STUDIES ON MICROBIAL PRODUCTION OF LIPOXYGENASE INHIBITOR

A thesis submitted to the

UNIVERSITY OF MYSORE

for the award of the degree of

DOCTOR OF PHILOSOPHY IN BIOTECHNOLOGY

By

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DECLARATION

I hereby declare that the thesis entitled "STUDIES ON MICROBIAL PRODUCTION OF LIPOXYGENASE INHIBITOR" submitted to the University of Mysore for the award of the degree of DOCTOR OF PHILOSOPHY is the result of the research work carried out by me in the Discipline of Fermentation Technology and Bioengineering, Central Food Technological Research Institute, Mysore, India, under the guidance of **Dr Avinash P Sattur** during the period April 2005- June 2008.

I further declare that the work embodied in this thesis had not been submitted for the award of degree, diploma or any other similar title.

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CERTIFICATE

I hereby certify that the thesis entitled **"STUDIES ON MICROBIAL PRODUCTION OF LIPOXIGENASE INHIBITOR"** submitted to the University of Mysore for the award of the degree of DOCTOR OF PHILOSOPHY by **Mr. CHIDANANDA.C**, is the result of the research work carried out by him in the discipline of Fermentation Technology and Bioengineering, Central Food Technological Research Institute, Mysore, India, under my guidance during the period April 2005-June 2008.

(**Avinash P Sattur**)

Abstract

The thesis reports isolation of several fungal cultures from forest soil and screening of the metabolites for their ability to produce inhibitors against lipoxygenase. Of about 74 isolates, one isolate CFTRI-A-24 found to produce consistently high yields of potent inhibitor, was characterized as *Penicillium frequentans*. The LOX inhibitor was purified using chromatographic techniques and was identified as "Sclerotiorin". The inhibition kinetics, mode and mechanism of LOX inhibition, its comparative evaluation to standard compounds, *in vitro* and *in vivo* (animal) models evaluation as antioxidant additive and other various biological properties of the inhibitors such as antioxidant, antimicrobial, aldose reductase inhibition, platelet aggregation inhibition, antimutagenic and anticancer property are reported. A medium was designed with optimum physical and nutritional parameters for the efficient production of sclerotiorin with better yields in a lab scale process. Effective recovery of the inhibitor in an economical method was also designed. An effort was made to evaluate sclerotiorin for the possible application as an antioxidant additive in commercial oil sample.

ACKNOWLEDGEMENTS

I take this opportunity to express my sincere gratitude to my guide Dr. Avinash P Sattur, who guided and inspired me throughout the course. It gives me immense pleasure to thank him for his constant support, encouragement, critical suggestions, constructive criticism and for inculcating the right scientific temperament in me. Without his guidance this study would have not been possible.

I am grateful to Dr S. Divakar, Head, FTBE department for his constant encouragement and help extended during my course. I also extend my sincere gratitude to Dr. N.G. Karanth and Dr. M.C. Misra, Former heads of the department for their support and encouragement.

I thank Dr. V. Prakash, Director, CFTRI, for providing me an excellent infrastructure to carryout my investigation.

My sincere thanks to Dr. L. Jagan Mohan Rao, Dr. T.R. Shamala, Dr. K. Srinivasan and Dr. T.P. Krishnakanth for the timely guidance and help rendered during, structure elucidation, culture characterization, animal study experiments and platelet aggregation experiments respectively.

I also extend my heartfelt thanks to Dr. S.G. Prapulla, Dr. M.S. Thakur and to the entire staff and students of Fermentation Technology and Bioengineering department who have helped me at various times during this study.

It gives me a great feeling to thank and acknowledge all my lab mates, Suresh, Saleem, Chakri, Mamatha Veeresh, Deepak kulkarni, Naveen Babu, Mohan and Sekhar, Without whom the work would have been definitely not interesting.

I specially thank Mamatha and Somanna for their timely help rendered during the study.

What's life without friends, my special thanks to all my buddies and pals, Mahesh, Sandesh, Naveen, Tippeswamy, Sharath, Monu, Abhi, Reddy, Girish, Sanchi, Vasu, Sanju, Ashwin, Jyothi, Neeta and Manju, who were with me through the ups and downs of my life.

I would continue to remain forever grateful to all my fellow colleagues and friends who were associated with me.

Last but not the least, I wish to acknowledge the support rendered by Council of Scientific and Industrial Research (CSIR) in the form of Senior Research Fellowship.

I owe my gratitude to my parents for their blessings and unconditional love and support. I extend my thanks to my brother Sadu as well for his moral support.

Chidananda. C

Dedicated to
My Beloved Parents …

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INTRODUCTION

1.1.Enzyme inhibitors:

Microbial biodiversity is a precious source for modern biotechnology and holds potentially innovative and sustainable solutions to a broad range of problems for modern society. It constitutes an infinite pool of novel solutions in fields of biotechnology. Microorganisms are known to produce number of biologically active substances and secondary metabolites like antibacterial, antifungal, antiviral and anti tumor (cytotoxic) substances. Apart from these other well-known biologically active agents are also produced such as plant growth regulatory factors, enzymes, bioinsecticides, bioherbicides, biopesticides, immune suppressants and eukaryotic enzyme inhibitors.

Today, one of the high growth areas in the field of fermentation technology is the search for a new bioactive molecules from microbial sources. One such group of bioactive metabolites produced by fermentation is low molecular weight enzyme inhibitors. Since many pharmaceutical/biological agents are known to inhibit specific enzymes and several diseases are associated with abnormal enzyme activities, this concept of enzyme specific "target enzyme inhibitors" has yielded exceptional pharmaceutical compounds of microbial origin. Already now, several of these microbial inhibitors have been introduced in health practice (Umezawa, 1982).

One of the well known commercial successes has been lovastatin a fungal metabolite used as cholesterol-lowering agents in humans. Lovastatin (also known as mevinolin) is a potent competitive inhibitor of 3-hydroxy-3-methylglutaryl- coenzyme A (HMG-CoA) reductase from liver produced from genus *Aspergillus*. Other well-known enzyme inhibitors include clavulanic acid, a penicillinase-inhibitor that protects penicillin from inactivation by resistant pathogens and acarbose, an inhibitor of intestinal glucosidase, which is produced by an Actinomycete of the genus *Actinoplanes*. Acarbose decreases hyperglycemia and triglyceride synthesis in adipose tissue, the liver and the intestinal wall of patients suffering from diabetes, obesity and type IV hyperlipidemia. Some of these novel compounds of pharmacological and medical importance are listed in Table 1 (Vandamme, 1994; Lam, 2007).

Table 1: Microbial enzyme inhibitors and metabolites of pharmacological / medical interest (Vandamme, 1994; Lam, 2007)

Microbial Enzyme Inhibitor	Producing strain	Target enzyme	Target disease
Arylomycin	*Streptomyces* sp.		Anti-infective
Aldostatin	*Pseudorotium zonatum*	Aldose reductase	Diabetes
Genestein	*Streptomyces* sp	α-Glucosidase	Diabetes
Trestatin	*Streptomyces dimorohgenes*	α-Amylase	Diabetes
Asperlicin	*Aspergillus alliaceus*	Cholecystokinin-antagonist	Ulceritis
Muraymycin	*Streptomyces* sp		Anti-infective
Ancovenin	*Streptomyces* sp	Angiotensin-converting enzyme	Hypertension
Lovastatin	*Aspergillus terreus*	HMG-Co-A-Reductase	Hypercholesteremia
Triacrin	*Streptomyces* sp	Acetyl CoA-synthase	Hypercholesteremia
Ramoplanin	*Actinoplanes* sp		Anti-infective
Purpactin	*Penicillium* sp	Acetyl CoA- cholesterol -acyl transferase	Hypercholesteremia
Squalistatin	*Streptomyces* sp	Squalene synthase	Hypercholesteremia
Leupeptin	*Streptomyces roseus*	Serine protease	Inflammation & Pancreatitis
Lipstatin	*Streptomyces toxytricini*	Lipase	Obesity
Nocathiacins	*Nocardia* sp.		Anti-infective
Arisugacin	*Aspergillus terreus*	Acetylcholinesterase	Dementia
Erbstatin	*Streptomyces* sp	Tyrosine kinase	Tumor
Epocarba-zolins A & B	*Streptomyces* sp	5-Lipoxygenase	Tumor
Temsirolimus	*Streptomyces hygroscopicus*		Anticancer
Ixabepilone	*Sorangium cellulosum*		Anticancer
Irofulven	*Clitocybe illudens*		Anticancer
Vorinostat	*Streptomyces hygroscopicus*		Anticancer

1.2. Lipoxygenases:

Lipoxygenases (LOXs) (linoleate: oxygen oxidoreductase EC 1.13.11) are a family of non-heme, non-sulfur iron containing dioxygenases. The enzyme catalyzes the addition of molecular oxygen to unsaturated fatty acids containing cis, cis, 1,4-pentadiene systems to give cis, trans-hydroperoxydiene (Gardner, 1991). LOXs are ubiquitous in nature and widely found in plants and mammals. LOXs have been reported from algae, molds and bacteria (Gerwick, 1994; Brash, 1999; Porta and Rocha-Sosa, 2001).

1.2.1. Nomenclature of lipoxygenases:

Lipoxygenases enzyme classification is linoleate: oxygen oxidoreductase (for plant LOX) and arachidonate: oxygen oxidoreductase (for mammalian LOX). Historically LOX are classified according to their positional specificity of the dioxygenation of their most common substrates linoleic acid (LA) (C18:2) in plants, and arachidonic acid (C20:4) in mammals.

Plant LOXs are mostly classified based on their positional specificity for LA dioxygenation i.e. reacting at the ω-6 position of the substrate (as counted from the tail end of the fatty acid). Linoleic acid is oxygenated at either C-9 or C-13 position of the fatty acid resulting in the formation of corresponding 9-hydroperoxy or 13-hydroperoxy derivatives of LA (Feussner and Wasternack, 2002). Thus on the basis of product formation, soy LOX-1 is also designated as 13-LOX and potato LOX as 9-LOX. In case of animals, hydroperoxy derivatives are formed at carbon number 5, 8, 12 or 15, when arachidonic acid is the substrate. Hence these isozymes are often called as 5-, 8-, 12- or 15- lipoxygenases (Maccarone et al., 2001).

When necessary, the stereoconfiguration is specified (e.g. 12S-LOX and 12R-LOX). In mammals, isozymes that exhibit the same position-specificity are named additionally after the prototypical tissue of their occurrence (e.g. platelet-type, leukocyte type and epidermis-type 12S-LOX) (Brash, 1999).

1.2.2. Classification of lipoxygenases:

EC 1.13.11.12 lipoxygenase (linoleate: oxygen 13-oxidoreductase) Linoleate + O_2 = (9Z, 11E, 13S)-13 hydroperoxyoctadeca-9, 11-dienoate

EC 1.13.11.31 arachidonate 12-lipoxygenase (arachidonate: oxygen 12-oxidoreductase) Arachidonate + O_2 = (5Z, 8Z, 10E, 12S, 14Z)-12-hydroperoxyicosa-5, 8, 10, 14-tetraenoate

EC 1.13.11.33 arachidonate 15-lipoxygenase (arachidonate: oxygen 15-oxidoreductase) Arachidonate + O_2 = (5Z, 8Z, 11Z, 13E, 15S)-15-hydroperoxyicosa-5, 8, 11, 13-tetraenoate

EC 1.13.11.34 arachidonate 5-lipoxygenase (arachidonate: oxygen 5-oxidoreductase) Arachidonate + O_2 = leukotriene A_4+ H_2

EC 1.13.11.40 arachidonate 8-lipoxygenase (arachidonate: oxygen 8-oxidoreductase) Arachidonate + O_2 = (5Z, 8R, 9E, 11Z, 14Z)-8-hydroperoxyicosa-5, 9, 11, 14-tetraenoate

EC 1.13.11.45 linoleate 11-lipoxygenase also called manganese lipoxygenase (linoleate: oxygen 11-oxidoreductase) Linoleate + O_2 = (9Z, 12Z)-(11S)-11-hydroperoxyoctadeca-9, 12-dienoate

1.2.3. Structure of Lipoxygenases (LOXs):

Plant lipoxygenases have been widely studied since 1930s and much of the knowledge gained through them has contributed to the advancement in the mammalian LOX research (Shibata and Axelrod, 1995). Soybean LOXs are the well-established and intensively studied proteins in this group of enzymes. LOX-1 is the first isozymes of soy whose X-ray crystallographic structure has been determined to 1.4 Å and 2.3 Å resolutions by two different groups (Boyington et al., 1993; Minor et al., 1996). Till date rabbit reticulocyte 15-LOX is the only mammalian LOX for which the three-dimensional X-ray structure has been obtained (Gillmor et al., 1997; Choi et al., 2008).

Lipoxygenases have been purified and characterized from both plant and animal tissues (Siedow, 1991; Ford-Hutchinson et al., 1994). The sequences of lipoxygenases have been reported and many have been cloned and expressed as active proteins (Skrzypczak-Jankun et al., 2000; Kuhn and Thiele, 1999). They range in length from 923 residues (LOX-2 from rice) to 661 residues (rabbit reticulocyte 15-lipoxygenase). The amino acid sequences between plant and mammalian LOX enzymes show considerable homology. The soybean lipoxygenase isozymes, LOX-1 and LOX-3, are 72% identical in their amino acid sequences, but share only 25% sequence homology to any mammalian 15-LOX. Overall, sequence identity between plant and mammalian pairs of lipoxygenase isozymes is 21-27%, while plant pair sequence identity ranges from 43-86%, with mammalian pair sequences at 39-93% identity.

The highest level of sequence identity between lipoxygenases from plants and mammals lies in the area of the catalytic domain containing the non-heme iron atom. Mammalian lipoxygenases are 165-261 residues shorter than the plant lipoxygenases and were believed to lack a N-terminal β barrel due to the fact that similarities in the sequence identity of the first 200 residues between pairs of plants and animals never exceeds 15%. Comparisons between various mammalian lipoxygenase cDNAs have recently been reviewed. The similarities in sequence data across species lead to the assumption of similar 3 dimensional structures and the comparison of soybean LOX-3 with rabbit 15-LOX confirms that plant and mammalian enzymes share the same topology and overall architecture despite differences in size.

Although, the catalytic mechanism of all lipoxygenases may be same, there are significant differences in the properties of plant and animal enzymes. For example, the soybean enzyme is a soluble, cytosolic protein, where as the 5-LOX from rat and human leukocytes is activated by binding to a specific membrane associated activating protein called 5 lipoxygenase activating protein (FLAP) (Ford-Hutchinson et al., 1994). Therefore, although it is highly likely that the lipoxygenases employ the same catalytic mechanism there are significant variations among them in the regulation of the activity of the enzyme from different organisms.

1.2.4. Soybean LOX-1:

Soybean contains mainly three isozymes (sharing 70% sequence homology) designated as LOX-1, LOX-2 and LOX-3, which exhibit diversity in their enzymatic behaviour, the regio and stereo specificities and pH optima (Axelrod, 1974). These three isozymes further differ with respect to their isoelectric points, showing values of 5.68, 6.25 and 6.15 for LOX-1, LOX-2 and LOX-3, respectively (Siedow, 1991).

LOX-1 contains 839 amino acid residues with a molecular weight of 94,262 daltons (Figure 1 and 2) (Boyington et al., 1993). The corresponding values for LOX-2 and LOX-3 are 865 (97,053) and 857 (96,541) respectively. LOX-1 has an unusual pH optimum of 9 to 10 that is not shared by other lipoxygenases. LOX-1 also shows a much higher degree of regioselectivity and stereoselectivity in comparison with LOX-2 and LOX-3. LOX-1 oxygenates linoleic and linolenic acids to 13(S)-hydroperoxyoctadecadienoic acid (13-HPOD) and with arachidonic acid. The oxidation occurs at 15^{th} carbon giving 15(S)-hydroperoxyeicosatetraenoic acid (15-HPETE).

Several reports have indicated that the specificity of LOX may be altered *in vitro* by changing the physical state of the substrate (Kuhn et al., 1990) and conformational state of enzyme (Pourplanche et al., 1994). Further, it has been reported that 12- and 15-LOXs from plants and animals are sensitive to the location of the reactive 1,4-diene moiety relative to the methyl terminus, but not the carboxylate of the fatty acid substrate (Kuhn et al., 1985, 1990).

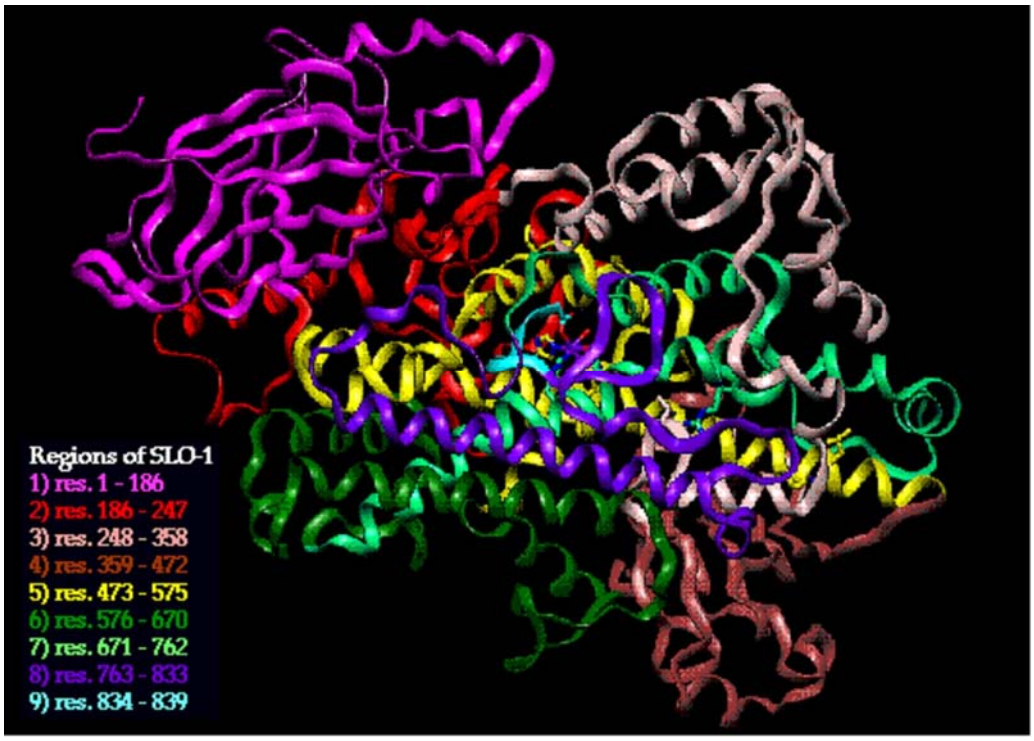


Figure 1. Tertiary Structure of SLO-1

Soybean Lipoxygenase-1 structure has been described as composed of two domains (Boyington et al., 1993) or as composed of five domains (Minor et al., 1996). This figure shows 9 regions. Region 1 is the beta sheet part, and is roughly coincident with what both crystallographic groups have called domain 1 which is a smaller N-terminal domain. The rest of these region constitute the larger C- terminal domain namely, regions 2, 4, 6 and 8 wrap around the outside of the protein, and regions 3, 5, 7 and 9 form the core of the protein and provide all the residues at the interior surface of the fatty-acid-binding cavity and the residues which bind the non-heme iron atom.

Reference: Brookhaven Protein Structure Databank

http://www.haverford.edu/chem/Scarrow/SLO/ribbon.gif

1.2.5. Structure of mammalian lipoxygenase: rabbit reticulocyte 15-LOX:

Generally mammalian LOXs are smaller molecule (75-80 kDa) when compared to that of plants (94-104 kDa). But the structural configuration overall is very similar and especially the catalytic domain match is too high and almost super imposable (Coffa et al., 2005). For a long time it was believed that LOXs did not occur in animals but in 1974 the formation of (12*S*, 5*Z*, 8*Z*, 10*E*, 14*Z*)-12-hydroxyeicosa-5, 8,10,14-tetraenoic acid (12-HETE) was described, when human thrombocytes were incubated with exogenous arachidonic acid (Hamberg and Samuelsson., 1974). This discovery marked the starting point of animal LOX research and over the following years more and more LOX isoforms were discovered. In 1975 a LOX was detected in rabbit reticulocyte, which was capable of oxidizing phospholipids and biomembranes (Schewe et al., 1975)

Rabbit reticulocyte 15-LOX (EC1.13.11.33) is the only mammalian lipoxygenase, whose 3D structure is been published (Figure 3) (Gillmor et al., 1997; Choi et al., 2008). The enzyme is elliptic cylinder in shape and consists of two domains- one small N-terminal domain (110 residues with 8 β-barrels) and a C-terminal catalytic domain (Kuhn et al., 2005). The large catalytic domain consists of 18 helices, which are interrupted by a small β-sheet sub-domain. A random coil without structure, links both domains. The center of C-terminal domain consists of 2 long helices. It contains four of the five-protein ligands-His361, His366, His541 and His 545 that coordinate non-heme catalytic iron. The fifth protein ligand is the carboxyl ate of the C-terminal residue lle663 while water may be the sixth ligand required for enzyme catalysis. These five ligands coordinate the iron with excellent octahedral geometry. The substrate-binding cavity is boot shaped and is directly accessible from the surface of the protein (Kuhn et al., 2005). Arg403, Gly407and leu597line its entrance and side chains of Phe353, lle418 and lle593 define th bottom of substrate binding cavity. Arg403 might interact with carboxylic group of the fatty acid substrate. While other residues have been identified as sequence determinants for the positional specificity of the enzyme.

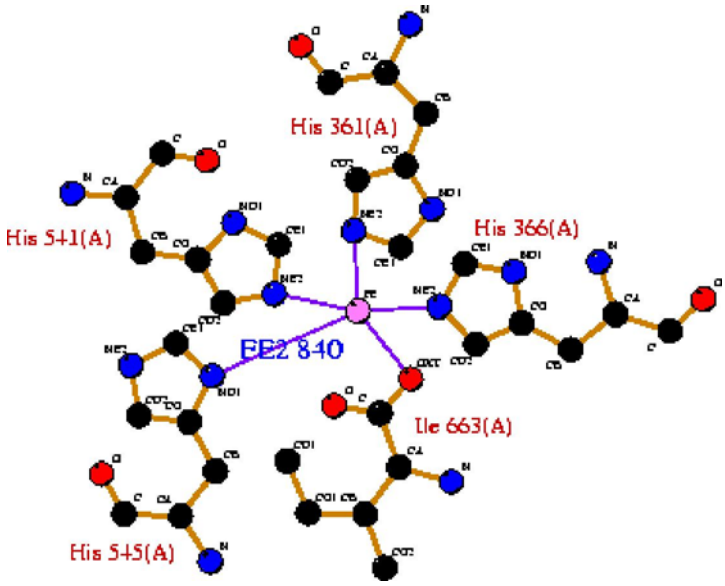


Figure 2. Active site of soybean LOX-1

Histidine ligands to the active site iron (Pink colours)

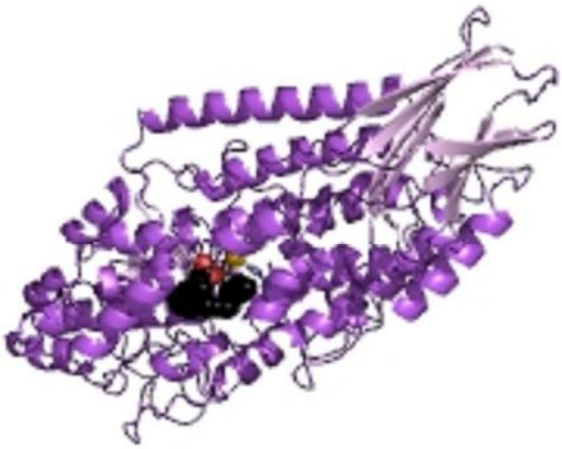


Figure 3. Three-dimensional structure of Rabbit reticulocyte 15-LOX

(PDB No-1lox, Gillmor et al., 1997)

1.2.6. Assays for lipoxygenases:

The assays are mainly based on the end products formed by oxidation of cis, cis, 1,4-pentadiene systems to cis, trans-hydroperoxydiene by enzyme catalysis. Generally three methods have been practiced to study lipoxygenase-catalyzed reaction.

