures were harvested for antibacterial activity after 5, 7, 10, 15 and 20 days of incubation period.

As a blank, pure ethyl acetate was used. Compounds produced by *C. asperatum* were tested for antibacterial activity against all bacterial strains. Production of secondary metabolite from *C. asperatum* commenced from 5th day of incubation and continued to increase confirming the inverse relationship as reported by Stanier *et al.*, (1981) between the rate of mycelial growth and biosynthesis of many secondary metabolites of fungi. Maximum production of antibacterial compound was found after 15 days of incubation time. After 15 days of incubation, metabolite production was depleted, it might be due to enzymatic cleavage of the molecule or a conversion to other related compounds or both (Alberts *et al.*, 1990).

Besides being dependent on fungal species, the production of metabolites is also influenced by media (Sunesson *et al.*, 1995). Therefore in the present study, other factor studied was effect of media on metabolite production. Different media have different constituents which directly or indirectly help for production of metabolites. Maximum production of metabolite has found in media having 3% malt extract broth (Table 33). Sunesson (1995) reported that a large number of metabolites could be identified, but are produced by only one species and often on specific medium.

Media	Inhibition zone(mm) (*E. coli*)
Malt extract broth	6.0
Potato dextrose broth	NG
Saubrouds broth	4.5
Soyabean meal	3.5
Malt glucose broth	4.7
Yeast peptone broth	5.0

NG- no growth.

Table 33: Various media used for growth and metabolite production.

According to Grayer and Kokubun (2001), the media for fungus growth and metabolite production should be close to natural habitat of the fungus, as it has strong influence on biosynthetic pathway. Different media have different constituents which directly or indirectly contribute in the production of metabolites (Mendgen and Deising, 1993; Grayer and Kokubun, 2001). Mendgen and Deising (1993) and Turner (1971) also suggested that mutation can also hinder metabolite production in a particular media.

Variability of metabolite production for one single isolate can also vary under a given set of fermentation condition as individual component of media has different effect on metabolic pathway of organism (Keller and Hohn, 1997). This could be due to some portions of the mycelium having different biochemical pathways and thus producing different compounds (Turner, 1971).

4.9 Static condition:

After 15 and 20 days of incubation under static condition, ehtyl acetate fraction of fungi showed less antibacterial compound as compared to that of shaking condition. Therefore, further studies on the static condition was discontinued .

4.10 Exudate analysis:

Old culture (35-45 days) of *Chrysosporium asperatum* is characteristically produce brownish color exudates. After 30 days of incubation, brownish exudate secreted by the culture was collected in eppendorf centrifuge tube. That exudate was further anaysed for antibacterial testing by bioassay from TLC plate. Nature of this exudate was found to be water soluble, which suggests that it is non-volatile in nature.

Different combinations of solvent systems were also explored for the efficient seperation of bioactive compounds. Among the different solvents used, ehtyl acetate: methanol (4:1) solvent system was found to be suitable. When the TLC plates were

observed under UV light (254 and 360nm), separated bands were found to be devoid of any fluorescence.

TLC of the exudate was performed and the plates were developed in 5% sulphuric acid (prepared methanol). For visualisation, TLC plates were heated at 70 °C till the yellowish bands were visible. These bands were scrapped out from the TLC plates and dissolved in ethanol. These fractions were further checked for its antibacterial activity as described earlier. Pure ethanol was used as control. Though, isolated compounds showed positive results against all the strains but the antibacterial effect was more pronounced against *P. aeruginosa* (Figure 26).

Figure 26: Zone of growth inhibition against *P. aeroginosa* and no inhibition in case of *Salmonella* sp.

It is well known fact that *Pseudomonas aeruginosa* is an important prevalent pathogen in hospitals that often causes infections after surgeries (Jung *et al.*, 2005). Moreover, many strains of this bacterium have developed drug resistance towards many antibiotics, e.g. penicillins, some cephalosporins and cotrimoxazole (Jung *et al.*, 2005). Fungal crude extracts, especially collected from static flask condition, showed considerable inhibitory activity against *P. aeruginosa*, suggesting its potential use as a new anti- *P. aeruginosa* antibiotic producer.

Standardisation of solvent system for compound isolation was done. After running through the solvent system the TLC plates were treated with 5% sulphuric acid (in methanol) as a chromogenic reagent (Figure 27). Appearance of yellow colour after spraying with the chromogenic reagent, indicates that the antibacterial activity of compound may be belonging to macrolides groups of antibiotics (Stahl, 1969).

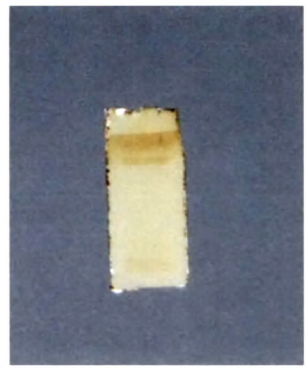


Figure 27: Yellow band observed after spraying with 5% sulphuric acid in methanol.

From the another set of TLC plate, same band was scrapped out and it was then eluted in ethanol to obtain the UV spectra. Fraction was filtered and eluted in ethanol. Maximum absorbance of the eluted compound was observed at 202nm in UV visible spectrophotometer (Figure 29).

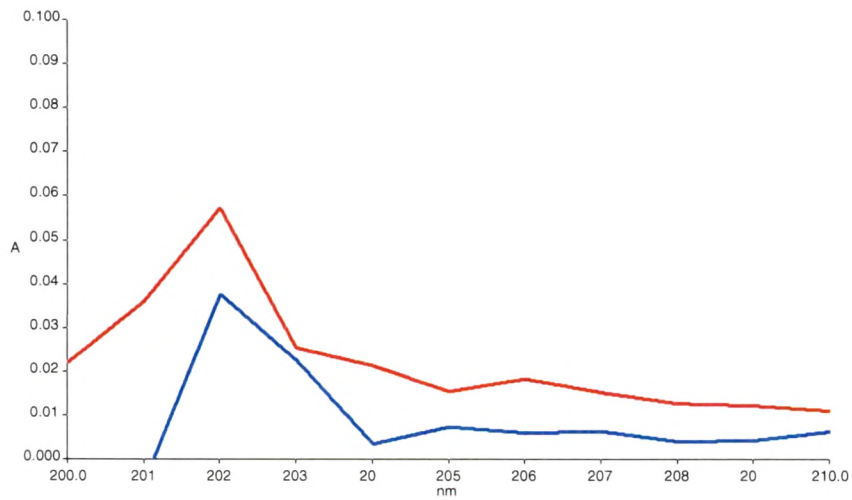


Figure 29: UV spectra of fraction eluted in ethanol (Blue line).
UV spectra of fraction diluted in ethanol (Red line).