1.2.6.1. Polarographic method:

Dioxygenase activity is characterized by uptake of oxygen, which is one of the reactant in the assay system. This mechanism is measured polarographically using Clark electrode (Schewe et al., 1986)

1.2.6.2. Spectroscopic method:

The increase in the absorbance at 234 nm due to the formation of end products conjugated dienes by the catalytic activity of lipoxygenase is measured using a simple spectrophotometer (Axelrod et al., 1981). This method is more sensitive, accurate and convenient for routine assay of lipoxygenase.

1.2.6.3. Radio labeled assay:

The products formed from the lipoxygenase catalysis are quantified using a radio labeled 14C* or 3H* substrate. This method is more sensitive but requires an additional step to purify and quantify the end product by TLC or HPLC (Schewe et al., 1986)

1.2.7. Catalytic mechanism of lipoxygenases:

Lipoxygenase contain non-heme iron as co-factor, which is covalently bound to the protein. Iron exists in one of the two oxidation states; Fe^{2+} (inactive or a resting enzyme) and Fe^{3+} (active form). In both the forms, the iron is in high spin state and plays an important role on oxidation of fatty acids. In most of the proposed mechanisms, the metal ion oscillates between Fe^{3+} and Fe^{2+} during a catalytic cycle. The iron atom is relatively difficult to remove from lipoxygenase. Reducing agents and *o*-phenanthroline are required: neither agent is effective alone (Siedow, 1991; Nelson et al., 1994).

1. Product activation model: this models supports the hydro peroxides formed is required to activate the enzyme by oxidizing the Fe^{2+} to Fe^{3+}. In this hypothesis only the Fe^{3+} form is active.

2. Substrate inhibition model: In this model both Fe2+ and Fe3+ are active and are inhibited by substrate to a noncatalytic site. Here the lag period reflects the displacement of substrate in the regulatory site by the products they accumulate.

Two schemes have been proposed to explain the mechanism of catalysis by lipoxygenase. The first scheme proposed by (Nelson et al., 1990), the 1-4 diene moiety of the substrate, is oxidized by Fe^{3+} and a proton as abstracted by a base/ water molecule to form pentadienyl radical a free radical intermediate and free Fe^{2+} enzyme. The radical reacts with molecular oxygen to produce a peroxyl radical which abstracts the electron from the metal, regenerating Fe^{3+} enzyme and producing a peroxidase anion. The peroxidase receives the proton from the base water yielding a hydro peroxide product.

The second scheme proposed by (Corey and Nagata, 1987), the Fe^{3+} assists in the de protonation by making a direct bond with resulting radical (organoiron intermediate). The path continues with the insertion of dioxygen in to the Fe-C bond by cleavage of the Fe-O bond. This mechanism offers an explanation for the stereo and regiospecificity of the enzyme (Figure 4).

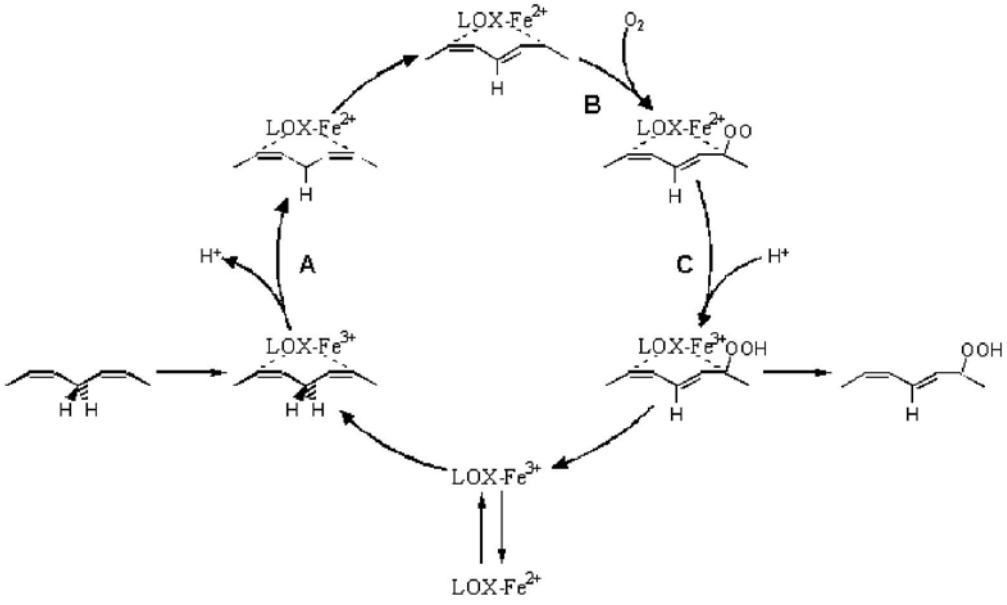


Figure 4. Mechanism of lipoxygenase catalyzed reaction

The most accepted hypothesis for the lipoxygenase reaction is the radical hypothesis (Kuhn *et al.*, 1986; Kuhn and Thiele, 1999) according to which the reaction consists of three major steps (Figure 5)

- Hydrogen is abstracted from the bisallylic methylene. The specificity of the hydrogen abstraction, both positional- and stereo-selectivity (-S, -R), depends upon the orientation of the substrate at the active site and by the specificity properties of the enzyme.
- Re-arrangement of the radicals leading to the formation of a cis-trans conjugated diene system. The direction of the rearrangement [-2] or [+2] is determined again by the orientation in the active site.
- The molecular dioxygen is inserted sterospecifically forming a peroxy radical, which is subsequently reduced to hydroperoxide anion.

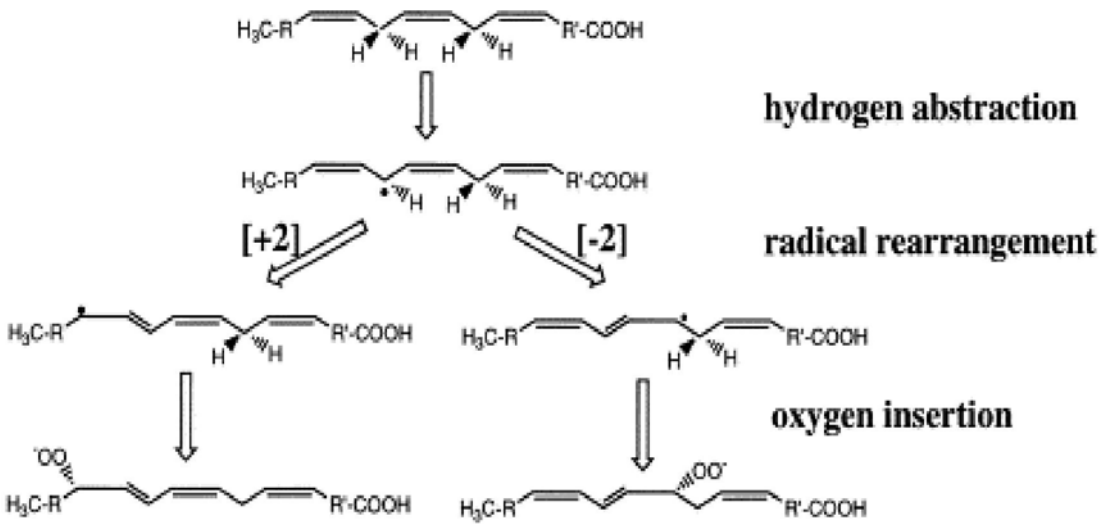


Figure 5. Radical mechanism of lipoxygenase catalyzed reaction

1.2.8. Biological role of lipoxygenases in plants.

These enzymes are most common in plants where they may be involved in a number of diverse aspects of plant physiology including growth and development, organogenesis, pest resistance, and responses to wounding (Gardner, 1991; Grechkin, 1998). The plant fatty acids, linoleic acid and linolenic acid, are converted by 9- or 13-lipoxygenase, giving rise to a multitude of oxylipins. These compounds are involved in stress responses. Some oxylipins have direct antimicrobial properties, whereas others may act as regulators of plant defense (Table 2) (Porta and Rocha- Sosa, 2002).

Table 2: LOX metabolites in plants having biological activity

Compound/product	Origin	Activity
9-and 13-HPOD or HPOT	Lox	Development of Hypersensitivity cell death
OPDA	Aos	Signaling in wounding and pathogen attack, tendril coiling
Jasmonic acid	Aos	Signaling in stress and tendril coiling
Colneleic and Colnelenic acids	DES	Antifungal
(C6) volatile (aldehydes and alcohols)	HPL	Signaling in wound healing, attracters to enemies or herbivores,antimicrbial.
Traumatin	HPL	Signaling in wound healing
(Z)-jasmone	Aos	Herbivore repellant and attract enemies of Herbivores, plant defense signaling
(13S)- Hydroperoxy-(9Z-11E)-octadecadienoic (13-HPOD)	Lox	Inhibitors of mycotoxin synthesis

Aos =Allene oxide synthase, HPL= Hydroperoxide lyase, LOX= Lipoxygenase, DES= divinyl ether synthase (Porta and Rocha- Sosa, 2002).

1.2.9. Beneficial attributes of LOXs in Foods:

LOXs are responsible for lipid peroxidation of polyunsaturated fatty acids promoting rancidity or off odor in foods of both plant and animal origin, which in turn reduces the nutritive value and functional property of the food (Grechkin, 1998). It is of interest due to their dual role in the genesis of volatile flavor and aroma compounds in plant products and to their ability to form free radicals, which can then attack other constituents such as vitamins, colours, phenolics and proteins. Hexanal, derived from the hydroperoxidation of linoleic acid is primarily responsible for the "green bean" flavour of defatted soy flour. Owing to its extremely low flavor threshold, LOX-2 is believed to be the isoenzyme mainly responsible for the generation of n-hexanal in soybeans (Matoba et al., 1985). Similarly, the off flavors in sweet corn have been attributed to the lipoxygenase activity.

LOX activity has been reported to be higher in leaves used for making high quality black tea than in those used for lower quality products (Robinson et al., 1995). Some of the LOX catalyzed volatile compounds have shown to bring characteristic banana flavour (Mac Leod and Ames, 1988). Some of the most important volatile compound 2-hexanal, *trans*-2-hexanal, 12-oxo-*trans*-dodecenoic acid and trans-2, *cis*-nonadienal are reported as cucumber aroma compounds. These are formed from LOX catalysed peroxidation of linoleic acid and linolenic acids (Grosch and Schwarz, 1971). Nona-*cis, cis*-3,6-dienal and nona-*cis*-6-enal have been claimed to be important aroma compounds in melons (Vick and Zimmerman, 1987).

The use of lipoxygenases has been extended to the flavour industry involving the various precursor molecules for food flavours (Whitehead et al., 1995). LOXs have the ability of cooxidation LOXs to prematurely release peroxyl radical is associated with the capacity to catalyze the oxidation of sensitive molecules such as thiol containing proteins, antioxidants (including vitamins) and pigments. Cooxidation requires the presence of polyunsaturated fatty acids. LOXs in peas and beans have been reported to have a high cooxidation activity (Grosch et al., 1976). Therefore lipoxygenases have commercial

importance in baking industry for bleaching carotenoids and modifying the rheological properties of the dough (Frasier, 1979).

1.2.10. Arachidonic acid metabolism in mammals:

In mammals series of enzymes namely, cyclooxygenases (COX-1, COX-2), monooxygenases (MO) and lipoxygenases (LOXs) are involved in metabolism of (C20)/arachidonic acid (AA). These enzymes are responsible for the formation of numerous lipid mediators/regulators known as eicosanoids, prostaglandins (PGs) and thromboxanes (TXs) (Figure 6). These lipid-derived mediators are capable of producing a multitude of physiologic effects in the local environment. They play an important role in a variety of signaling pathways both in physiological and pathophysiological conditions (Parker, 1987; Funk, 1996;Yamamoto et al., 1997).

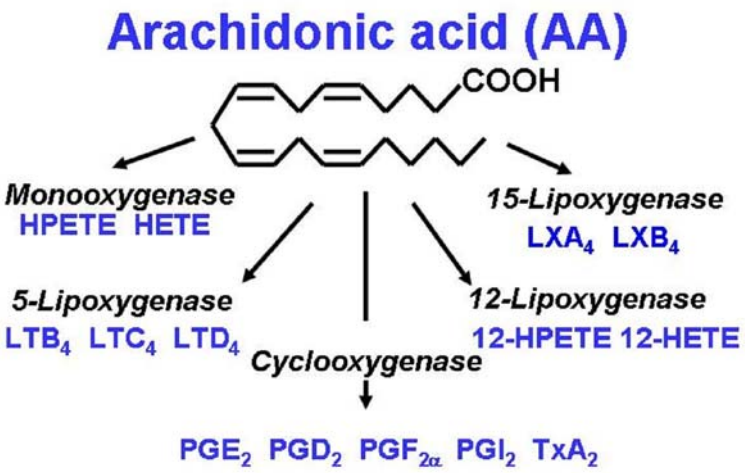


Figure 6. Major pathways involved in arachidonic acid metabolism in mammals

1.2.11. Biological role of lipoxygenases in animals:

Lipoxygenases carryout the first step in arachidonic acid cascade in converting AA to leukotrienes (LTs). The dual lipoxygenation of arachidonic acid and regio-specific enzymatic reactions between oxygen and polyunsaturated fatty acids forms a new class of compounds by either the 15-lipoxygenase, 5-lipoxygenase or the 12-lipoxygenase

producing eicosanoids known as lipoxins/oxylipins. These lipoxins are most frequently with the S-configuration of hydroperoxyeicosatetraenoic acids (5-HPETE, 12-HPETE or 15-HPETE) and dihydroxyeicosatetraenoic acids (HETEs) by peroxidases, leukotrienes (LTC_4) by lipoxygenase, hydrase, glutathione S-transferase (GST), and lipoxins by lipoxygenase (Figure 7).

Lipoxins and leukotrienes have potent biological roles like leukotactic and myotropic effects, they also constitute mediators of anaphylactic and inflammatory disorders (Samuelsson et al., 1987). Lipoxygenase products such as dihydroxy fatty acid leukotriene B_4 (LTB_4) and 9S-hydroxyeicosatetraenoic acid (9S-HETE) are ligands for G-protein coupled receptors and a nuclear hormone receptor which regulate pathway involved in inflammation and lipid homeostasis (Forman et al., 1997; Yokomizo et al., 1997), all of these endogenous lipid mediators are known to produce their biological effects by activation of specific cloned receptors.

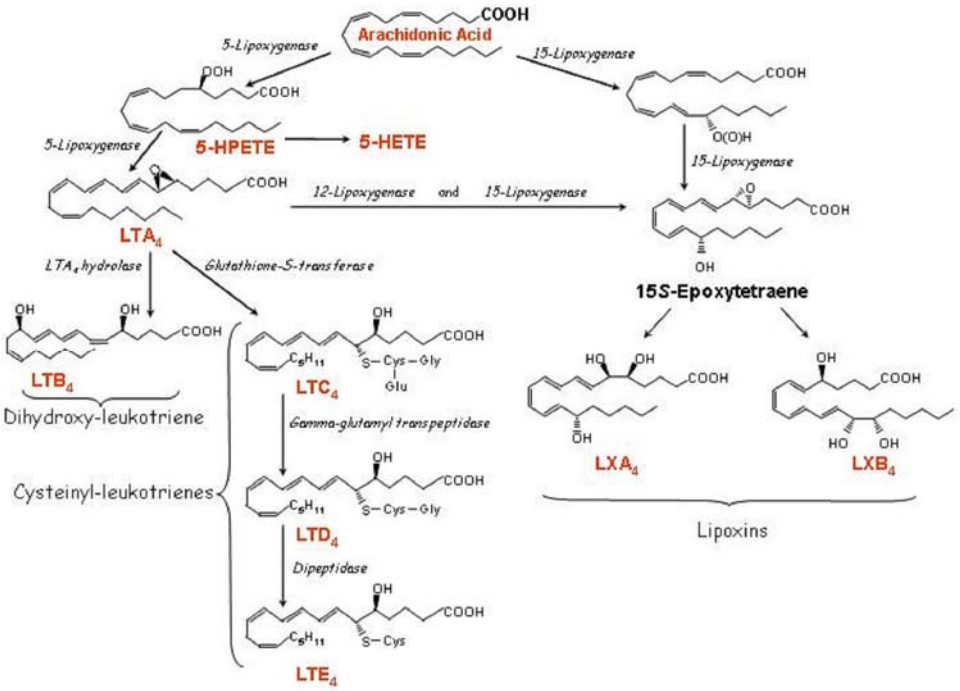


Figure 7. Lipoxygenase catalyzed arachidonic acid metabolism

1.3. LOXs as pharmacological target:

In mammals, the products of lipoxygenase-catalyzed reactions are responsible for variety of human disorders, such as atherosclerosis, allergy, inflammation, asthma, and hypersensitivity (Steinburg, 1999; Prigge et al., 1997). Leukotrienes (LTs) and lipoxins have been the major mediators of anaphylactic and inflammatory disorders (Samuelsson et al., 1987). Lipoxygenase activities and the end products of the enzyme catalysis are involved in atherosclerosis, inflammatory bowel disease, psoriasis, and other immune system disorders (Table 3). 12-LOX is over expressed in the pathological lesions of inflammatory bowel disease and psoriasis (Shannon et al., 1993). The ability of 15-LOX to oxidize LDL is suggestive that this enzyme plays an important role in pathogenesis of asthmatic or inflammatory potencies (Steinberg, 1999; Samuelsson et al., 1987).

Although most attention has focused on PGs and other COX-derived metabolites, mounting evidence suggests that LO-catalyzed products, LTs, and HETEs also exert profound biological effects on the development and progression of human cancers. For example, 12-LO mRNA expression has been well documented in many types of solid tumor cells, including those of prostate, colon, and epidermoid carcinoma (Honn et al., 1994; Chen et al., 1994).

1.3.1. 5-lipoxygenase in inflammatory diseases:

5-lipoxygenase (5-LO) catalyzes the conversion of AA into 5(S)-hydroperoxyeicosatetraenoic acid (5-HPETE) and to leukotriene (LTA_4) (Figure 7) (Samuelsson et al., 1987; Funk, 2001). The unstable intermediate LTA_4 can be further converted into LTB_4 by LTA_4 hydrolase activity (Haeggstrom, 2004) or into LTC_4 by LTC_4 synthase and the LTC_4 synthase isozymes $MGST_2$ or murine $MGST_3$ (Schroder et al., 2003). LTB_4 is a potent chemoattractant for neutrophils, eosinophils and monocytes leading to adherence of phagocytes to vessel walls, neutrophil degranulation and release of superoxide anions. LTC_4 and its metabolite, LTD_4, are potent bronchoconstrictors that increase vascular permeability and stimulate mucus secretion from airways.

Table 3: Biologically active lipids as therapeutic targets in the field of Inflammation (Rader and Daugherty, 2008)

Target	Type of therapy	Biomarkers
TXA_2 receptor	Antagonist	TXA_2
PGE_2 receptors (EP1 and EP3)	Antagonist	None
PGD2 receptor (DP1)	Antagonist	Niacin-associated flushing
5-LO	Inhibitor	LTB_4
FLAP	Inhibitor	LTB_4
LTA_4 hydrolase	Inhibitor	LTB_4
LTB_4 receptors (BLT_1andBLT_2)	Antagonist	None
15-LO	Inhibitor	LTE_4
Secretory PLA_2 (groups IIa, V andX)	Inhibitor	Secretory PLA_2 activity
Lipoprotein-associated PLA_2	Inhibitor	Lipoprotein-associated PLA_2 activity

1.3.1.1. Asthma:

Experiments with 5-LO (−/−) mice in models of asthma and allergic airway inflammation confirmed that 5-LO plays an essential role in the development of airway hyper responsiveness and that LT are involved in eosinophil recruitment after allergen challenge (Funk, 1996; Irvin et al., 1997). Further reports obtained from clinical studies with 5-LO inhibitors and CysLT receptor antagonists drugs showed significant improvement of asthma. Thus, the concept of LT as important mediators in asthma has been confirmed CysLT1 receptor antagonists and 5-LO inhibitors reduce both the early and late phase of bronchospasm and reduce airway hyperreactivity (McGill and Busse, 1996; Claesson and Dahlen, 1999).

Zileuton, a well-known 5-LOX inhibitor and other CysLT1 receptor antagonists like montelukast, zafirlukast and pranlukast have shown significant improvement in reversing the process of asthma (Bell et al., 1992). Remarkable progress has been made in the development of selective antagonists that target LT receptors. The CysLT

antagonists, montelukast, BAY-u9773 and zafirlukast have been shown to be clinically efficacious in chronic asthma and have been successfully introduced into market (Kemp, 2003; Tudhope et al., 1994).

1.3.1.2. Inflammatory skin disease, psoriasis, bowel disease and rheumatoid arthritis:

5-LO metabolites suggested that these mediators could be involved in inflammatory skin diseases such as atopic dermatitis and psoriasis. (Janssen-Timmen et al., 1995), and there have been several clinical trials and case reports with low numbers of patients on the application of CysLT1 and BLT1 receptor antagonists in inflammatory skin diseases with varying therapeutic success (Wedi and Kapp, 2001) Zileuton the only FDA approved drug which is an 5-LOX inhibitor has shown significant improvement in atopic dermatitis and chronic urticaria (Woodmansee and Simon, 1999) whereas this compound and other the 5-LO inhibitors R68151 and R85355 were inactive in psoriasis (Degreef et al., 1990; Van de Kerkhof et al., 1996). Hence it is difficult to say the involvement 5-LO catalysis in the skin disease.

Similarly the role of LT in other inflammatory diseases like rheumatoid arthritis or inflammatory bowel diseases is much less clear, but in study on mice with FLAP-deficient, the severity of collagen-induced arthritis was substantially reduced when compared with wild type or heterozygous animals. Suggesting that LT play an essential role in both the acute and chronic inflammatory response in mice (Griffiths et al., 1997). Zileuton or the FLAP inhibitor MK-591 did not lead to significant therapeutic benefits in patients with ulcerative colitis (Hawkey et al., 1997; Roberts et al., 1997) and rheumatoid arthritis (Weinblatt et al., 1992).

1. 3.1.3. Atherosclerosis and cardiovascular disease:

Clinical trials have confirmed that certain lipoproteins and the renin–angiotensin–aldosterone system are important in the pathogenesis of atherosclerotic cardiovascular disease (CVD). An increased production of LT was observed in ischemic patients, in humans after cardiopulmonary bypass, and in patients undergoing aortic aneurysm repair,

and LT receptor antagonists or 5-LO inhibitors showed cardio-protective effects in various animal models using rats, cats or dogs (Vila, 2004). Application of LT antagonists or various LT synthesis inhibitors are able to improve experimental CVD states in animals, and a clinical trial with a FLAP inhibitor suggests a benefit in the treatment of CVD (Figure 8) (Rader and Daugherty, 2008).

In mice, an early study suggested that deficiency in 5-LO reduces the development of atherosclerosis in mice (Mehrabian et al., 2002). In addition, antagonism of the LTB_4 receptors BLT_1 and BLT_2, or deletion of the genes encoding these, was reported to reduce atherosclerosis in mice (Qiu et al., 2006). Thus, inhibition of the 5-LO–LTB_4 pathway could be a therapeutic approach to atherosclerosis. Indeed, a FLAP inhibitor has entered clinical development for the treatment of atherosclerotic cardiovascular disease (Hakonarson et al., 2005).

Four classes of drugs are currently under development as antiasthma or antiinflammatory therapy, which interfere with LT synthesis or activity (Figure 8).

1) The 5-1ipoxygenase inhibitors directly block the catalytic activity of 5-1ipoxygenase it self.
2) The FLAP inhibitors displace arachidonate from its binding site on the FLAP molecule and prevent it from providing arachidonate to 5-1ipoxygenase as a substrate for LT synthesis. Both these classes of drugs block the synthesis of the cysteinyl LTs and of LTB_4.
3) The activity of cysteinyl LTs (LTC_4, LTD_4, and LTE_4) at the LTD_4 receptor can be blocked by competitive cysteinyl LT receptor antagonists
4) Blockade of LTB_4 activity may be achieved by competitive antagonists at the LTB_4 receptor.

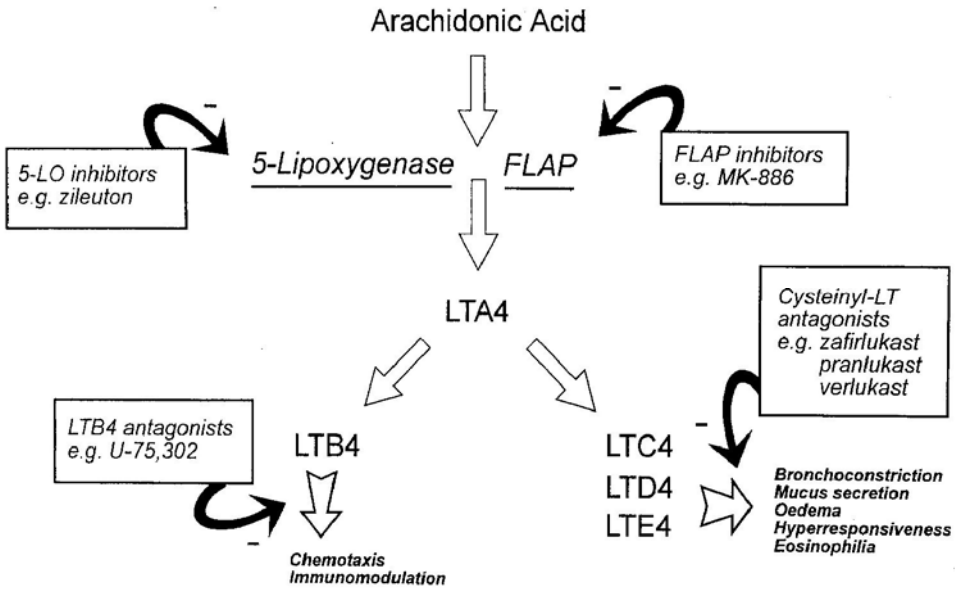


Figure 8. The possible targets in anti-asthma and anti-inflammatory therapy directed against LT synthesis and activity (Holgate et al., 1996)

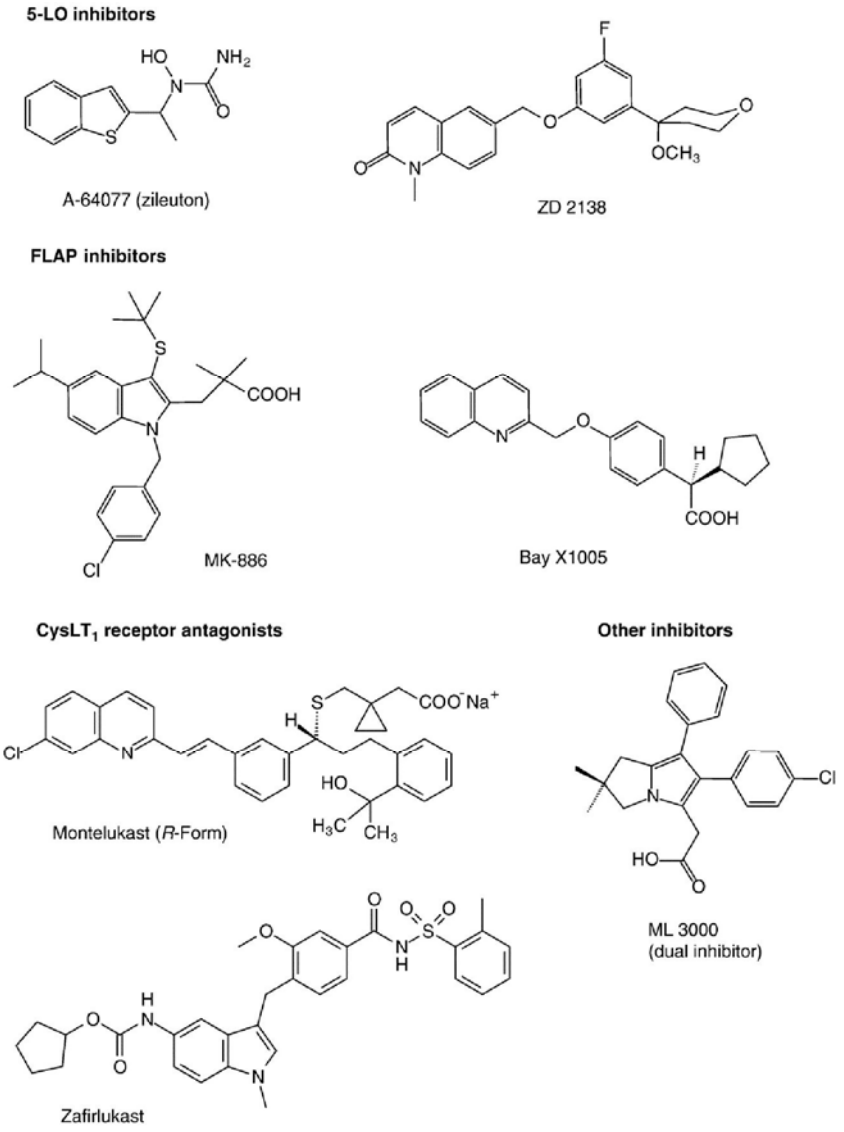


Figure 9. Anti leukotriene drugs

1.3.2. 5-lipoxygenase in cancer:

Accumulating evidence suggests a role for the 5-LO pathway in tumor cell proliferation and survival, implying that cancer may become a novel indication for anti-LT therapy. LO-catalyzed products, LTs, and HETEs also exert profound biological effects on the development and progression of human cancers. For example, 12-LO mRNA expression has been well documented in many types of solid tumor cells, including those of prostate, colon, and epidermoid carcinoma (Honn et al., 1994; Chen et al., 1994). Currently pharmacological agents that specifically inhibit the LO-mediated signaling pathways are now commercially available to treat inflammatory diseases such as asthma, arthritis, and psoriasis (Figure 9). These well-characterized agents are considered good candidates for clinical chemoprevention studies. Major targets are aimed at inhibition of LO activity (5-LO and associated enzymes, or 12-LO and leukotriene receptor antagonism

In order to hold such a postulate or to prove the involvement of 5-LO cascade in the carcinogenesis, one would expect that following criteria be fulfilled in the cancerous cell lines

1. Presence of enzymes required for LT biosynthesis as well as LT receptors
2. Over-expressed in transformed cells or tissues
3. 5-LO product formation occurs at these sites
4. Exogenous addition of 5-LO products stimulates cancer cell proliferation and survival
5. Pharmacological or genetic inhibition of the 5-LO pathway inhibits growth of cancer cells and induces apoptosis.

Expression of enzymes and receptors of the 5-LO pathway is restricted to certain cell types, where as 5-LO protein was detected in cancer cell lines of animal or human origin including brain (Boado et al., 1992), breast (Przylipiak et al., 1998; Avis et al., 2001), colon (Wachtershauser et al., 2000), esophageal mucosa (Zhi et al., 2003; Hoque et al., 2005), lung (Avis et al., 1996), kidney (Matsuyama et al., 2005), mesothelium (Romano et al., 2001), pancreas (Ding et al., 1999; Hennig et al., 2002), and prostate (Myers and

Ghosh, 1999). Increased formation of 5-LO products was observed and interestingly, untransformed cells of the respective healthy organs or tissues did not exert significant 5-LO protein or mRNA levels and had markedly less amounts of 5- LO with respect to overexpressed cancer cell lines. Similar observation was made in the tissue of patients suffering from adenocarcinoma, breast and pancreatic cancer (Gupta et al., 2001; Jiang et al., 2005; Hennig et al., 2005).

Besides 5-LO, FLAP was also found to be enhanced in a panel of human epithelial cancer cell lines (Hong et al., 1999). Various enzymes involved in the synthetic pathway of 5-LO products and respective receptors were found abundantly expressed (Ohd et al., 2003). Addition of 5-LO products like 5(S)-HETE enhanced the growth of human pancreatic, breast, and lung cancer cell lines (Ding et al., 1999 and 2005; Avis et al., 2001 and 1996).

1.3.2.1.Anticancer therapy with LOX inhibitors:

1.3.2.2. Breast cancer: Markedly elevated levels of COX and LOX metabolites were reported in many of the breast cancer cell lines compared with a non-cancerous breast epithelial cells lines (Przylipiak et al., 1998; Avis et al., 2001). Further arachidonic acid stimulates the breast cancer cell lines *in vitro* leading to increased secretion of both LOX and COX products such as 12-HETE, 15-HETE and PGE2. This effect could be completely blocked by adding esculetin, an inhibitor of 5- and 12-LOX (Liu et al., 1994).

1.3.2.3. Lung cancer: 5-HETE stimulates the growth of several lung cancer cell lines (Avis et al., 1996). While cells treated with 5-LOX inhibitors such as NDGA, AA-861 and MK-886 (Avis et al., 2005) showed decreased proliferation, the COX inhibitor, aspirin, had little effect. This suggests that 5-LOX pathway inhibitors have a chemopreventive activity in lung carcinogenesis (Moody et al., 1998).

1.3.2.4. Prostate cancer: LOX-inhibitors such as A-63162, MK-886 and AA-861 effectively blocked prostate tumor proliferation induced by arachidonic acid, suggesting

that; LOX products are essential in modulating prostate DNA synthesis (Ghosh and Myers, 1997).

1.3.2.5. Colon cancer: 12(s)-HETE mediates the release and secretion of cathepsin B, a cysteine protease involved in tumour metastasis and invasion particularly in colon cancer cells. While normal intestinal cells have a limited capacity to synthesize leukotrienes, a platelet type 12-LOX mRNA has been recovered from a human colon carcinoma cell line and was found to have a role in colon tumor proliferation. (Bortuzzo et al., 1996) have reported the *in vitro* inhibition of human cancer cell line proliferation using SC41930, a competitive LTB_4 antagonist

In order to confirm the implication of the 5-LO pathway in to the pathophysiology of cancer, many investigators applied pharmacological tools such as inhibitors of 5-LO (Kennedy et al., 2003; Hoque et al., 2005; Li et al., 2005), FLAP inhibitors (Anderson et al., 2000; Fan et al., 2004), LTA_4 hydrolase antagonists (Chen et al., 2003, 2004) and LT antagonists (Ding et al., 2005) that essentially attenuated the effects attributed to 5-LO and its metabolites, thus, blocking cell proliferation and inducing apoptosis *in vitro* and *in vivo*. The therapeutic value of pharmacological inhibitors of the 5- LO pathway are also supported by various animal models of cancer (Rioux and Castonguay, 1998; Hennig et al., 2005). Despite the strong potential of anti-LT for prevention and treatment of cancer only a few clinical trials have been launched. A phase II study of the LO and thromboxane A_2 synthetase inhibitor CV6504 proposed therapeutic benefit in advanced pancreatic cancer (Ferry et al., 2000), and a current phase II trial with the LTB_4 antagonist LY293111 are in progress (Ding et al., 2005)

1.4. Classification of LOX inhibitors:

Lipoxygenase inhibitors can be classified on the basis of their pharmacological action into (I) Inhibitors of LT biosynthesis (II) Inhibitors of LT action (Werz and Steinhilber, 2006). In general LOX inhibitors can be broadly classified as (a) redox inhibitors, (b) non-redox inhibitors, (c) FLAP inhibitors, (d) LTB_4 receptor antagonists,

(e) LTD_4 receptor antagonists, (f) antioxidants and (g) substrate analogs (Steele et al., 1999).

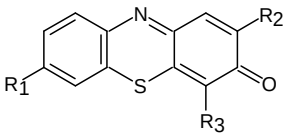
1.4.1. Redox inhibitors:

Many compounds by virtue of their redox potentials can act as alternative substrates for 5-LOX either by 5-LOX-induced oxidation of the native drug or by oxidation of products resulting from metabolic reduction of a prodrug. These compounds, also called as redox inhibitors, may be further classified based on their chemical compositions as Phenothiazines, Quinones and Hydroquinones, Phenolic compounds, Pyrazolidinones, Hydroxamic acids and N-hydroxyureas

Redox-active 5-LO inhibitors comprise lipophilic reducing agents like nordihydroguaiaretic acid (NDGA), caffeic acid, flavonoids (e.g. cirsiliol), coumarins, or compounds such as phenidone (Ford-Hutchinson et al., 1994). These drugs act by reducing/chelating the active site iron, thereby uncoupling the catalytic cycle of the enzyme and are highly efficient inhibitors of 5-LO product formation *in vitro* and *in vivo*. However, they possess only poor selectivity for 5-LO and exert severe side effects (e.g. methemoglobin formation, genotoxicity and interference with other biological redox systems or by the production of reactive radical species (McMillan and Walker, 1992). Moreover, most of the redox-class inhibitors lack suitable oral bioavailability. Rational development of potent and orally active 5-LO inhibitors with weak redox properties, such as AA-861, BW755C, or L 656,224 still led to unspecific compounds that could not enter the market due to severe side-effects (Ford-Hutchinson et al., 1994).

Therefore this class of drug has not been developed further, except some compounds such as lonapalene and docebenone, which display clinical efficacy for psoriasis and arthritis by the topical route

Phenothiazines: Screening efforts at Merck identified potent inhibitors under this class. e.g., L-615919 and L-615392 (Guindon et al., 1987). These compounds act as potent inhibitors of leukotriene synthesis in polymorphonuclear granulocytes.

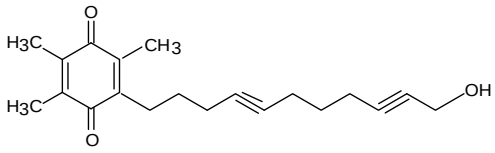


L-615919; R_1, R_2 = H, R_3=Cl

L-651392; R_1, R_2 = CH_3, R_3 = Br

(Iron chelator)

Quinones and Hydroquinones: One of the most widely studied redox inhibitors is AA-861 also called as docebenone or 2,3,5-trimethyl-6-(12-hydroxy-5,10-dodecadiynyl)-1,4-benzoquinone. This is a lipophilic quinone structurally resembling coenzyme Q developed by Takeda chemical industries Ltd. (Japan). It is a potent competitive inhibitor of 5-LOX but has no effect on either 12-LOX or COX at concentrations less than 10 μM (Yoshimoto et al., 1982). In addition to its anti-inflammatory property, it strongly suppresses the induced skin tumor, human leukemia cell lines and human lung cancer cell lines.

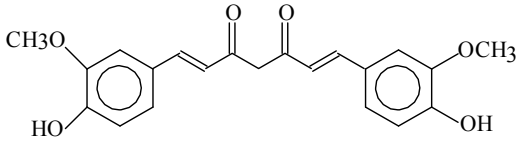


AA-861 or Docebenone

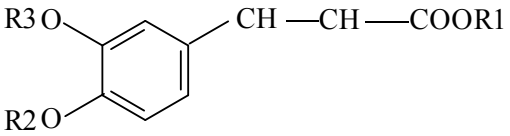
(Iron chelator)

Phenolic compounds: They may be further classified as *natural* phenolics (curcumin, caffeic acid/methyl ester, NDGA, vignafuran etc.) and *synthetic* phenolics (L-670630, TMK-688 and DUP-654).

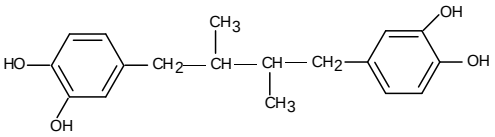
Curcumin (diferuloyl methane) is a naturally occurring polyphenolic phytochemical isolated from rhizome of the plant *Curcuma longa*. It possesses anti-inflammatory property and inhibits the formation of 5 (S)-, 8 (S)-, 12 (S)- and 15 (S)-HETE in mouse epidermis represents the assumption that, curcumin binds to the soybean LOX-3 active site and inhibiting the LOX activity (Skrzypczak-Jankun et al., 2000).



Curcumin

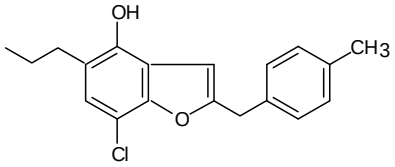


Caffeic acid/methyl ester



NDGA

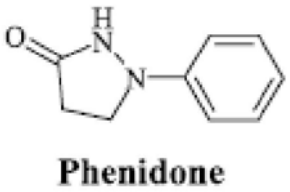
Observation of the activity of the natural phenolic LOX inhibitors resulted in the synthesis of potent, specific and orally active phenol 5-LOX inhibitors such as L-656224 (Lau et al., 1989), L-670630, TMK-688 and DUP-654.



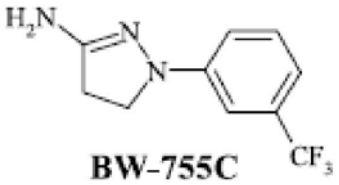
L-656224

(Iron chelator)

Pyrazolidinones: Phenidione and its synthetic prototype analogue BW-755C, apart from their potent *in vitro* 5-LOX inhibitory activity and high redox potentials, show severe toxic effects such as induction of methemoglobin in blood.



Phenidone

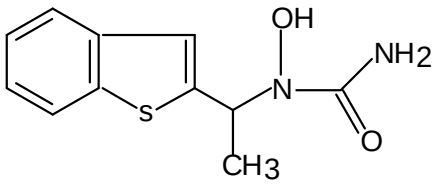


BW-755C

Hydroxamic acids and N-hydroxyureas:

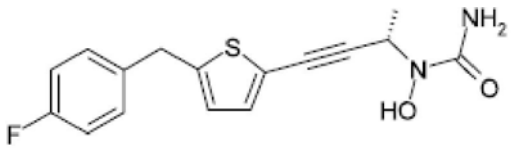
BW-A4C, a hydroxamic acid, selectively and potently inhibits 5-LO in intact granulocytes (Tateson et al., 1988) (IC_{50} 40nM) but is rapidly inactivated *in vivo*.

Zileuton, (A-64077), [N-(1-benzo (b)-thien-2yl) ethyl-N-hydroxyurea] is the first generation N-hydroxyurea series, a specific 5-LOX inhibitor developed by Abbott, represents the first leukotriene inhibitor to show clinical efficacy in humans (Knapp, 1990; Carter et al., 1991) (IC_{50} 0.1-1 μM) the only 5-LO inhibitor that could enter the market. This inhibits 5-LOX via iron chelation but is devoid of 12- and 15-LOX inhibitory activity. In addition to asthma, it has also been evaluated to treat ulcerative colitis.



Zileuton- A-64077 (Iron chelator)

The primary drawbacks of zileuton are its relatively short activity duration and high effective dose. Search for the similar compounds with greater potency has led to the discovery of second-generation N-hydroxyurea derivatives such as A78773 and A79175. Other representatives, ABT- 761 (Brooks et al., 1995), has shown more potent than zileuton in animal models (IC_{50} 120nM) of bronchospasm and possessing a longer oral half life (up to 16 hr) (Lehnigk et al., 1998), as well as the orally active N-hydroxyurea LDP-977 (CMI-977)



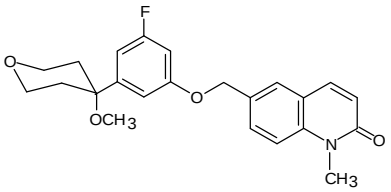
Atreleuton
(ABT-761)

Zileuton is currently approved by the FDA for the prophylaxis and chronic treatment of asthma in adults and children 12 years of age and older. The currently FDA approved dosing schedule for zileuton is 600 mg tablet four times a day. For convenience, it may be taken with meals and at bedtime and it is recommended that zileuton be taken regularly, even during symptom-free periods.

1.4.2. Non-redox inhibitors:

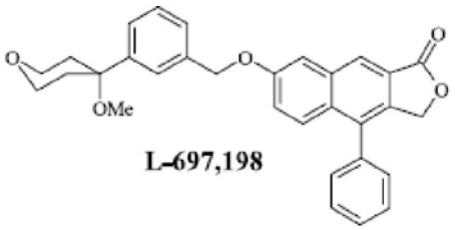
Compounds in this series, also called as competitive inhibitors do not act as reducing substrates in the 5-LOX-catalyzed decomposition of lipid hydroperoxides. They inhibit the 5-LOX-catalyzed reaction of reducing agents with lipid hydroperoxides and the turnover-dependent inactivation of 5-LOX. Hence, they can be considered to be true

nonredox inhibitors (Kusner et al., 1994). e.g. ZD2138 (synthetic) and Jucticidin C (natural product). The former developed as a synthetic hybrid by Zeneca, a selective 5-LOX inhibitor of the methoxytetrahydropyran series, is devoid of redox and iron-ligand binding properties.



ZD-2138 (Synthetic hybrid)

With poor aqueous solubility and short half lives they are orally active compounds ZD 2138 or its ethyl analogue ZM 230487 have shown to inhibit 5-LO product synthesis in whole blood (IC_{50} 20–50 nM) (Crawley et al., 1992). Other hybrid molecule of a methoxytetrahydropyran and a naphtalenic lignan lactone L-697,198 inhibits LTB4 generation in human PMNL with an IC_{50} of 1.5 nM, and is orally active (Ducharme et al., 1994). In spite of the high potency observed by these compounds these non-redox-type 5-LO inhibitors depends on the stimulus, the signalling pathway of 5-LO activation Ca2+ or phosphorylation (Werz et al., 1998; Fischer et al., 2003).

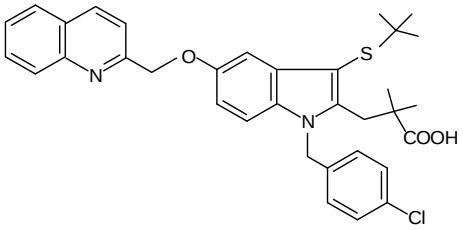


L-697,198

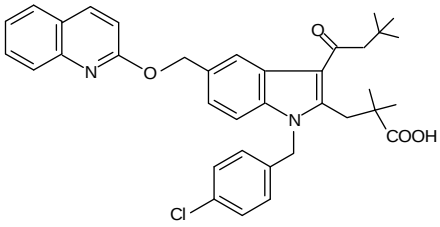
1.4.3. FLAP (five lipoxygenase activation protein) inhibitors:

Extensive screening by Merck on indole compounds derived from COX inhibitors, indomethacin and sulindac led to the development of MK-886, the first FLAP inhibitor to reach clinical evaluation (Gillard et al., 1989). This was believed to work by binding to an arachidonic acid binding site on FLAP, facilitating the transfer of the substrate to 5-LOX. MK-0591, a novel 2-indolealkanoic acid derivative, represents the second generation FLAP inhibitor developed by Merck (Prasit et al., 1993). Like MK-886, MK-0591 blocks 5-LOX activity by binding to FLAP, thereby preventing 5-LOX

translocation and activation. Studies on the 2-quinolylmethyloxy phenyl residue of the compound REV 5901 led to the discovery of BAY-X1005, a potent inhibitor of 5-LOX to treat asthma. This Bayer company's molecule lacks 12-LOX and COX inhibitory activity and is devoid of antioxidant activity (Muller-Peddinghaus et al., 1993). With the recognition of the two separate classes of drugs that can bind to FLAP, a novel class of hybrid structures based on the indole and quinoline classes of inhibitors and termed as quindoles was developed (Mancini et al., 1992). e.g. L-691831.



MK-0591



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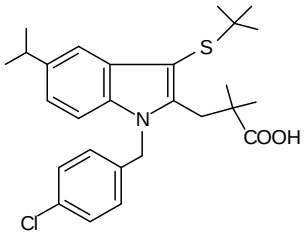
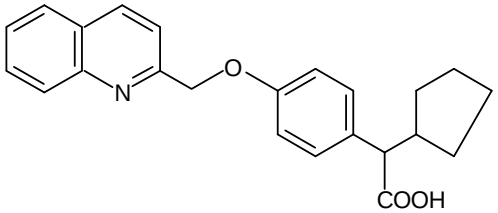


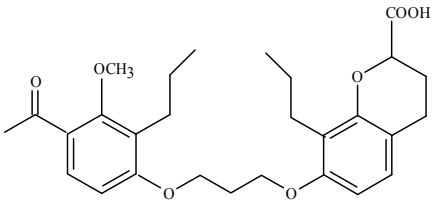
MK-886



BAY-X-1005

1.4.4. LTB_4 receptor antagonists:

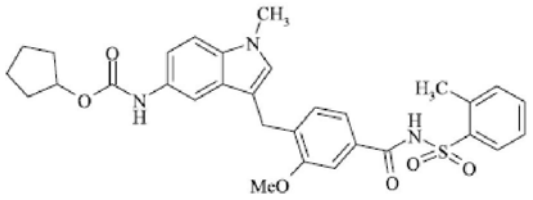
SC 41930, a first generation LTB_4 receptor antagonist developed by Searle demonstrated potency in a variety of inflammatory models. Interestingly, the discovery that SC 41930 inhibits f-MLP-induced superoxide release prompted further research to develop agents with greater potency and selectivity (Djuric et al., 1989). Second generation agents derived from structural analogues of SC 41930 include monomethyl amide SC 53228 and thiazole analogue SC 50605 have demonstrated substantial improvements in pharmacological profiles compared with the first generation agent SC 41930.



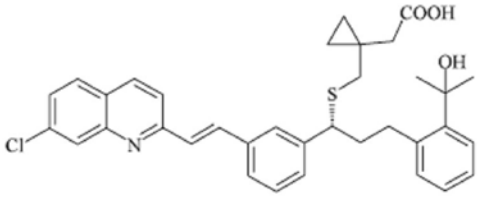
SC-41930

1.4.5. LTD_4 receptor antagonists:

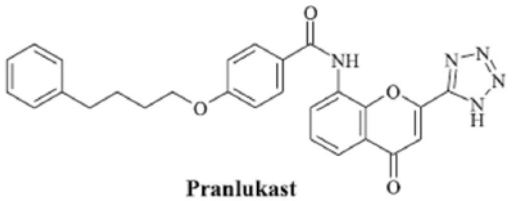
Ultair also called as N-(4-oxo-2-(1H-tetrazol-5-yl)-4H-1-benzopyran-8-yl)-4-(4 phenylbutoxy)-benzamide, synthesized by Smithkline Beecham, was the first LTD4 antagonist to be introduced in 1995 to treat asthma. This was followed by Zeneca's Zafirlukast or ICI-204219 in 1996, for treating mild to moderate asthma conditions. While intensive efforts to develop drugs from LT biosynthesis inhibitors have been much more fruitful and are been rewarded by following marketed compounds (Krell et al., 1990). Remarkable progress has been made in the development of selective antagonists that target LT receptors. The CysLT antagonists montelukast and zafirlukast have been shown to be clinically efficacious in chronic asthma and have been successfully introduced into market (Kemp, 2003).



Zafirlukast



Montelukast



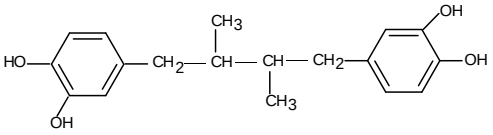
Pranlukast

1.4.6. Antioxidants:

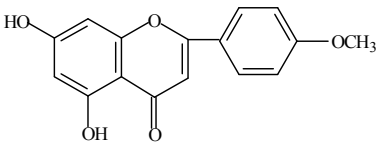
The presence of large amounts of unsaturated fatty acids (60%) in soy phosphatidylcholine makes it prone to enzymatic and non-enzymatic lipid peroxidation. As lipoxygenase enzyme generates peroxide free radicals, one of the modes of action of a

lipoxygenase inhibitor could be due to antioxidant nature of the compound and many of the natural antioxidants are known to inhibit lipoxygenase by interacting with the radicals generated in the lipid peroxidation process (Yasumoto et al., 1970). Free radicals such as superoxide anions ($O_2^{\cdot -}$) hydroxyl radical ($^{\cdot}OH$), malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) trigger the process of Lipid peroxidation (LPO), a well-known phenomenon occurring in both plant and animal systems (Summerfield and Tappel, 1983) The term "reactive oxygen species" (ROS) is often used to denote all oxygen–derived species radicals such as superoxide ($O_2^{\cdot}$), hydroxyl ($^{\cdot}OH$), peroxyl/alkoxyl ($ROO^{\cdot}$) and nonradicals such as hydrogen peroxide (H_2O_2), hypochlorus acid (HOCl) and singlet oxygen ($^1O_2$) (Mc Cord, 1993).

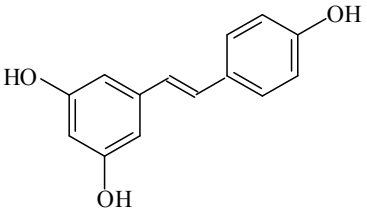
Cells are equipped with several defense systems to combat the detrimental effects of ROS and lipid peroxidation products, which are either enzymatic viz., superoxide dismutase, catalase, glutathione-peroxidase, (Yu, 1994). or non-enzymatic, viz., antioxidants and ROS quenchers such as vitamin E / α-tochopherol, β-carotene, ascorbic acid and caffeic acid. Lipoxygenase inhibitors such as NDGA, n-propyl gallate, Quercetin, Resveratrol (Pinto et al., 1999), Eugenol and BHT are antioxidants that function by reacting with free radical intermediates of the reaction (Yasumato et al., 1970). α-Tochopherol (Grossman and Waksman, 1984) chlorophyll form an enzyme inhibitor complex, there by inhibiting the LOX-1 activity (Cohen et al., 1984).



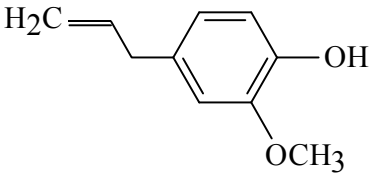
NDGA



Quercetin



Resveratrol



Eugenol

1.4.7. Substrate analogs:

A number of monoenoic fatty acids which resemble the fatty acid substrate but differ in cis, cis, 1-4 pentadiene system are shown to be competitive inhibitors of LOX-1 (Angelo and Ory, 1984). Fatty acids such as, linolelaidic acid, a trans isomer of linoleic acid are not oxygenated by lipoxygenases. Some of the trans fatty acids are competitive inhibitors of lipoxygenase (Veldink et al., 1977), implying that trans fatty acids are also bound to the enzyme. This could be due to the sterically hindered trans configuration, obstructing the initial removal of hydrogen atom (Funk et al., 1987).

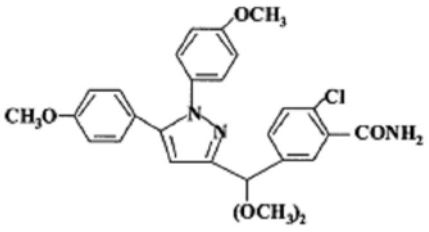
1.4.8. Other LOX inhibitors

1.4.8.1. Dual inhibitors of COX and LOX

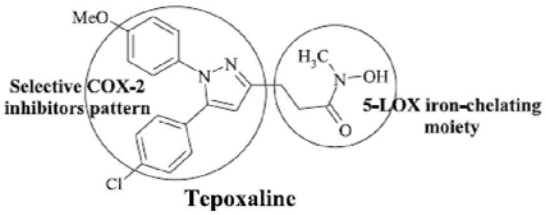
Today one of the leading approaches in the anti inflammatory therapies are dual inhibitors of COX/5-LOX (Celotti and Laufer, 2001; Bertolini et al., 2001; Fiorucci et al., 2001). With good selectivity toward COX-2 inhibitors like ER-341225, Which is an orally active dual inhibitor of COX/5-LOX. Tepoxalin, a dual inhibitor compound was discontinued in clinical Phase II, exhibited anti-inflammatory activity in rats (Argentieri et al., 1994).

Among dual inhibitors, a novel class of non-antioxidant compounds has been described; the most potent and well balanced dual inhibitor of COX/5-LOX is ML3000 ([2,2- dimethyl-6-(4-chlorophenyl)-7-phenyl-2,3-dihydro-1H-pyrrolizine- 5-yl]-acetic acid; a derivative of the phenyl moiety at the 6-position of the pyrrolizine ring (Laufer et al.,1994), now in clinical Phase III. While inhibitors of COXs and 5-LOX in combination have been proved to be more effective than either class of drugs used alone (Nutter and Medvedeff, 1996).

A list of LOX inhibitors and their current status is compiled in Table 4.

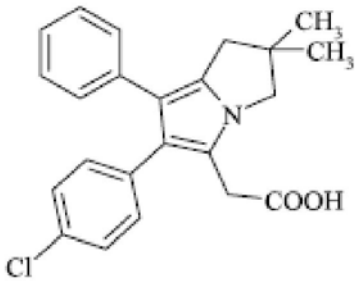


ER-34122



Tepoxaline

IC_{50} COX = 4.2 μM
5-LOX = 1.7 μM



Licofelone
(ML-3000)

IC_{50} COX-1 = 0.16 μM
COX-2 = 0.37 μM
5-LOX = 0.21 μM

1.5. Lipoxygenase inhibitors from various sources:

A literature search on the isolation of LOX inhibitors from natural sources revealed a number of reports since 1986, mainly from plants. However, the isolation of such inhibitors from microbial sources gained momentum only in early 1990s. Many such wide rage of LOX inhibitors from plant and marine sources are listed in Table 5. These compounds include a variety of basic ring systems such as flavonoids, alkaloids and terpenoids. Among the LOX inhibitors from microbial sources, one of the earliest reports is from *Pseudomonas*. *Streptomycetes* genus has the highest number of reports than any other microbial source (Table 6)

Table 4: LOX inhibitors and their status

Compound	Company	Target	Status
Zileuton Leutrol Zyflo	Abbott	5-LO	FDA approved 12/96, Launched in the United States January 1997 (asthma)
ABT-761	Abbott	5-LO & 12LO	Phase-III (asthma)
AA-861	Takeda	5-LO & 12LO	Discontinued Phase-II (asthma and allergy)
ZD-2138	Zeneca	5-LO	Discontinued Phase-II (asthma)
MK-886	Merck	FLAP	Discontinued Phase-I (asthma)
MK-059	Merck	FLAP	Discontinued Phase I (asthma)
BAY-X1005	Bayer	FLAP	Discontinued Phase-II (asthma and cardiac failure)
SC 41930	Searle	LTB_4 receptor	Discontinued Phase-II (asthma and colitis)
SC 5328	Searle	LTB_4 receptor	Discontinued Phase-I (asthma and ulcerative colitis)
Pranlukast	SmithKline	LTD_4 receptor	Approved and launched
Ultair/ ONO-1078	Beecham/Ono Pharmaceuticals		In Japan 1995 Phase III (asthma) and Phase I (Pediatric asthma) in UK&US
Zafirlukast Accolate	Zeneca	LTD_4 receptor	Approved in 1996 in United states
Montelukast Singulair	Merck	CysLT receptor	FDA Approved
Celecoxib celebrex	Pfizer	COX-2	FDA Approved
Refecoxib vioxx	Merck	COX-2	FDA Approved
CJ-13610	Pfizer	5-LO	Preclinical

Table 5: Lipoxygenase inhibitors from plant and marine sources

Name and source of inhibitor	Target enzyme	Reference
***Plant*:**		
Flavonoids	soybean 15-LOX	Ratty et al., 1988; Robak et al., 1988; Alcaraz et al., 1986; Alcaraz and Ferrandiz, 1987
Curcumin from *Curcuma longa*	soybean 15-LOX	Began et al., 1998
Prenyl flavone and artonin E	animal 5-LOX	Reddy et al., 1991
Ursolic acid from flowers of *Culluna vulgaris*	potato 5-LOX & soybean 15-LOX	Simon et al., 1992
Ardisiaquinone from wood of *Ardisia sieboldii*	animal 5-LOX	Fukuyama et al., 1994
Barbamine & oxycanthine alkaloids from *Mahonia aquifolium*	animal LOX & antioxidant	Bezakova et al., 1996
Phenolics from olive	animal 12-/5-LOX	Kohyama et al., 1997
Trans veratrol, viniferin, luteolin from *Paeonia lactiflora* seeds	soybean 15-LOX	Kim and Oh, 1999
Sinensetin & nobiletin from Orange peel	soybean 15-LOX & free radical scavenger	Malterud and Rydland, 2000
Resveratrol from grapes	animal LOX	Pinto et al., 1999
Triacontanol from *Spinacea oleracea* leaves	soybean 15-LOX & antioxidant	Ramanarayan et al., 2000
Extracts of *Boswellia*	animal 5-LOX	Safayhi et al., 2000; Sailer et al., 1996.
Abietic acid	soybean 15-LOX	Ulusu et al., 2002
Boswellic acid	animal 5-LOX	Lalithakumari et al., 2006.
***Marine*:**		
Dicranin from moss *Dicranum scoparium*	soybean 15-LOX	Borel et al., 1993
Terpenoids from sponge	human 15-LOX	Carroll et al., 2001
Terpenoids from sponge	human 15-LOX	Amagata et al., 2003

Table 6: Lipoxygenase inhibitors from microbial sources

Name and source of inhibitor	Inhibition / Activity	Reference
Fungi:		
St-1, from fungus F-124	radical scavenger	Kobayashi et al., 1993
Aspergillus terreus	LOX & antioxidant	Ishikawa et al., 1996
Aspergillus oryzae	*in vivo* antioxidant	Matsuo, 1997, Kawasumi et al., 1999
USF-3506A from fungi	indophenol reducing & DPPH scavenging	Ito et al., 1999
Lipids from fungi	antioxidant	Gvozdkova et al., 2000
Carbazole, phenazine & dicarbonyl compounds from fungi	free radical scavenger	Abe, 2000
Falconensones A & B from Ascomycetes, *Emiricella falconensis*	antioxidant & DPPH scavenger	Takahashi et al., 2000
Aspergillus candidus	antioxidant	Yen et al., 2001
Asperenone from *Aspergillus niger*	soybean 15-LOX	Rao et al., 2002a
Nigerloxin from *Aspergillus niger*	antioxidant/15-LOX	Rao et al., 2002b
Bacterial:		
2-n-heptyl-4-hydroxyquinoline -N-oxide by *Pseudomonas methanica*	animal 5-LOX	Kitamura et al., 1986a
Streptomycetes:		
3-methoxy tropolane from *Streptoverticillum hadanonense* KY11449	animal 12-LOX	Kitamura et al., 1986b
Catechol derivatives from *Streptomyces* sp.	animal LOX, anti-inflammatory & antiallergy	Sato et al., 1992
Nitrosoxacins A, B & C	animal 5-LOX	Nishio et al., 1993
Epocarbazolins A & B from *Streptomyces* sp	rat 5-LOX	Nihei et al., 1993
Isodecyl, isoundecyl, isolauryl -5-hydroxy anthranilates from *Streptomyces* sp AA2807	cell lines 5-LOX	Ohkuma et al., 1993
USF-19A from *Streptomyces* sp	cell lines 5-LOX & soybean 15-LOX	Komoda et al., 1995
Tetrapetalone from *Streptomyces* sp	soybean 15-LOX	Komoda et al., 2004

1.6. Microbial bioactive metabolites:

For more than thousands of years, microorganisms have been used to produce products such as bread, beer and wine. Microbial biotechnology in an advanced phase began during World War I and resulted in the development of the products like acetone-butanol and glycerol fermentations, followed by processes yielding, compounds like, citric acid, vitamins and antibiotics. In the early 1970s, traditional industrial microbiology was merged with molecular biology to yield many biopharmaceutical products, which are of high value, such as human growth hormone, interferons and many more.

Today, microbiologically derived products such as, Antibacterial agents like erythromycin A, vancomycin, penicillin G, streptomycin and tetracycline; antifungal agents amphotericin B and griseofulvin; the cholesterol-lowering agent lovastatin; anticancer agents daunorubicin, mitomycin C and bleomycin; and immunosuppressant rapamycin, mycophenolic acid and cyclosporine A and many more such products of microbial origin have reached the market without requiring any chemical modifications. These examples clearly demonstrate the remarkable ability of microorganisms to produce drug-like small molecules (Table-1) (Vandamme, 1994; Butler, 2005).

Microorganisms are important for many reasons, particularly because they produce things that are of value to us (Demain, 1990). These can be very large materials (e.g. proteins, nucleic acids, carbohydrate polymers, even cells) or smaller molecules and are usually divided in to metabolites that are essential for vegetative growth (*primary*) and those that are inessential (*secondary*). Industrial microbiology explores and screens for a 'wasteful' strain that will overproduce a particular compound that can be isolated and marketed. The main reason for the use of microorganisms to produce compounds that can otherwise be isolated from plants and animals, or synthesized by chemists, is the ease of increasing production by environmental and genetic manipulation; 1000-fold increases have been recorded for small metabolites (Demain, 1988).

1.6.1. Primary metabolites:

Primary metabolites are the small molecules of living cells; they are intermediates or end products of the pathways of intermediary metabolism, building blocks for essential macromolecules. Primary metabolism is generally divided into four metabolic areas: (i) sugar metabolism (including the citric acid cycle and associated pathways), (ii) lipid metabolism, (iii) amino acid and protein metabolism, (iv) nucleotide and nucleic acid metabolism and (v) metabolism of cofactors (Betina, 1994).

Some of the primary metabolites used in the food industries include: alcohols (ethanol), amino acids (monosodium glutamate, lysine, threonine, phenylalanine, tryptophan), organic acids (acetic, propionic, succinic, fumaric, lactic), polyols (glycerol, mannitol, erythritol, xylitol), polysaccharides (xanthan, gellan), sugars (fructose, ribose, sorbose) and vitamins (riboflavin (B_2), cyanocobalamin (B_{12}), biotin).

1.6.2. Secondary metabolites:

Microbially produced secondary metabolites have no function in the growth of the producing cultures although, in nature, they are essential for the survival of the producing organism. As a group that includes antibiotics, other medicinals, toxins, biopestisides and animal and plant growth factors, they have tremendous economic importance. Secondary metabolites are produced by certain restricted taxonomic groups of organisms and are usually formed as mixtures of closely related members of a chemical family (Demain, 1992). Most of these metabolites are biosynthesized from one or more general metabolites by a wider variety of pathways than is available in general metabolism (Bennet and Bentley, 1989). Since secondary metabolites are derived from the intermediates of primary metabolism. Several connecting links exist between primary and secondary metabolic pathways. The key primary metabolites which lead to production of secondary metabolites in microorganisms are: saccharides, shikimic acid and/or aromatic amino acids, nonaromatic amino acids, C_1 compounds, fatty acids (acetic and propionic), citric acid cycle intermediates, purines and pyramidines (Demain, 1992).

In microorganisms, the secondary metabolites are synthesised by various pathways such as polyketide (e.g. aflatoxins, tetracyclines and anthracyclines), terpenoid or isoprenoid (e.g. trichodermin and sterols), shikimate-chorismate (e.g. chloramphenicol, phenyl alanine, tyrosine and tryptophan), metabolites derived from amino acids (e.g. bacteriocins, penicillins and cephalosporins) and metabolites derived from saccharides (e.g. streptomycins, neomycins, gentamycins and kanamycins) (Betina, 1994).

One of well-known group of the secondary metabolites are antibiotics. Most of these antibiotics are classified by, targets, such as DNA replication (actinomycin, bleomycin and griseofulvin), transcription (rifamycin), translation by 70-S ribosomes (chloramphenicol, tetracycline, lincomycin, erythromycin and streptomycin), transcription by 80-S ribosomes (cyclohexamide), transcription by 70- and 80-S ribosomes (puromycin and fusidic acid), cell wall synthesis (cycloserine, bacitracin, penicillin, cephalosporin and vancomycin) and cell membranes (surfactants including: polymyxin and amphotericin; channelforming ionophores, such as linear gramicidin; and mobile carrier ionophores, such as monensin) (Strohl, 1997).

1.7. Production of microbial metabolites through fermentation:

The microbial metabolites, which have a great commercial importance to us can be produced in three different ways, 1) the bioactive molecule can be produced directly by fermentation, 2) the fermentation product can be used as starting material for subsequent chemical modification/derivatization, 3) thirdly the molecules can be used as lead compounds for a chemical synthesis. However, the chemical composition of these microbially produced natural products, apart from their biological actives have an intriguingly complicated/complex structures, sometime including stereochemical diversities, which in many instances will make chemical synthesis of these compounds close to impossible at least when seen from an economical perspective (Clardy and Walsh, 2004).

The semi-synthetic approach can generate analogs by modifying the existing functional groups of natural products. Although this approach leads to less diversity in

terms of structural variety, it has certainly yielded many exciting lead natural product molecules with improved properties over the parent compounds (Butler and Buss, 2006). Tigecycline, a semi-synthetic analog of tetracycline, was approved by the FDA for the treatment of bacterial infections. This semi-synthetic analog overcomes the two major resistance mechanisms of tetracycline: drug-specific efflux pump acquisition and ribosomal protection (Stein and Craig, 2006).

Parasitic diseases in animals (e.g. coccidiostats and anti helminthics) came from the screening of synthesized compounds followed by molecular modification, which were ineffective, but compounds produced by microbial fermentation and used both with and without chemical modification were effective such as, Avermectins especially its derivative, Ivermectin, one of the leading compounds from *Streptomycete* a non antibiotic agent effective against helminthes, nematodes and arthropods (Burg et al., 1979; Demain, 1998).

Rifamycins and ansamycins produced from *Streptomyces* spp. fermentation have lead to a basic backbone skeleton for the production of Rifampicin, a semi synthetic derivative of rifamycin that is effective against Gram-positive bacteria (including *Mycobacterium tuberculosis*) (Heep et al., 1999).

The polyketide class of fermentation products encompasses a wide diversity of chemical structures and biological activities. Important products include macrolide antibiotics (erythromycin, josamycin, tylosin), antifungal polyenes (amphotericin B), parasiticides (avermectin, milbemycin), and immunosuppressive agents (FK-506, rapamycin) (Kirst et al., 1998).

Infectious disease is the second leading cause of death worldwide and there is an immense need to discover new drugs to combat drug-resistant pathogens. In addition to the anti-infective area, natural products have also had a major impact on cancer chemotherapy (Newman et al., 2003). Today more than >60% of the approved drugs for cancer treatment are natural products of which nearly >30% compounds are of microbial

origin, which are undergoing various stages of clinical development as anticancer agents. Ixabepilone (Denduluri , 2007) and temsirolimus have progressed to the advance Phase III stage (Duran et al., 2003).

Today, microbially produced polyethers such as monensin, lasalocid and salinomycin dominate the coccidiostat market and are also the chief growth promoters in use for ruminant animals. Production of surfactin antibiotic by the *Bacillus* sp utilizes simple and expensive medium by SmF (Peypoux et al., 1999).

1.8. Regulation of metabolites production in fermentation:

1.8.1. Carbon regulation:

Carbon utilization by microorganism for growth and secondary metabolism are very different. For example, glucose is usually an excellent source for growth but it has been shown to interfere with the biosynthesis of actinomycin, benzodiazepine alkaloids, cephalosporin, chlortetracycline, ergot alkaloids, erythromycin, kanamycin, oleandomycin, penicillin, but not with aflatoxin production. Oligosaccharides, polysaccharides, and oils are often more convenient carbon sources for fermentations leading to secondary metabolites (Luchese and Harrigan, 1993).

1.8.2. Nitrogen regulation:

The effects of nitrogen sources on secondary metabolism are conditioned by several factors including the type of metabolic pathway, the producing organism, the type and concentration of nitrogen source and whether cultures are stationary or submerged. Usually, complex production media include a protein source and defined media, with a slowly assimilated amino acid as the nitrogen source to obtain high production of secondary metabolites (Demain, 1992). Negative effects of ammonium salts have been reported in the production of cephalosporin, penicillin, erythromycin, chloramphenicol, refamycin, streptomycin and tylosin

Amino acids, e.g. L-aspargine and L-arginine have been proved to be the best nitrogen sources for β-lactam antibiotics production by *Cephalosporium acremonium* and

Streptomyces clavuligerus. Both stimulatory and inhibitory effects of amino acids on bacitracin production by *Bacillus licheniformis* were observed. Amino acids affected antibiotic production whether they were constituent aminoacids of bacitracin, stereo isomers of constituent amino acids or non-constituent amino acids (Betina, 1994).

In defined medium, optimal production of gibberellic acid occurred at 22.5 mM ammonium sulphate (Bruckner and Blechschmidt, 1991). In complex medium containing corn steep liquor, the maximum gibberellic acid titer was measured in medium containing 19 mM ammonium sulphate.

In media with different C:N ratios under submerged conditions, two maxima of citrinin production were observed. The first maximum lies in the trophophase, the other at the beginning of the idiophase. The position of maxima, as well as the quantity and quality of the secondary metabolites can be regulated by changing the C:N ratio (Betina, 1994).

1.8.3. Inorganic salts, trace elements and precursor regulation

Inorganic phosphate is known to exert suppressive effects on the synthesis of many secondary metabolites. Antibiotics susceptible to phosphate regulation belong to different chemical groups including aminoglycosides, tetracyclines, peptides, macrolides, polyene macrolides, β-lactam etc. Industrial fermentations of these antibiotics are frequently carried at concentrations of inorganic phosphate that are limiting for growth. While phosphate in the range 0.3 to 300 mM generally supports extensive growth, concentrations of 10 mM and above suppress the biosynthesis of many antibiotics (Demain 1992).

It is known that several trace elements are essential for microbial growth because of their involvement in metalloenzymes or as enzyme activators. In secondary metabolism, zinc, iron and manganese are the most important trace elements. It has been shown that zinc is an essential element for aflatoxin biosynthesis. The omission of zinc resulted in no detectable versicolorin production by a blocked mutant of *Aspergillus*

parasiticus (Betina, 1994). Maximal aflatoxin production was in a medium containing 0.8 mg of zinc per liter and the minimal requirement was found to be 0.4 mg per liter.Reports on the effects of metals other than zinc on aflatoxin biosynthesis are often conflicting. Trace elements like manganese, magnesium and vanadium were found to favor the biosynthesis of hydroxylated aflatoxin. In submerged cultures, calcium has been found to be important for the production of other fungal metabolites such as verruculogen, griseofulvin and penitrems (Sekiguchi and Gaucher, 1977).

It is known that the available cell precursor levels may regulate antibiotic production, especially when the specific synthetases are already active in the cells. The competition between primary and secondary metabolism for precursors in *Streptomyces clavuligerus* were α-amino adipate a precursor of cephamycin-C, is formed from lysine and thus competes with protein synthesis for a supply of this amino acid (Betina, 1994).

The addition of a side chain depends on the availability of suitable precursors in the medium, such as phenyl acetic acid or phenoxy acetic acid. The precursors are presented to acyltransferase as acyl-Co-A derivatives. If phenyl acetic acid is added to the production medium as the precursor, benzyl penicillin is predominantly formed (Turner, 1992). Thus the intensity of bezyl penicillin production is increased when this limiting precursor is available in the medium.

Another limiting precursor is lysine in the caphamycin-C production by *Streptomyces clavuligerus.* The biosynthesis of cyclosporin-A, the main product among cyclic 11-membered peptides produced by filamentous fungus *Tolypocladium inflatum* is critically effected by the addition to the medium of exogenous amino acids which are members of the cyclosporin ring.

1.9. Scope of the current investigation

Lipoxygenase catalyzes the regio-and stereospecific oxygenation of polyunsaturated fatty acids containing a cis,cis-1,4-pentadiene system in to their corresponding conjugated hydroperoxydiene. It is responsible for lipid peroxidation of

polyunsaturated fatty acids promoting rancidity or off odor in foods of both plant and animal origin, which in turn reduces the nutritive value and functional property of the foods. In mammals, the products of lipoxygenase-catalyzed reactions are responsible for variety of human disorders, such as atherosclerosis, allergy, inflammation, asthma, hypersensitivity and cancer.

Hence inhibitors against lipoxygenase may have a dual application in food industry as antioxidants, and in health sectors with a pharmacological benefit. Although a number of investigations have been carried out on the isolation of LOX inhibitors by synthetic, plant and microbial sources, only a few compounds were reported from fungi. Moreover, there are only a few systematic studies on the screening, isolation, characterization and media optimization for the production of LOX inhibitors from fungi.

In spite of the development of a large number of synthetic compounds for LOX inhibition, only a few compounds have actually gone to the clinical marketing stage. Further, there is a need for the isolation of new and novel metabolites from the available natural sources, so as to clearly understand the basic nucleus or functional groups of the compounds responsible for the inhibitory activity against LOX thus leading to the development of powerful inhibitors, which can actually reverse the LOX catalysis. With this background the current investigation was started.

The thesis entitled "Studies on Microbial Production of Lipoxygenase Inhibitor" consists of three major chapters. Chapter-1 Introduction and review of literature, Chapter-2 Materials and Methods and Chapter-3: Results and Discussion, which is subdivided in to five sections.

The thesis reports isolation of several fungal cultures from forest soil and screening of the metabolites for their ability to produce inhibitors against lipoxygenase. Of about 74 isolates, one isolate CFTRI-A-24 found to produce consistently high yields of potent inhibitor, was characterized as *Penicillium frequentans*. The LOX inhibitor was purified using chromatographic techniques and was identified as "Sclerotiorin". The

inhibition kinetics, mode and mechanism of LOX inhibition, its comparative evaluation to standard compounds, *in vitro* and *in vivo* (animal) models evaluation as antioxidant additive and other various biological properties of the inhibitors such as antioxidant, antimicrobial, aldose reductase inhibition, platelet aggregation inhibition, antimutagenic and anticancer property are reported. A medium was designed with optimum physical and nutritional parameters for the efficient production of sclerotiorin with better yields in a lab scale process. Effective recovery of the inhibitor in an economical method was also designed. An effort was made to evaluate sclerotiorin for the possible application as an antioxidant additive in commercial oil sample.

CHAPTER–2

MATERIALS AND METHODS

2.1. Screening of fungal cultures:

2.1.1. Isolation of fungi from forest soil flora:

About 15 terrestrial soil samples were collected in sterile plastic bags from various places in the Agumbe forest, Western Ghats, Karnataka, India. The soil samples were suspended in sterile water (1:10, w/v) and homogenized for 30 min. The supernatant was subsequently serially diluted in sterile water. 0.2 ml of 10^2 – 10^3 dilution was plated on to potato dextrose agar (PDA) screening medium, and incubated at 28 ^oC in an inverted position. After the 4th day fungal colonies formed on these plates were sub cultured to a fresh PDA slant.

2.2. General fermentation conditions:

2.2.1. Submerged fermentation (SmF) of fungal isolates for the production of lipoxygenase inhibitors:

1000 ml distilled water containing 200 gm of peeled potato slices was boiled for 30 minutes and filtered. To the cooled filtrate 20 gm of dextrose was added and pH was adjusted to 5.2with 0.1 N HCl and final volume was made up to 1000 ml with distilled water. 100 ml of this medium was dispersed in to 500 ml Erlenmeyer flasks and autoclaved for 15 minutes at 121 ^oC. 2 loopful of spores from a five-day-old mature culture slant were transferred aseptically to the medium and the flasks were incubated on a rotary shaker operating at 200 rpm at 30 ^oC for 5 days

2.2.2. Solid State Fermentation (SSF) of fungal isolates for the production of lipoxygenase inhibitors:

Basal wheat bran medium was prepared by adding 10 gm of wheat bran to a 250 ml Erlenmeyer flask, moistened with 10 ml of mineral salt solution (0.007 % (w/v) each of zinc sulphate, copper sulphate and ferrous sulphate in 0.2 N HCl) and 5 ml of distilled water. This medium was autoclaved at 121 ^oC for 60 minutes and cooled to room temperature before inoculation.

Pellets were transferred from a three day old SmF medium to the sterile SSF medium. The flasks were incubated in a slanting position at 30 ^oC. After a day's growth,

the bran was disturbed by gentle tapping and incubation was continued undisturbed till 7 days.

2.2.3. Submerged fermentation of *Penicillium frequentans* CFTRI A-24 for the production of sclerotiorin:

To a 5 day old slant, 15ml sterile 0.1% Tween 20 (v/v) solution was added. The conidial spore suspension was prepared by gentle scraping of the surface of the aerial mycelium with a sterile loop to liberate spores. 1 ml of this spore suspension containing 1.8x 10^6 cfu/ml, was inoculated to 500 ml Erlenmeyer flask containing 100 ml of production medium. The flasks were kept for fermentation at 30 ^oC for 5 days on a rotary shaker operating at 200 rpm.

2.3. General extraction conditions:

2.3.1. Extraction of crude lipoxygenase inhibitors from SmF broths:

After the end of the fermentation 100 ml of ethyl acetate (v/v) was added to each flask and the whole broth was agitated for 3 hours followed by filtration through cheesecloth to separate biomass from the broth. The immiscible organic solvent layer from the broth was separated using a separating funnel. Anhydrous sodium sulfate was added to remove traces of moisture and the organic solvent was distilled to yield a crude residue that was flushed with nitrogen to remove traces of solvent and used for further work.

2.3.2. Extraction of crude lipoxygenase inhibitors from SSF:

After the end of the fermentation culture flasks were treated with ethyl acetate (1:10, w/v) and kept on a rotary shaker for 3 hours at 200 rpm at 30 ^oC. The bran was then filtered through a muslin cloth and the organic layer was separated from aqueous layer by a separating funnel. Traces of moisture were removed by adding anhydrous sodium sulphate. The organic layer was subjected to vacuum distillation. To yield a crude residue, which was transferred to a pre-weighed eppendorf. The crude residue was flushed with nitrogen to remove traces of solvent and used for further work.

2.3.3. Extraction of sclerotiorin from Smf broth:

After the end of 5 days of fermentation 100 ml of ethyl acetate (v/v) was added to each flask and the whole broth was agitated for 3 hours followed by filtration through cheesecloth to separate biomass from the broth. The biomass was vacuum dried and ground to powder and was extracted with ethyl acetate (1:10, w/v) in a soxlet apparatus to yield a reddish crude compound. The immiscible organic solvent layer from the broth was separated using a separating funnel. Both the solvent fractions were pooled and 1 gm of anhydrous sodium sulfate was added to remove traces of moisture. Finally the crude compound was concentrated by distillation and was transferred to a dry preweighed petridish to yield reddish crude residue.

2.4. Media composition:

2.4.1. Medium for screening of fungal isolates

Potato starch	200 gm
Dextrose	20 gm
Rose Bengal	30 mg
Chloramphenicol	300 mg
Agar	15 gm
Distilled water	1000 ml
pH	5.20

2.4.2. Medium for maintenance of isolates (PDA)

Potato infusion	200 gm
Dextrose	20 gm
Agar	15 gm
Distilled water	1000 ml
pH	5.20

This medium was autoclaved at 121 ^{o}C at 15 lbs for 15 min. All the isolated cultures were maintained on PDA slants and sub cultured every fortnight.

2.4.3. Different production media used for the production of sclerotiorin from *Penicillum frequentans* CFTRI-A-24:

Potato dextrose broth

Potato infusion	200 gm
Dextrose	20 gm
Distilled water	1000 ml
pH	5.20

Sucrose broth

Sucrose	20 gm
NaCl	10 gm
Yeast extract	10 gm
Distilled water	1000 ml
pH	5.20

Modified sucrose broth

Sucrose	30 gm
NaCl	15 gm
Yeast extract	10 gm
Distilled water	1000 ml
pH	5.20

Czapek-Dox Broth

Sucrose	30 gm
$NaNO_3$	2 gm
KH_2PO_4	1 gm
$MgSO_4$	0.5 gm
KCl	0.5 gm
$FeSO_4$	0.01 gm
Distilled water	1000 ml
pH	7.30

Glycerol Broth

Glycerol	20 ml
Glucose	20 gm
Malt extract	20 gm
Soybean peptone	10 gm
CaCO3	10 gm
NaCl	10 gm
Distilled water	1000 ml
pH	7.30

Production media –1 (Matsuzaki et al., 1995a)

Sucrose	20 gm
Glucose	10 gm
Corn steep liquor	10 gm
Meat extract	5 gm
Yeast extract	5 gm

KH_2PO_4	1 gm
CaCO3	3 gm
Trace metal solution	10 ml ($FeSO_4$, $MgSO_4$, $ZnSO_4$, $CuSO_4$, $CaCl_2$ at 1% w/v)
Distilled water	1000 ml
pH	6.0

Production media –2 (Nam et al., 2000a,b)

Glucose	20 gm
Yeast extract	2 gm
Peptone	5 gm
KH_2PO_4	1 gm
$MgSO_4$	0.5 gm
Distilled water	1000 ml
pH	6.0

Production media –3 (Arai et al., 1995)

Soluble starch	15 gm
Yeast extract	2 gm
KH_2PO_4	1 gm
$MgSO_4$	0.5 gm
Agar	1 gm
Distilled water	1000 ml
pH	6.0

Production media –4 (Arai et al., 1995)

Soluble starch	30 gm
Glycerol	10 gm
Soybean meal	20 gm
Yeast extract	3 gm
KCl	3 gm
$CaCO_3$	2 gm

KH_2PO_4	2.5 gm
$MgSO_4$	2.5 gm
Distilled water	1000 ml
pH	6.0

2.4.4. Media composition for Ames test:

Nutrient agar

Glucose	10 gm
Beef extract	3 gm
Peptone	5 gm
NaCl	5 gm
Agar	15 gm
Distilled water	1000 ml
pH	7.4

Vogel –Borner medium (VB) (50x)

$MgSO_4$	10 gm
Citric acid monohydrate	100 gm
K_2HPO_4	500 gm
$NaHNH_4PO_4.4H_20$	175 gm
Distilled water warm	1000 ml

Top agar

Agar	6 gm
NaCl	6 gm
Distilled water	1000 ml

Minimal glucose medium

40% Glucose	50 ml
VB medium	20 ml
Agar	15 gm
Distilled water	930 ml

(40% glucose and VB medium added at the end)

Histidine/biotin solution

D-Biotin	30.5 mg
L-histidine	26.2 mg
Distilled water	250 ml

(Filter sterilized and stored in glass vials)

2.5. Biological Assays:

2.5.1.Soybean lipoxygenase assay (Axelrod et al., 1981)

2.5.1.1Purification of soybean lipoxygenase:

Soybean LOX-1 was isolated according to the method of Axelrod (1981) with some modifications (Sudharshan and Rao, 1997).

2.5.1.2. Extraction of lipoxygenase from Soybean seeds:

Soybean seeds were finely ground in a blurr mill and then defatted with hexane. The defatted soybean meal was stored in an airtight container at 4 ^{o}C. The defatted soybean meal (60 g) was extracted with 600 ml of ice-cold sodium acetate buffer (0.2 M, pH 4.5) for 1 hour. The suspension was centrifuged at 6000 rpm for 30 min. The pH of the supernatant solution was adjusted to 6.8 with 10 M NaOH and centrifuged for 30 min at 6000 rpm. All steps were performed at 4 ^{o}C.

2.5.1.3. Ammonium sulphate precipitation:

To this, ammonium sulphate was added (with constant stirring) to 30% saturation. The resultant precipitate was removed after centrifugation at 6000 rpm. To the clarified supernatant, ammonium sulphate was added to raise the saturation to 60%. The protein fraction that precipitated at this state was dissolved in sodium phosphate buffer (20 mM, pH 6.8). This solution was dialyzed against the same buffer with three changes of 3 liters.

2.5.1.4. Anion exchange chromatography:

The dialyzed protein was centrifuged at 6000 rpm for 30 min and the supernatant was chromatographed on DEAE-Sephadex A-50 column (25 x 4 cm), which was pre-equilibrated with sodium phosphate buffer (20 mM, pH 6.8). A column was developed with a linear gradient formed from phosphate buffer (20 mM, pH 6.8) to (70 mM, pH 6.8) and enzyme was eluted with a linear gradient of phosphate buffer (170 mM to 240 mM, pH 6.8). The fraction containing maximum lipoxygenase activity was pooled and the solution was made to 70% saturation by slow addition of ammonium sulphate. After

1 hour, the precipitate was centrifuged, dissolved in phosphate buffer (20 mM, pH 6.8) and was dialyzed against the same buffer with three changes.

2.5.1.5. Molecular sieve chromatography:

The dialyzed protein solution was loaded on a Sephadex G100 column (90 x 2.5 cm), which was pre-equilibrated with phosphate buffer (20 mM, pH 6.8). The enzyme was eluted with the same buffer. The protein fractions, which showed maximum activity, were pooled, concentrated using an Amicon ultrafiltration cell with 30 kD cut-off membrane. The purified enzyme had a specific activity of 150 μmoles/min/mg of protein. The enzyme concentration was determined by measuring the absorbance at 280 nm and using a value $E^{1\%} = 14$ (Pistorius and Axelrod, 1974). SDS-PAGE and specific activity measurements confirmed the purity of LOX-1 (Figure10).

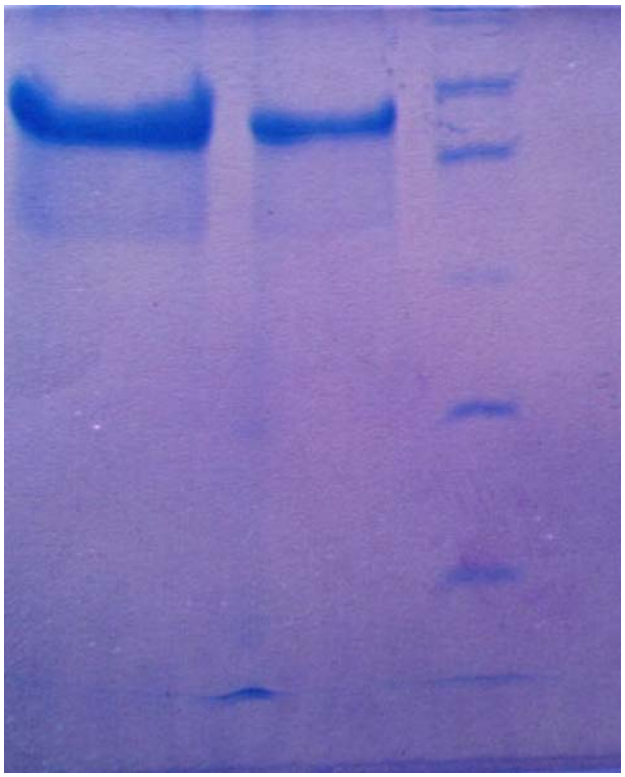
2.5.1.6. Preparation of linoleic acid substrate:

Linoleic acid (70 mg) with equal amount of Tween-20 were weighed and dissolved in 4 ml of oxygen-free water. The resulting suspension was clarified by adding 0.2 M NaOH. The final volume was made up to 25 ml with borate buffer (0.2 M, pH 9.0) and stored in 2 ml capacity Eppendorf tubes under nitrogen at –80 ^{o}C until used (Axelrod et al., 1981).

2.5.1.7. Soybean lipoxygenase assay:

The assay was carried out by monitoring the appearance of cis, trans-hydro peroxide derivative at 234 nm. The 3 ml assay mixture contained 2.955 ml sodium borate buffer (0.2 M, pH 9.0), 20 μl enzyme (0.18 units), 10-20 μl inhibitor dissolved in DMSO (the inhibitor was incubated with enzyme for 2-3 minutes at room temperature). Reaction was initiated by addition of 150 μM substrate. The control sample received equal volumes of DMSO without the inhibitor. The relative activity was expressed as the percentage of the enzyme activity in relation to the control without inhibitor used in the assay.

One unit of enzyme activity is defined as the amount of enzyme required in forming 1 μmol of the product/min/at 30 ^{o}C under the given assay conditions.



Lane 1 **Lane 2** **Lane 3**

Figure 10. SDS-PAGE purification of LOX-1

LOX-1, 1mg/ml diluted with SDS sample buffer (1:1) and heated on boiling water for 90 sec. Electrophoresis was done on 12% polyacrylamide gel. Gel was stained with coomassie brilliant blue. Lane 1 and Lane 2 are LOX-1; Lane-3 molecular weight marker

2.5.2. 5-Lipoxygenase (5-LO) assay (Aharony and Stein, 1986)

2.5.2.1. Isolation of human polymorphonuclear leukocytes (PMNLs):

About 9 ml of human blood was collected from a healthy individual (after seeking consent), in tubes containing 1 ml tri sodium citrate (3.8%, w/v) as anticoagulant. About 3 ml of Ficoll–Histopaque reagent (Sigma Aldrich, Saint Louis, USA) was taken in a 10 ml glass centrifuge tubes. On top of this reagent equal volume of anticoagulated blood was layered by gently pouring along the sides of the tubes. Care was taken such that the bottom reagent layer was undisturbed or did not mix with the blood. This was centrifuged at 1600 rpm for 30 min at room temperature. After the end of centrifugation the buffy layer rich with platelets and PMNLs that appears just below the upper plasma layer was separated. 2-2.5 ml of the buffy layer was mixed with 1-1.5 ml of 3% NaCl and centrifuged at 7000 rpm for 10 min at 4 ^{o}C. The supernatant was discarded and the white precipitate which resulted after centrifugation was again dispersed in 1 ml of 1.8% NaCl solution and centrifuged at 7000 rpm for 10 min at 4 ^{o}C. The white pellet was dispersed in 1-2 ml of phosphate buffer saline (0.1 M, pH-7.4 with 0.9% NaCl) and centrifuged at 7000 rpm for 10 min at 4 ^{o}C. This step was repeated for 3-4 times in order to wash the PMNLs (Boym, 1976).

2.5.2.2. Isolation of 5-Lipoxygenase (5-LO):

The PMNL cells were suspended in 0.5-1 ml of phosphate buffer saline (0.1 M, pH-7.4) and sonicated for 30 seconds at 20 KH_Z at 4 ^{o}C to release the cytosolic 5-lipoxygenase enzyme in the solution. The supernatant was used as source of the enzyme. Protein content was estimated by Lowry's method (Lowry et al., 1951) against bovine serum albumin as standard.

2.5.2.3. Substrate and reagent preparation:

The substrate stock solution (10 mM) was prepared by taking 3.32 µl of arachidonic acid in 1 ml oxygen free distilled alcohol. These vials were flushed with nitrogen and stored at– 20 ^{o}C till use.

10.14 mg of of Adenosine triphosphate (ATP) was dissolved in 1 ml distilled water and stored at 4 ^{o}C (stock 20 mM).

1.54 mg of Dithiothreitol (DTT) was dissolved in 2 ml distilled water and stored at 4 ^{o}C (stock 5 mM).

6.65 mg of $CaCl_2$ was dissolved 10 ml distilled water (stock 6 mM).

2.5.2.4. 5–LO assay:

The assay mixture contained phosphate buffer (0.1 M, pH-7.4), 10 µl of DTT, 10 µl of ATP, 50 µl $CaCl_2$, 15 µl of arachidonic acid and 8 µg of 5-LO enzyme in 3ml. The enzyme activity was measured as 5-hydroxyeicosa tetraenoic acid (5-HETE) formed at 234 nm. 10 µl of the inhibitor dissolved in DMSO was incubated with 5-LO enzyme for 3 minutes at room temperature prior to the initiation of the reaction with the substrate. The control received equal volumes of DMSO without inhibitor. All assays were performed in triplicates and the mean value is represented. The inhibition of 5-LO activity was expressed as percent inhibition over control.

2.5.3. Human Platelet Aggregation Assay (Brammer et al., 1983)

2.5.3.1. Preparation of platelet rich plasma (PRP):

Human blood was drawn by venipuncture from a healthy volunteer (who had not taken any medication for 15 days prior) after seeking consent. Blood was transferred to test tube containing anticoagulant 3.8% tri-sodium citrate (9:1 v/v). The anticoagulated blood by centrifugation at 1100 rpm for 20 min and the supernatant PRP was transferred to polypropylene tube or siliconised glass tubes and stored at 37 ^{o}C. The aggregation experiment was performed within two hours of blood collection.

2.5.3.2. Preparation of Epinephrine, Collagen and ADP:

2.49 mg of epinephrine was suspended in 1 ml of saline (0.9% w/v sodium chloride solution) and 0.7 N HCl was added drop wise till a clear solution was obtained and final was made up to 6.8 ml with water. This stock solution was stored frozen until use.

5 mg of collagen in 10 ml of 0.1N acetic acid was kept undisturbed for 18 hours and homogenized to get a clear solution.

2.05 mg of adenosine diphosphate was dissolved in 2 ml of distilled water.

2.5.3.3. Platelet Aggregation Assay:

Platelet aggregation was monitored turbidometrically at 37 ^{o}C in a Dual Aggregometer (Chrono-Log, USA) operating at 1000 rev/min coupled with an Omniscribe recorder (Coulter, USA). The assay mixture contained 450 µl of PRP, aggregation inducers (8 µl ADP or 10 µl collagen or 8 µl epinephrine) and 2-10 µL inhibitor sample dissolved in DMSO (2 mg/ml stock). Aspirin (1 mg/ml) was used as positive standard. Controls received the same quantity of solvent. PRP was pre incubated with inhibitors for 1 min for 37 ^{o}C with constant stirring of 1000 rpm at 37 ^{o}C. ADP was added to initiate the reaction and the progress of the aggregation was recorded for 5 min. The rate of aggregation was quantified by decrease in absorbance and expressed as percent change over control. All assays were carried out in duplicate and the mean value has been reported.

2.5.4.Rat lens aldose reductase assay (ADR) (Kim and Oh 1999)

2.5.4.1. Preparation of enzyme, substrate and reagents:

15 pairs of fresh rat eye lens (approximately 1.65 gm) were homogenized in sodium phosphate buffer (1:5, w/v) (0.135 M, pH 7.0) containing 0.5 mM PMSF and 10 mM β-mercaptoethanol. The homogenate was centrifuged at 8000 g for 30 min at 4 ^{o}C and the supernatant was used as enzyme source.

14.8 mg of DL-glyceraldehyde was dissolved in 1 ml of distilled water and stored at 4 ^{o}C (164 mM stock).

1.64 gm of lithium sulphate was dissolved in 5 ml distilled water (30 mM stock).

7.15 mg of NADPH was dissolved in 1 ml of distilled water and stored at 4 ^{o}C (10 mM stock).

2.5.4.2 ADR assay:

The assay mixture contained Na,K-phosphate buffer (0.135M,pH 7.0), 100 µL NADPH, 100 µL DL-glyceraldehyde, 100 µL lithium sulphate, 10 µl of inhibitor dissolved in DMSO. The reaction was initiated by addition of enzyme solution (0.125 units/ml of homogenate) and monitored by decrease in absorbance at 340nm. Control

sample received appropriate amount of DMSO, which had no inhibitory effect on enzyme. Inhibition was expressed as a percentage relative to solvent control

2.5.5. Antibacterial assay (Owais et al. 2005)

Food born pathogenic bacterial cultures (*Micrococcus luteus* NCIB 196, *Bacillus cereus* F 4810, *Bacillus subtilis* NCIB 3610, *Staphylococcus aureus* CFTRI-CI-01, *Klebsiella pneumoniae* CFTRI-CI-12, *Escherichia coli* NCIB 9132,*Yersinia enterocolitis* CFTRI-CI-19 *Listeria monocytogenes* CFTRI-CI-21,) were obtained CFTRI culture collection center and maintained on nutrient agar slants and were sub cultured every fortnight. For the agar well diffusion antibacterial assay, bacteria seed suspension was prepared in nutrient broth from an overnight grown stock culture. Nutrient agar plate were prepared by pour plate technique containing 0.2 ml stock inoculum of each culture (1.4x 10^7 cfu/ml). After the solidification of agar wells were made with a sterile borer. Different concentrations of sclerotiorin (stock 2 mg/ml DMSO) were added to the wells. Control well received the appropriate amount of solvent DMSO. Plates were incubated at 30 ^oC for 3 days and at 35 ^oC for 5 days. Plates were visually examined for zones of inhibition, expressed in millimeters.

2.5.6. Antifungal assay (Wilson et al., 2005)

Test Fungal cultures (*Aspergillus niger* CFTRI 1105, *Fusarium moniliforme* M3125, *Alternaria alternata, Penicillium* sp, *Candida albicans and Candida tropicalis* NCIM-3074 and 3118*)* were obtained from CFTRI culture collection center and University of Mysore. These cultures were maintained on PDA slants and were sub cultured every fortnight. For the agar well diffusion antifungal assay, fungal spore suspension was prepared in (0.1% Tween 20, w/v) from a 5 day old mature slants. Potato dextrose agar plates were prepared by spread plate technique, fungal spore suspension of each culture (1.8x 10^5 cfu/ml) was uniformly spread on the surface of the agar plates from a sterile cotton swab. After the solidification of agar wells were made with a sterile borer. Different concentrations of sclerotiorin (stock 2 mg/ ml DMSO) were added to the wells. Control well received the appropriate amount of solvent DMSO. Plates were

incubated at 30 ^{o}C for 3 days and at 35 ^{o} C for 5 days for *Candida sps*. Plates were visually examined for zones of inhibition, expressed in millimeters.

2.5.7. Mutagenicity test or Ames test (Maron and Ames, 1983).

2.5.7.1. Preparation of S9 fraction:

S9 fraction, is a post mitochondrial fraction extracted from Aroclor 1254 induced rat liver. The S9 cofactor mix was prepared by adding 2 ml of S9 fraction, 0.4 ml magnesium chloride (8 mM stock), 0.4 ml potassium chloride (33 mM stock), 1ml D-glucose-6- phosphate (120 mM stock) and 1ml Nicotinamide adenine dinucleotide phosphate (80 mM stock) prepared in sodium phosphate buffer (0.2 M, pH 7.4). All components were prepared and mixed just before the use.

2.5.7.2. Tester strain medium:

Nutrient broth was used for the regeneration of the lyophilized tester strains. *Salmonella typhimurium* TA 1535, TA 97a, TA 98 and TA 100 were obtained from Microbial Type Culture Collection numbered MTCC 1254B, 1357B, 1251B and 1252B respectively. The strains were maintained on nutrient agar slants (Mortelmans and Zeiger, 2000).

2.5.7.4. Mutagenicity Assay:

The tester strains from frozen lyophilized cultures were grown overnight for 12-16 hours at 37 ^{o}C at 100 rpm in nutrient broth. Different concentrations of sclerotiorin (stock 1 mg/ml DMSO) was added to 1.8 ml of top agar (0.6% agar and 0.6% sodium chloride) supplemented with S9 cofactor mix and 100 µL culture (2 - 5 X 10^{8} cell / ml and poured on to a plate containing minimal agar. The plates were incubated at 37 ^{0}C for 48 hours and the his^{+} revertant colonies were counted.

2.5.8. Human hepatocarcinoma (Hep3B) cell line experiment:

Human hepatocarcinoma (Hep3B) cells were obtained from National Center for Cell Sciences (NCCS, Pune, India). Cells were cultured in Dulbecco's modified Eagle Medium supplemented with 10% fetal bovine serum (Sigma Aldrich, Saint Louis, USA),

100 U/ml penicillin and 0.1 mg/ml streptomycin at 37 ºC in a humidified atmosphere containing 5% CO_2.

2.5.8.1.Cytotoxicity assay (Mosmann, 1983)

The cytotoxic effect was evaluated using the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were trypsinized for 10 min (Tripsin 1x, Sigma Aldrich, Saint Louis, USA) and seeded into 96-well plates at a density of 2 x 10^5 cells/ well. After 24 hours of growth at 37 ºC under 5% CO_2, the cells were treated with different concentrations of sclerotiorin (stock 1 mg / ml 0.5% DMSO) and incubated for two hours. MTT solution (1 mg/ ml) was added to the wells (10 µl/100 µl medium per well) and further incubated for another four hours. The medium was then removed and 200 µl of (DMSO) was added to each well. The absorbance was read at 570 nm in microplate ELISA reader (Versamax, microplate reader, USA). Control cells were maintained without the addition of the test sample. The absorbance of the untreated cells was considered as 100%. The assay was done in triplicates and the data are expressed as the mean percentage of viable cells as compared to the untreated cells.

2.5.8.2. Antiproliferative assay (Mosmann, 1983)

The assay was carried out in a similar fashion as that in cytotoxicity assay except that the cells were incubated for 24 and 48 hours with the test sample at the end of which MTT assay was carried out. At least three repeats for each sample were used to determine the cell proliferation.

2.5.8.3. Morphological assessment of apoptosis (Chodon et al, 2007)

To assay nuclear morphology (apoptotic nuclei), Hep3B cells were (2 x10^5) seeded on glass cover slips placed in a 12 well tissue culture plate incubated in a humidified atmosphere of 5% CO_2 at 37 ºC. At 80% confluence the cells were treated with 40 ppm of sclerotiorin for different time intervals (30 to 120 minutes). At the end of the treatment, the cells were washed in phosphate buffered saline (PBS) and stained with an equal mixture of ethidium bromide and acridine orange. The nuclear morphology of cells were visualized by a fluorescence microscope (Leica).

2.5.9. Evaluation of antioxidant property of sclerotiorin *in vivo*:

2.5.9.1. Animal treatment:

Sclerotiorin suspended in 0.25ml of 5% defatted milk powder containing 3% gum acacia, was intragastrically administered at 10, 25 and 50 mg / kg body weight to groups of adult male mice (each weighing around 30 g) starved overnight. The compound was thus administered on alternate days for a week. Twelve hours after the final dose on the seventh day, carbon tetrachloride (CCl_4) was intraperitonially administered at 1.5 ml / kg body weight. Vitamin-E was used as positive control in this study. The animals were sacrificed 12 hours after carbon tetrachloride administration. Appropriate control without any exposure to sclerotiorin and a solvent control were maintained in parallel.

2.5.9.2. Estimation of antioxidant metabolites:

5% (w/v) liver homogenate was prepared with 0.15 M KCl and centrifuged at 500 g for 10 min. The cell-free supernatant was used for the assays.

2.5.9.2.a. Lipid peroxides estimation (Yagi 1984)

Plasma lipid peroxides were estimated by the fluorimetric measurement of thiobarbituric acid complex. The fluorimetric measurement was carried out at an excitation wavelength of 515 nm and emission wavelength of 553 nm and compared with the standards prepared by reacting 0.5 nmol 1,1,3,3-tetraethoxypropane with TBA reagent.

Liver lipid peroxides were determined by photometric measurement of thiobarbituric acid complex (Ohkawa *et al*.1979), involving extraction of end product complex into *n*-butanol. Absorbance of butanol extract was measured at 532 nm, values were compared with those of tetraethoxy-propane, which is used as standard treated similarly.

2.5.9.2.b. Ascorbic acid estimation (Omaye et al., 1973)

Ascorbic acid in serum and liver homogenate were estimated spectrophotometrically by measuring the 2,4-dinitrophenylhydrazone derivative of

dehydroascorbic acid. 100 µl of serum and 200 µl liver homogenate were mixed with 1 ml of ice cold 10% TCA and centrifuged for 6000 rpm for 20 min and to the 0.5 ml of supernatant 0.1 ml of 2,4-. dinitrophenyl hydrazine-thiourea-$CuSO_4$reagent (DTC) reagent was added and incubated at 3 hours at 37 ^oC. Reaction was terminated by adding 750 µl of ice cold 65% H_2SO_4 and the absobance was recorded 520 nm.

2.5.9.2.c. Glutathione estimation (Beutler et al., 1963)

Glutathione in serum and liver homogenate were estimated spectrophotometrically. 300 µl of serum or liver homogenate samples were mixed with 0.9 ml of distilled water and 3 ml of precipitation solution, This mixture was allowed to precipitate for 10 min and filtered through whatman filter paper no-1. To 1 ml of filtrate, 4 ml phosphate solution and 1 ml 5,5'-dithio-bis(2-nitrobenzoic acid) DTNB (Ellmans reagent) was added and the absorbance was recorded at 412 nm.

2.5.9.2.d. Thiols estimation (Sedlock and Lindsay, 1968)

Thiols in serum and liver homogenate were estimated spectrophotometrically. 100 µl of serum or liver homogenate samples were mixed with 0.6 ml of tris-HCl buffer (10 mM, pH 8.2), 0.04 ml of DTNB reagent (10 mM) and 3.16 ml methanol. This mixture was mixed well and allowed at room temperature for 10 min. centrifuged at 5000 rpm for 10 min and the final absorbance was recorded at 412 nm.

2.5.9.3. Antioxidant enzymes:

5% (w/v) liver homogenate was prepared with 0.15 M KCl and centrifuged at 500 g for 10 min. The cell-free supernatant was used for the assay of activities of catalase superoxide dismutase (SOD) and peroxidase.

2.5.9.3.a. Catalase assay (Aebi 1984)

1 ml of liver homogenate was added to 1.9 ml of phosphate buffer (50 mM, pH 7.4). The reaction was initiated by the addition of 1 ml of hydrogen peroxide (30 mM). Blank without liver homogenate was prepared with 2.9 ml of phosphate buffer and 1 ml of hydrogen peroxide. The decrease in optical density due to decomposition of hydrogen

peroxide was measured at the end of 1 min against the blank at 240 nm. Catalase activity was expressed as the amount of enzyme that decomposes 1 μM H_2O_2 per mg protein.

2.5.9.3.b. Superoxide dismutase assay (Beauchamp and Fridovich 1971)

0.5 ml of liver homogenate was mixed with 1 ml of 50 mM sodium carbonate, 0.4 ml of 24 μM nitro blue tetrazolium NBT, and 0.2 ml of 0.1 mM EDTA. The reaction was initiated by adding 0.4 ml of 1 mM hydroxylamine hydrochloride. Zero time absorbance was recorded at 560 nm followed by recording the absorbance after 5 min at 25^0 C. A control was simultaneously run without liver homogenate. SOD activity was expressed as the amount of enzyme required to inhibit the reduction of NBT by 50%. The specific activity was expressed in terms of units per mg protein.

2.5.9.3.c. Peroxidase assay (Nicholos 1962).

0.5 ml liver homogenate was mixed with 1 ml of 10 mM potassium iodide solution and 1 ml of 40 mM sodium acetate solution. The absorbance of potassium periodide was read at 353 nm, which indicates the amount of peroxidase. Twenty ml hydrogen peroxide (15mM) was added and the change in the absorbance in 5 min was recorded. Peroxidase activity was expressed as the amount of enzyme required to change the OD by 1 unit per min. The specific activity was expressed in terms of units per mg protein.

2.5.9.4. Plasma non-specific enzymes:

Alanine aminotransferase (SGPT) and aspartate aminotransferase (SGOT) activities were determined in serum using test kits (Span Diagnostic, Surat, India).

2.5.9. 4.a. SGPT:

Assay was estimated by monitoring pyruvate formed when α-ketoglutrate reacts with L-alanine, mediated by SGPT. Pyruvate is made to react with 2,4-Dinitrophenyl hydrazine to form hydrazone producing brown colour in alkaline medium which was

monitored calorimetrically at 505 nm. The SGPT content was calculated using standard graph with pyruvate as standard.

2.5.9.4.b. SGOT:

Assay was estimated by monitoring oxaloacetate formed when α-ketoglutrate reacts with L-aspertate, mediated by SGOT. oxaloacetate is made to react with 2,4-Dinitrophenyl hydrazine to form hydrazone producing brown colour in alkaline medium which was monitored calorimetrically at 505 nm. The SGOT content was calculated using standard graph with oxaloacetate as standard.

2.5.9.4.c. Alkaline phosphatase (Taussky and Shorr, 1953)

Assay was estimated by measuring the inorganic phosphate liberated at pH 9.5 by using β-glycerophosphate as substrate. A total volume of 1 ml assay mixture contained triethanol amine buffer (0.04 mM, pH 9.5) 0.2 ml of plasma as enzyme source and 40 µl of β-glycerophosphate. Assay mixture was incubated at 37 ^{o}C for 15 min and reaction was terminated by adding 0.5 ml of 20 % TCA. The absorbance of inorganic phosphate liberated was read at 660 nm.

2.5.10. Total antioxidant activity by Phospho-molybdate method (Prieto et al., 1999)

Various aliquots of sclerotiorin (10 mM stock in DMSO) were mixed with 1ml of phospho-molybdate reagent (28 mM sodium phosphate and 4 mM ammonium molybdate in 0.6 M H_2SO_4). The tubes were capped and incubated at 95 ^{o}C for 90 minutes and the samples were cooled to room temperature and absorbance was recorded at 695 nm. The control contained 1 ml of reagent and equal volumes of DMSO. Same concentrations of butylated hydroxy toluene (BHT) a synthetic antioxidant was used as a standard. The activity was expressed in terms of molar absorption equivalent range

2.5.11. Free radical scavenging activity by 1,1-Diphenyl-2-picrylhydrazyl (DPPH) assay (Abe et al., 2000)

The assay mixture consisted of 2 ml ethanolic solution of sclerotiorin (1 mM stock in ethanol) and 2 ml acetate buffer (0.1 M, pH 5.5) followed by 1 ml of 0.5 mM

ethanolic DPPH solution. The mixture was vigorously shaken and incubated at 28 ^{o}C for 30 minutes. The absorbance was measured at 517 nm. The free radical quenching ability of the inhibitor was determined as a percentage decrease in the absorbance with respect to control. The control received appropriate quantity of the solvent without any inhibitor. The effective dose ED_{50} value was determined as the concentration of the inhibitor required to reduce 50% of the absorbance with respect to control.

2.5.12. Antioxidant ability in a liposome model system:

2.5.12.1. Preparation of egg liposome:

15 ml freshly beaten egg yolk was dispersed in 100 ml cold acetone to obtain about 2 gm precipitate, which was washed with three times in 100 ml cold acetone till the precipitate turned white. 300 mg of this precipitate was dispersed in 100 ml phosphate buffer (10 mM, pH 7.4) and sonicated for 15 min at 4 ^{o}C in an ultra sonicator (Bendelin sonoplus, Berlin, Germany). This liposome mixture stock (3 mg protein/ml) was stored at 4 ^{o}C.

2.5.12. 2. Lipid peroxidation assay by TBARS method (Buege and Aust., 1978)

Various aliquots of sclerotiorin (5 mM stock in DMSO) were added to 1 ml liposome mixture and 200 µl of 400 mM ferric chloride and 200 µl of 400 mM ascorbic acid. The final assay volume was made to 2 ml with phosphate buffer (20 mM, pH 7.4) and incubated for one hour at 37 ^{o}C. The reaction was stopped by adding 1 ml of 1% thiobarbituric acid (TBA) and 1 ml of 2% of trichloro acetic acid (TCA) and mixed thoroughly. The reaction mixture was boiled for 15 minutes and cooled to room temperature. The precipitate formed was removed by centrifuging the samples at 1000 g for 15 min. The absorbance of the supernatant was measured at 535 nm against blank sample that contained all the reagents except liposome mixture. The corresponding control samples contained the appropriate volumes of DMSO the IC_{50} value was determined as the concentration of the sclerotiorin required to reduce 50% of the absorbance with respect to DMSO control.

2.5.13. Metal binding assay (Carter, 1971)

To various aliquots of sclerotiorin (10 mM stock in DMSO), 20 µl of freshly prepared 2 mM $FeCl_2$ was added followed by addition of 400 µl ferrozine reagent (5 mM stock). The total volume was adjusted to 1ml with methanol. The samples were incubated at room temperature for 10 min and absorbance of a coloured Fe^{2+}- ferrozine complex formation was recorded at 562 nm. Equal aliquots of DMSO served as control, while EDTA was used as a positive control.

2.5.14. Evaluation of antioxidant property of sclerotiorin *in vitro*:

2.4.14.1. Oil emulsion model (Cillard et al., 1980)

An emulsion was prepared by adding 0.28 g linoleic acid/groundnut oil and 0.5 g of Tween-20 (v/v) in 100 ml of phosphate buffer (20 mM, pH 7.0,) and homogenized for 15 min. Different aliquots of sclerotiorin (stock 1mg/ ml in 0.5% Tween-20) was mixed with an equal volume fatty acid/oil emulsion to bring to a total volume to 5 ml. The blank tubes received 100 µl of dimethyl sulfoxide. The sample tubes were incubated in dark at 25 ^{o}C. Rate of oxidation was measured by monitoring the conjugated dienes formed at 234 nm by withdrawing 100 µl sample every 24 hours in to a 3 ml cuvette containing 2.9 ml borate buffer (20 mM, pH-9).

2.5.14.2. Oil alcohol suspension model (Yagi 1984)

Different concentrations of sclerotiorin (stock 1mg/ ml in 0.5% Tween-20) was added to the mixture containing 4 ml phosphate buffer (20 mM, pH 7), 2 ml distilled water, 2 ml ethanol and 2 ml of 2.5% solution of linoleic acid/groundnut oil in ethanol (w/v). The sample tubes were incubated in dark at 37^{o}C. Rate of oxidation was measured by monitoring the conjugated dienes formed at 234 nm by withdrawing 100 µl sample every 24 hours in to a 3 ml cuvette containing 2.9 ml borate buffer (20 mM, pH-9)

2.6. Analytical techniques:

2.6.1. Nuclear magnetic resonance spectroscopy (NMR)

NMR spectra were recorded at 500 MHz on a Brüker DRX-500 MHz spectrometer (500.13 MHz proton and 125 MHz carbon frequencies) at 27^0C. Proton and carbon 90° pulse width were 11.2 and 8.8 μs respectively. About 10 mg of the sample dissolved in $CdCl_3$ $-d_6$ was used for recording the spectra.Two-dimensional heteronuclear multiple quantum coherence transfer spectra (2D-HMQCT) were recorded in magnitude mode with the sinusoidal shaped z gradients of strength 25.7, 15.42 and 20.56 G/cm in the ratio of 5:3:4 were applied for duration of 1 ms each with a gradient recovery delay of 100 μs to defocus unwanted coherences. The t_1 was incremented in 256 steps. The size of the computer memory used to accumulate the 2D data was 4K. The spectra were processed using unshifted and π/4 shifted sine bell window function in F1 and F2 dimensions respectively.

2.6.2. GC-EIMS:

GC-EIMS data was generated using VG Auto Spec M mass spectrometer equipped with HP 5890 series II gas chromatograph under GC/EIMS conditions. Solid sample dissolved in $CHCl_3$ (HPLC grade) was used for recording GC/EIMS conditions BP-1 capillary column was used with the temperature program: 120 ^{o}C-10 ^{o}C / min -180 ^{o}C – 4 ^{o}C/ min - 280 $^\circ$C (15 min); injection at 250 ^{o}C; detector (FID) at 280 ^{o}C, 2 ml/min flow rate (N_2).

2.6.3. Infrared spectroscopy:

IR absorption spectra were obtained with a Perkin Elmer model 2000 Infrared Fourier-transform spectrophotometer using an attenuated total reflectance cell on a thin layer of the sample (1 mg/ml) dissolved in $CHCl_3$.

2.6.4. HPLC:

Sclerotiorin was dissolved in HPLC grade methanol (1 mg/ml), RP- C_{18} column (5 μm, 250 mm x 4.6 mm, Shimpack CLC-ODS) was used on a LC 10A system (Shimadzu, Japan). Acetonitrile concentration was increased after an initial delay of 2

minutes from 60 to 100% over a period of 30 min time program with a flow rate of 0.5ml/min at 362 nm.

2.6.5.Thin layer chromatography:

Qualitative TLC plates (20 x 80 cm) were prepared by making slurry of 2 gm of silica gel-G with 5 ml of water and spread over the plate manually on a 5 x 20 cm glass plate followed by air-drying. The plates were then activated in oven for 3 hours at 150°C. After activation, the TLC plates were spotted with crude extracts or purified compounds and run using a suitable mobile phase (Benzene: Diethyl ether, 9.5:0.5 v/v). For preparative TLC plates, the slurry was prepared by mixing 8 gm of silica gel-G with
20 ml of water.

2.6.6. Column chromatography:

Silica gel (60-120 or 100-200 mesh) was dried in an oven overnight at 150 °C. 20 gm of this was packed on to a glass column (65 x 1 cm) fitted with a G0 filter, in hexane with a flow rate of 1 ml/min. Elution of the crude extract was carried out using hexane, chloroform, ethyl acetate, methanol and various combinations of these solvents. Two bed volumes were taken as a fraction. Each fraction was analyzed by qualitative TLC and enzyme assay.

2.6.7. Melting point determination:

2 mg of compound was ground into fine powder and packed into a capillary tube sealed at one end. This tube was placed in a 230V PEW-thermal block and temperature was read manually using a mercury bulb thermometer.

2.6.8. Fluorescence spectroscopy:

Compound dissolved in acetonitrile (1mg/ml) concentration was subjected for survey scan on a Fluorescence spectroscopy. The wavelength was selected from 200–400 for excitation and far UV/visible range from 400-800 was selected for emissionspectra. Sclerotiorin shows an excitation maximum of 361 nm and Emission maxima of 600 nm.

CHAPTER–3

RESULTS AND DISCUSSION

SECTION 1:

SCREENING AND SELECTION OF FUNGAL CULTURE FOR LIPOXYGENASE INHIBITOR PRODUCTION

3.1. Introduction

The key to success in obtaining active substances from microorganisms depends on effective screening systems, sometimes needing as much as an year or two to devise a satisfactory one (Jong and Donovick, 1989).

Biological screening most essentially requires the following: (i) the use of appropriate biological material such as bacteria, animal cells, enzymes and others for the detection of desired activity; (ii) the ability to assay a small quantity of substance easily and quickly; and (iii) the ability to devise various conditions for the successful isolation and cultivation of microorganisms or their metabolites (Berdy, 1985). If any of the above is inappropriate, it is difficult to obtain a desired substance.

One of the greatest barriers to the discovery of an active metabolite with any kind of *in vitro* or especially *in vivo* screen is sensitivity. The extreme dilution of the active material in broth or crude extracts results in activity being observed only when the compound is extremely potent. The ideal screen should be rapid, sensitive, inexpensive and suitable for high throughput, and would select only those compounds, which have potent activity. There is no single screening programme, however, capable of detecting all compounds desired (Berdy, 1985). In reality every screen will miss some useful activities (false negatives) and some of the selected compounds will turn out to be inactive (false positives). Moreover, majority of the compounds do not have the desired activity (true negatives) (Pearce, 1997). This is what makes discovery of new compounds an exciting area to work.

3.1.1. Selective isolation of fungi from forest soil samples:

About 15 terrestrial soil samples were collected in sterile plastic bags from various places of Agumbe forest, Western Ghats, Karnataka, India. Random selection of places was made for the soil sample collection. Sufficient care was taken while selecting places for soil collection such that the point of collection had minimum human interaction or presence.

There is probably no such thing as the best culture medium that will allow any fungus to express metabolite capability and it is impossible to predict what a freshly isolated fungus will require for metabolite accumulation. Selection of media is complex since the possible variations are so large. Simple media such as potato dextrose work very well as broth and agar, and this has been validated many times in our laboratory, with novel bioactive compounds being produced (Umezawa, 1982; Rao et al., 2002a,b).

Special screening medium was used for the selective isolation of fungi. The potato dextrose agar (PDA) medium, a well-known medium for fungi was acidified to around pH 4.0 to 5.0 by supplementing with (30 mg/L) rose bengal and (300 mg/L) chloramphenicol.

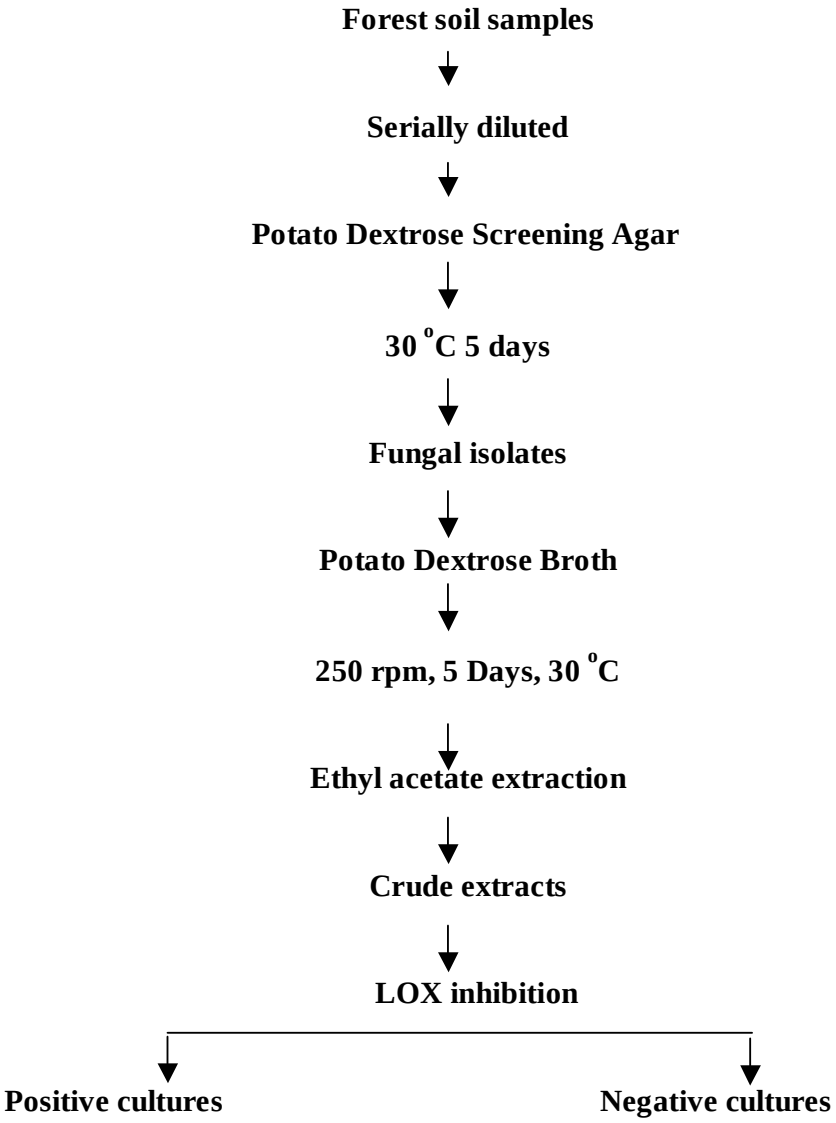
The incorporation of rose bengal in the screening medium not only suppresses the development of bacteria but also the spreading of molds such as *Rhizopus* and *Mucor*. Actinomycetes grow more slowly than most bacteria and fungi (8-10 days), hence they are likely to be masked in culture plates. However actinomycetes as a group are capable of growing in media containing low nitrogen, this problem was also solved by rose bengal incorporation. Actinomycetes appeared uniformly stained in the rose bengal incorporated plates with intense pink and few were pink-orange. This feature clearly distinguished actinomycetes from fungal colonies (Martin, 1950;King et al., 1979; Otitow and Glathe, 1968)

3.1.2. Primary screening of soil isolates for LOX inhibitor production:

Totally seventy-four (74) fungal soil isolates were isolated (Figure 11). All the cultures were fermented on Potato Dextrose Broth for 5 days. During the course of fermentation many parameters were visibly observed like, colour changes in the medium, pigment production, pellet morphology, size and pH changes at the end of fermentation.

At the end of 5 days of fermentation, each culture was extracted with ethyl acetate (1:1, v/v). Ethyl acetate was chosen as extraction solvent because of its immiscible nature with the aqueous phase and its partitioning ability of both hydrophilic and hydrophobic

compounds. The ethyl acetate extracts were then dissolved in DMSO (stock 2 mg/ 0.1 mL) and 0.2 mg of each sample was tested for inhibitory activity against LOX (Flowchart 1).



Flowchart 1. Screening of fungal forest soil isolates for LOX inhibition

Figure 11. Forest soil fungal isolates on PDA slants

The basic screen used in the present study was an *in vitro* cell free enzyme inhibition system for soybean 15–LOX assay by Axelrod (1981). Soybean lipoxygenase is a well-studied and well-characterized enzyme, whose crystal structure is known (Boyington et al., 1993; Minor et al., 1996). It is relatively stable enzyme of all the lipoxygenases (Shibata and Axelrod, 1995). The reagents and other chemicals used in this assay are relatively cheap and the method easy and reproducible to perform by spectrophotometry.

However the trickiest part of this assay is the substrate, linoleic acid. It is a highly unstable compound and undergoes nonspecific lipid peroxidation in presence of light and atmospheric oxygen. However, this can be controlled by using oxygen free water to prepare substrate stock solution and by storing at -80^0C. Further, linoleic acid has poor solubility in aqueous phase and tends to form aggregates. This can be countered by using Tween-20 as the surfactant and by converting it to its sodium salt (Sodium linoleate) by adding alkali. Keeping all these advantages and drawbacks in mind, Axelrod's method was adopted for the screening of inhibitory extracts. The results of this screening of extracts are given in Table 7.

Table 7: Primary screening of fungal isolates for LOX inhibition

Isolate no	% Inhibition
A-1	13.0
A-2	9.2
A-3	4.2
A-4	1.0
A-5	4.8
A-6	5.4
A-7	26.0
A-8	0.3
A-9	Nil
A-10	11.5
A-11	18.8
A-12	1.5
A-13	Nil
A-14	10.6
A-15	22.6
A-16	**56.0**
A-17	4.0
A-18	11.8
A-19	10.0
A-20	19.3
A-21	19.9
A-22	19.3
A-23	16.2
A-24	**70.0**
A-25	21.3

Isolate no	% Inhibition
A-26	19.2
A-27	5.0
A-28	**61.1**
A-29	12.3
A-30	22.0
A-31	13.5
A-32	21.4
A-33	22.8
A-34	28.4
A-35	31.2
A-36	26.2
A-37	16.1
A-38	**50.7**
A-39	26.8
A-40	**49.5**
A-41	20.3
A-42	26.1
A-43	26.9
A-44	15.7
A-45	13.8
A-46	25.6
A-47	22.0
A-48	16.9
A-49	11.7
A-50	4.0

Isolate no	% Inhibition
A-51	12.0
A-52	10.0
A-53	16.1
A-54	Nil
A-55	4.6
A-56	1.9
A-57	17.5
A-58	10.8
A-59	19.1
A-60	8.1
A-61	7.9
A-62	**55.3**
A-63	11.7
A-64	20.5
A-65	5.6
A-66	15.7
A-67	6.8
A-68	7.3
A-69	8.1
A-70	16.5
A-71	1.5
A-72	18.7
A-73	12.5
A-74	2.5

From the primary screening out of 74 isolates for LOX inhibition, cultures showing inhibition below 20% at 66 µg/ml were considered not potent and thus grouped as, inactive cultures. The results indicated that total 22 active cultures were showing above 20% inhibition. Out of these, 6 cultures, which were showing above 50% inhibition, were selected and subjected to secondary screening (Table 8).

Table 8: Primary screening of fungal cultures against LOX

Inhibition %	**< 20**	**Above 21-30**	**Above 31-49**	**50-60**	**> 61%**	**Total**
Active isolates	-	15	1	4	2	22
Inactive isolates	52					52
Total						74

As ethyl acetate alone does not extract all the metabolites from the fermented broth, the broth filtrates after the solvent extractions were also tested for LOX inhibition. As none of those culture filtrates showed inhibition, further studies were concentrated only on inhibition shown by the ethyl acetate extracts

3.1.3. Secondary screening of soil isolates for LOX inhibitor production:

From the primary screening experiment, 6 cultures showed inhibition towards lipoxygenase greater than 50%. These were subjected for secondary screening to check their consistency in LOX inhibitor production.

Each culture was grown as four replicates in SmF and SSF and followed the same protocol as described for primary screening. The inhibition shown by the four replicates of each culture are represented in Table 9. Only culture No A-24 showed reproducibility in inhibitor production in SmF and not in SSF. The remaining cultures did not show consistency in inhibition in SmF as well as SSF. Hence isolate A-24 was taken up for further studies.

Table 9: Secondary screening of ethylacetate extracts of fungal isolates for LOX inhibition

Name of the culture	% Inhibition concentration 66 μg/ml							
	Run-I		Run-II		Run-III		Run-IV	
	SmF	SSF	SmF	SSF	SmF	SSF	SmF	SSF
A-16	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
A-24	**69.0**	Nil	**70.0**	Nil	**71.0**	Nil	**69.0**	Nil
A-28	9.5	Nil	9.8	Nil	Nil	Nil	5.0	Nil
A-38	27.8	Nil	15.0	Nil	10.0	Nil	14.0	Nil
A-40	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
A-62	10.0	5.0	5.8	12.0	Nil	5.9	6.2	3.8

3.1.4. Stability study of isolate A-24 for the production of LOX inhibitor:

A-24 culture was subcultured for 5-6 generations. From each generation the culture was subjected for submerged fermentation (100 ml x 10 flasks) on potato dextrose broth. The ability of A-24 culture to produce LOX inhibitor consistently was observed from all the generation (Table 10).

Table 10: Stability of isolate A-24 in LOX inhibitor production.

Generation	% Inhibition
I	72.6
II	70.4
III	70.8
IV	69.8
V	71.2

The results obtained in this screening clearly indicated the consistency of A-24 culture to produce LOX inhibitor and gave sufficient confidence to go ahead with the characterization of the culture as well as the active compound involved in LOX inhibition.

3.1.5. Culture characterizations of isolate A–24:

Culture identification was done on the basis of morphological characteristics following incubation on

a) Czapek-Dox solution agar

b) Czapek-Dox solution agar with corn steep liquor

c) Malt extract agar.

The growth was followed for a period of 2 weeks on all the media (Ainsworth and Bisby, 1945). On Czapek-Dox solution agar the colonies formed were smaller (about 1 cm) and the surface was not evenly green. The culture appeared broadly zonate and radiantly wrinkled. When compared to malt extract agar plates, central area was sulcate and there was thinning appearance at margins. The exudate was present but was limited and was amber colored. The culture had a moldy odor and on reverse side of plate, showed shades of yellowish brown pigmentation. Colonies formed on corn steep agar plates were larger (about 3 cm) than that of Czapek- Dox agar solution and was more prominently radially wrinkled. Colour on reverse side of colonies was deeper yellowish brown when compared to malt agar and Czapek-Dox solution agar plates.

On the malt extract agar the colonies formed were large (about 2 cm) almost plane and velvety. Trace of zonation was seen at the margins and the reverse side of colony showed shades of yellowish brown coloration and the central area was sulcate. Conidial column broke away easily when the culture dish was tapped.

Microscopic observations showed that conidiophores formed were erect, unbranched, septate, arising from aerial hyphae and 100-200 μ by 3-3.5 μ in size. Conidia were produced in well-defined column up to 200 μ or more in length and conidia were globose to subglobose in shape and were 3-3.5 μ in size (Figure 12 and 13).

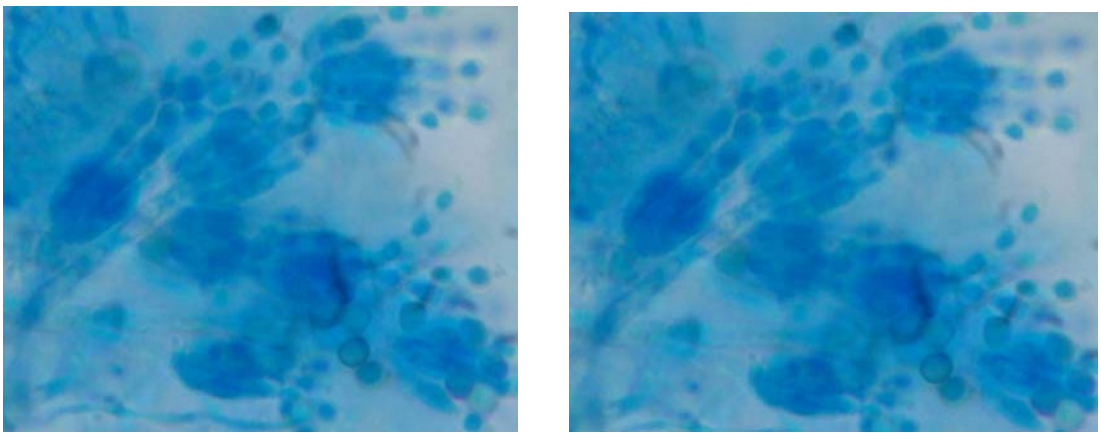


Figure 12. Microscopic observation of *Penicillium frequentans*, wet mount with cotton blue stain (40 x)

Penicillium sps are recognized by their dense brush-like spore-bearing structures called as conidiophores. Which can be simple or branched and are terminated by clusters of flask-shaped phialides. The spores (conidia) are produced in dry chains from the tips of the phialides, with the youngest spore at the base of the chain, and are nearly always green, which are an important feature for identifying *Penicillium* species (Raper and Thom, 1949; Samson and Pitt, 1986)**.** Based on the growth patterns and results, isolate A-24 was designated as *Penicillium frequentans* (Figure 14). The classification of the isolate as follows;

Kingdom: Fungi
Phylum: Ascomycota
Class: Eurotiomycetes
Subclass: Eurotiomycetidae
Order: Eurotiales
Family: Trichocomaceae
Genus: *Penicillium*
Species: *P. frequentans*

This culture is currently deposited at CFTRI culture collection center as CFTRI A-24.

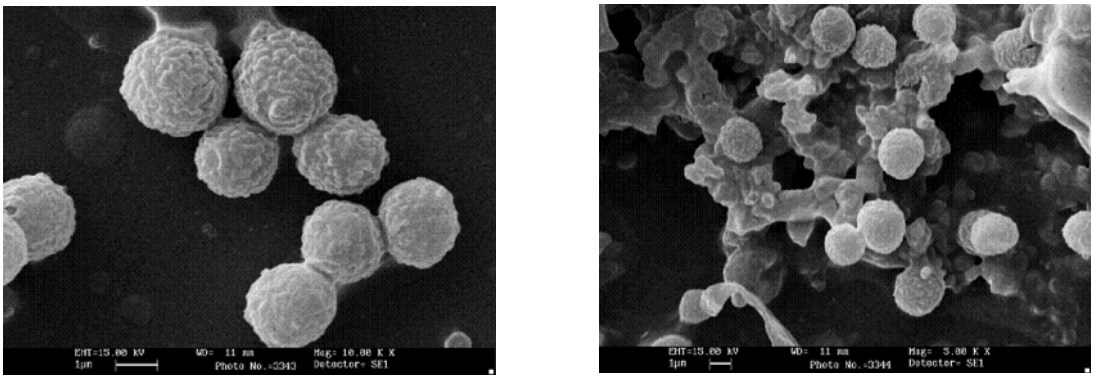


Figure 13. Scanning electron micrograph showing *Penicillium frequentans* spores

Figure 14. *Penicillium frequentans* isolate (A-24) on a 5-day old PDA slant

SECTION–2

PURIFICATION AND STRUCTURAL ELUCIDATION OF LOX INHIBITOR

3.2. Production of LOX inhibitor from *Penicillium frequentans* A-24 culture:

Fermentation was carried out in 3 liters of PDB, distributed into 30 x 100 ml medium in 500 ml Erlenmeyer flasks. After 5 days fermentation, the whole fermented broth was extracted into 100 ml (v/v) ethyl acetate in conical flask. The biomass debris obtained after filtration through nylon cloth was then further extracted with 500 ml ethyl acetate. All the extracts were pooled and dried with 100 gm of anhydrous sodium sulphate. The residue that remained after complete distillation of the solvent was 2 gm and showed 70 % inhibition against LOX at a concentration of 0.066 mg/ml of assay system.

3.2.1. Isolation and purification of the inhibitor:

1 gm crude extract was loaded on a silica gel column (60-120 mesh) of 15 ml bead volume and eluted with 100 ml hexane with a flow rate of 1 ml/min to remove the impurities. The elution solvent was then changed to hexane: ethylacetate (9:1, v/v), the fractions were collected on the basis of colour. 2 fractions, yellow and orange were collected. This was followed by hexane: ethyl acetate (1:1, v/v), hexane: ethyl acetate (1:9, v/v) and ethylacetate as shown in Table 11. All column fractions were subjected to distillation under reduced pressure. The residual compound for each fraction that remained after complete removal of solvent was dissolved in DMSO and tested for inhibitory activity. The inhibitory activities of all the fractions are as shown in Table 11.

The orange fraction (1b) (150 mg) of Hexane: Ethylacetate (9:1, v/v) appeared as yellowish crystalline powder upon distillation. This showed highest inhibition amongst the fractions (89% inhibition at 16 μg/ml assay). Therefore, this active fraction was taken for further purification by three methods of column chromatography, crystallization and preparative TLC.

3.2.2. Purification by successive column chromatography:

150 mg of active fraction (1b) was loaded on a silica gel column (60-120 mesh) of 15 ml bed volume and eluted with hexane (100 ml) to remove the impurities with a flow rate of 1 ml/min. The elution solvent was then changed to hexane: ethylacetate (9:1,

v/v). Out of the 2 fractions yellow colour inactive fraction was discarded and orange colour active fraction was collected. This process was repeated till a TLC pure compound (50 mg) was obtained.

3.2.3. Purification by crystallization:

To the active fraction (250 mg), 25 ml of warm methanol was added refluxed for 15 min and filtered on Whatman No. 1. The filtrate was left in dark at 27 ^{o}C for 2 days and was filtered on Whatman No-1 and dried at 27 ^{o}C for 24 hours to yield 50 mg of yellow crystalline compound. Structure determination was accomplished using this compound.

3.2.4. Purification by preparative TLC:

Active fraction (100 mg) was dissolved in 6 ml chloroform, spotted on to silica gel-G preparative TLC plates (5 x 20 cm) and developed on benzene: diethyl ether (95:0.5, v/v). The compound absorbed under UV at 361nm and had an Rf value 0.62. The compound was scraped and extracted in ethylacetate and filtered on Whatman No. 1. The filtrate was concentrated under vacuum to yield a TLC pure compound of 36 mg.

Table 11: Purification of LOX inhibitor by column chromatography

Mobile phase	Fraction number	Colour of the fraction	Fraction weight (mg)	% Inhibition
Hexane (100%) 100 ml	-	-	-	-
Hexane: ethyl acetate (9:1) 200 ml	1a 1b	Yellow Orange	28.5 150.8	46 89
Hexane: ethyl acetate (1:1) 100 ml	2	Reddish	36.2	33
Hexane: ethyl acetate (1:9) 100 ml	3	Light yellow	23.3	19
Ethyl acetate (100%) 100 ml	4	Dark brown	56.3	1.8
Methanol (100%) 100 ml	5	Dark	15.12	-

3.2.5. Modified method of Purification:

4.2 g of the crude was dissolved with MeOH (1:60, w/v). This mixture was gently warmed to dissolve the compound completely and cooled to room temperature. To this distilled water (2:1, v/v) was added with gentle stirring. This mixture was incubated on a rotary shaker at 100 rpm at room temperature for 3 hours. After 3 hours the turbid liquid was filtered on a Whatman filter paper No-1. Upon drying the filter paper at 50 ^{o}C, 1.8 g of inhibitor was obtained which was 96% GC pure. As this method is simple and easy to perform, further purification on large scale was carried out by this method.

3.2.6. Structural elucidation:

3.2.6.a. Nuclear Magnetic Resonance Spectroscopy (NMR):

^{1}H NMR spectra was recorded at 500 MHz (500.13 MHz proton and 125 MHz ^{13}C). 30 mg of solid sample dissolved in $CDCl_3$ was used for recording the NMR spectra at 27 ^{o}C. These assignments were recorded. ^{1}H NMR (500 MHz, $CDCl_3$): 7.94 (1H, s, H-1), 6.60 (1H, s, H-4), 6.08 (1H, d, *J* = 15.7 Hz, H-9), 7.06 (1H, d, *J* = 15.6 Hz, H-10), 5.71(1H, d, *J* = 9.7 Hz, H-12), 2.49 (1H, m, H-13), 1.34 (1H, m, H_a-14), 1.44 (1H,m, H_b –14), 0.87 (3H, t, *J* = 7.3 Hz, H-15), 1.01 (3H, d, *J* = 6.6 Hz, H-16), 1.85 (3H, s, H-17), 1.57 (3H, s, H-18), 2.17 (3H, s, H-20) and ^{13}C NMR (125 MHz, $CDCl_3$): 153.3 (C1), 158.8 (C2), 107.0 (C4), 139.3 (C5), 115.2 (C5), 192.4 (C6), 85.2 (C7), 186.6 (C8), 111.4 (C8a), 116.3 (C9), 143.5 (C10), 132.6 (C11), 149.5 (C12), 35.8 (C13), 30.7 (C14), 12.6 (C15), 20.8 (C16), 13.0 (C17), 23.2 (C18), 170.7 ($\underline{C}OCH_3$), 20.7 ($CO\underline{CH_3}$) detailed chemical shifts are given in Table 12 (Figure15-23).

3.2.6.b. GC-EIMS:

Solid sample dissolved in $CHCl_3$ was used for recording GC/EIMS. The compound had a RT value of 33 min on BP-1 column under the following temperature program (Figure 24 and 25).

Column temp:	120^0 C- 280^0 C
Injection temp:	250^0 C
Detection temp:	250^0 C
Nitrogen flow rate:	2 ml/min

EI-MS m/z; MS data showed: 390 [M^+], 348 [M^+ - $COCH_2$], 306 [M^+ - 2 X $COCH_2$], 291[306- CH_3] (Figure 26 and 27) .

Table 12: ^{1}H and ^{13}C NMR chemical shifts of compound

H*	Chemical shifts	MULTIPLICITY	J (Hz)	C** [ppm]
1	7.94	s		153.3
2				
3				158.8
4	6.60	s		107.0
4a				139.3
5				115.2
6				192.4
7				85.2
8				186.6
8a				111.4
9	6.08	d	15.66	116.3
10	7.06	d	15.66	143.5
11				132.6
12	5.71	d	9.74	149.5
13	2.49	m		35.8
14	1.34, 1.44	m,m		30.7
15	0.87	t	7.30	12.6
16	1.01	d	6.60	20.8
17	1.85	s		13.0
18	1.57	s		23.2
$\underline{C}OCH_3$				170.7
$CO\underline{CH}_3$	2.17	s		20.7

* : 500 MHz, ** : 125 M

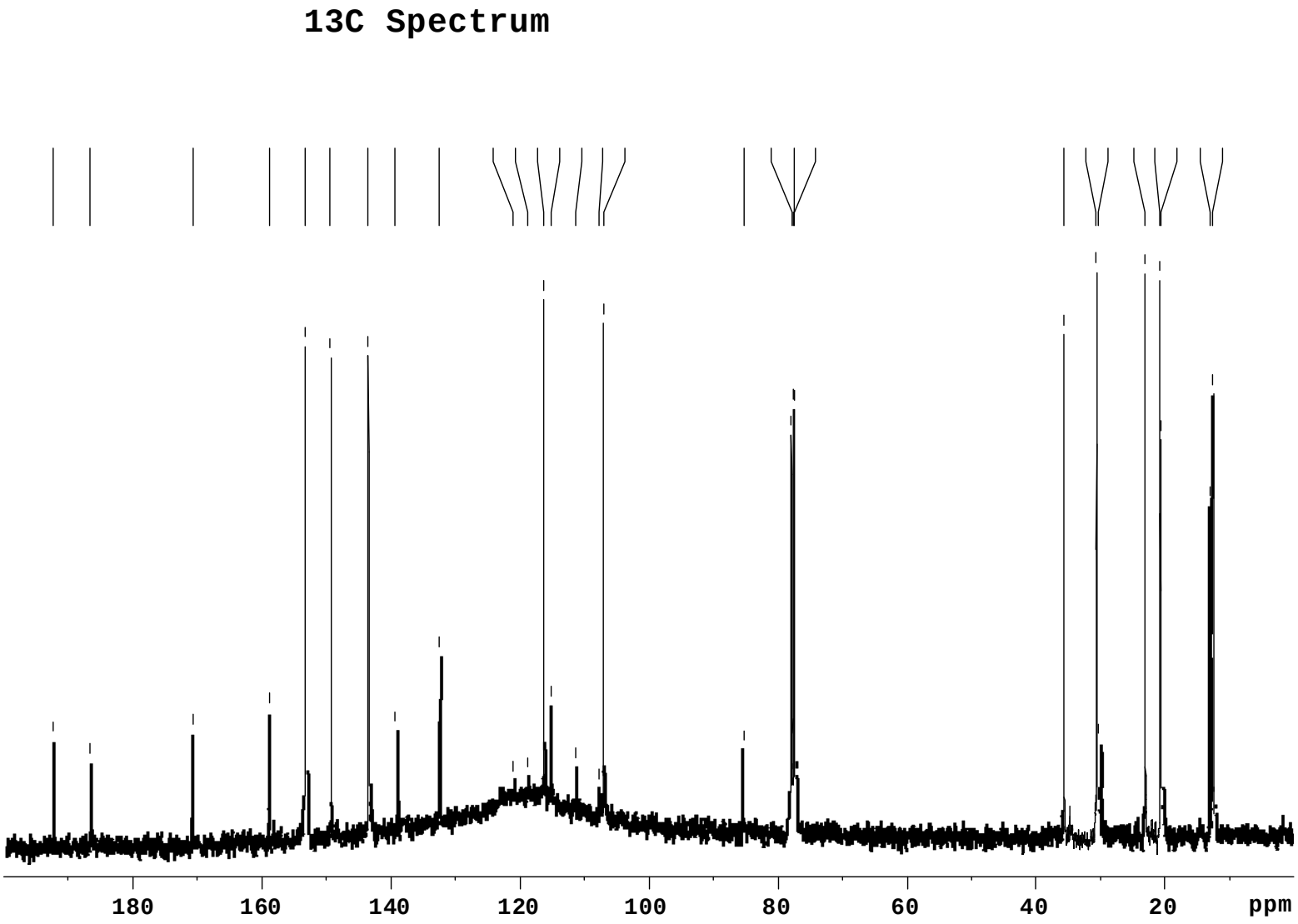


Figure 15. ^{13}C NMR spectrum of LOX inhibitor from *Penicillum frequentans* A-24 recorded in $CdCl_3$-d_6

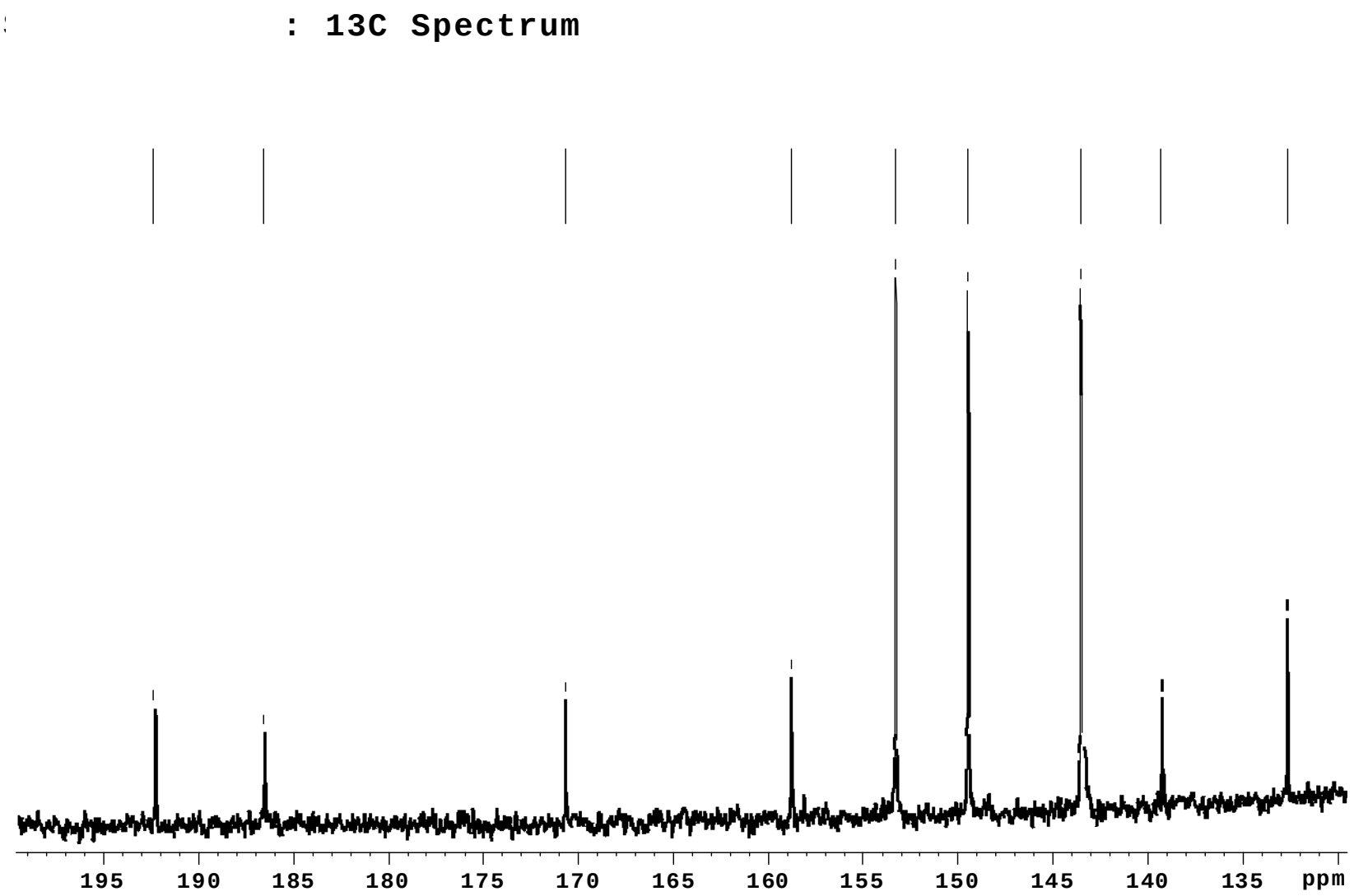


Figure 16. ^{13}C NMR spectrum of LOX inhibitor from *Penicillum frequentans* A-24 recorded in $CdCl_3$- d_6 (expansion of Figure 15)

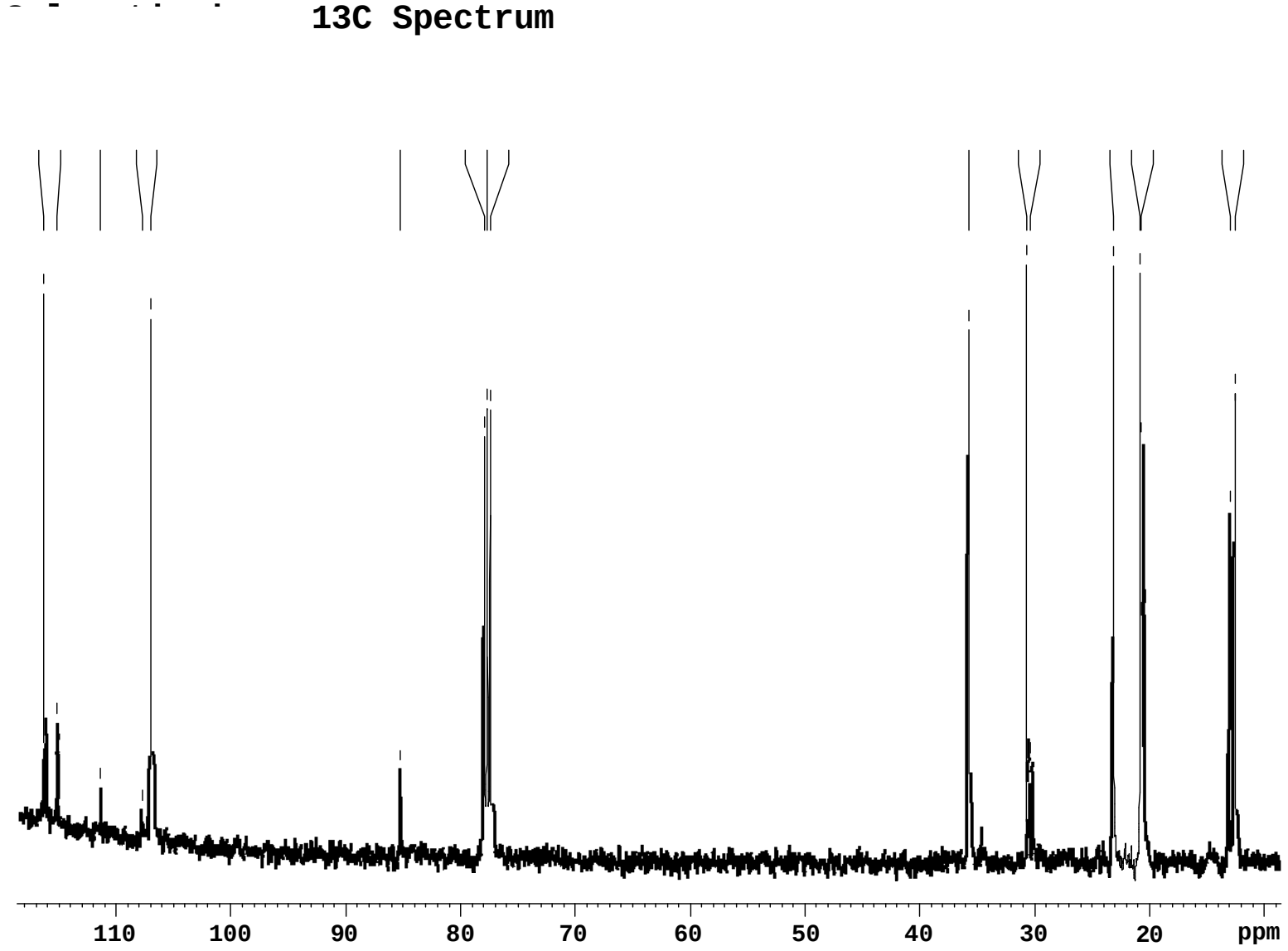


Figure 17. ^{13}C NMR spectrum of inhibitor from *Penicillum frequentans* A-24 recorded in $CdCl_3$- d_6 (expansion of Figure 15)

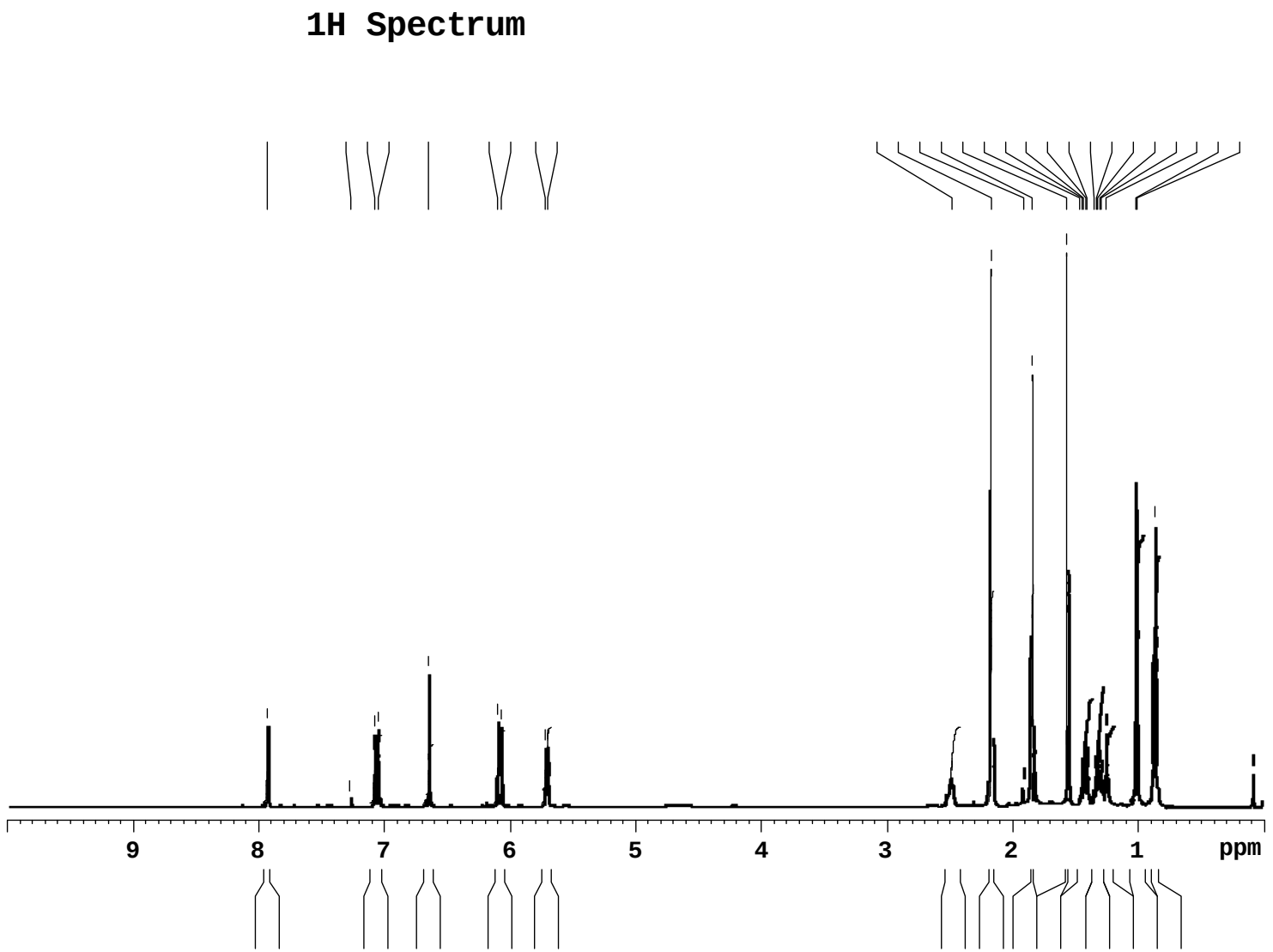


Figure 18. ^{1}H NMR spectrum of LOX inhibitor from *Penicillum frequentans* A-24 recorded in $CdCl_3$- d_6

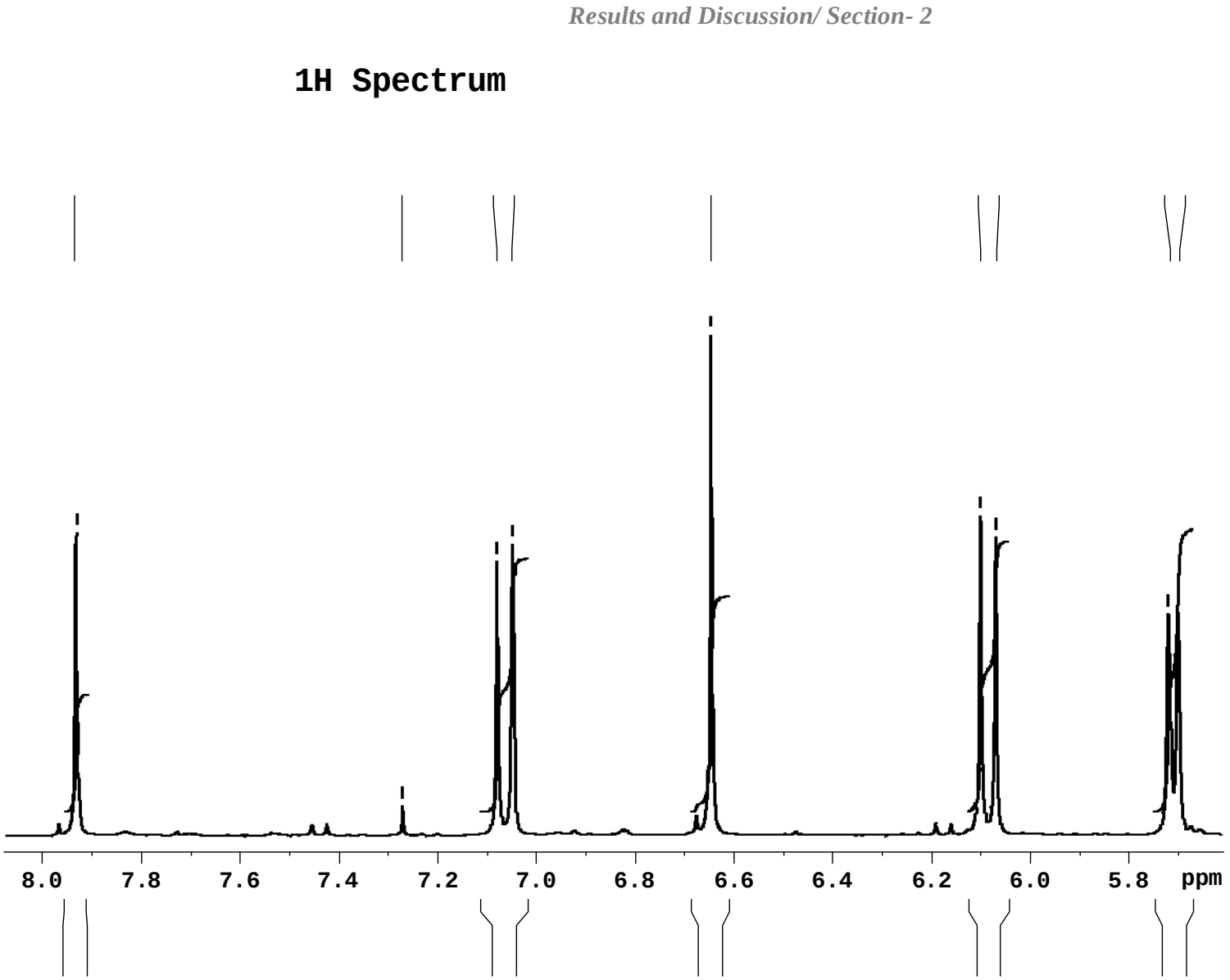


Figure 19. ^{1}H NMR spectrum of LOX inhibitor from *Penicillum frequentans* A-24 recorded in $CdCl_3$- d_6 (expansion of Figure 18)

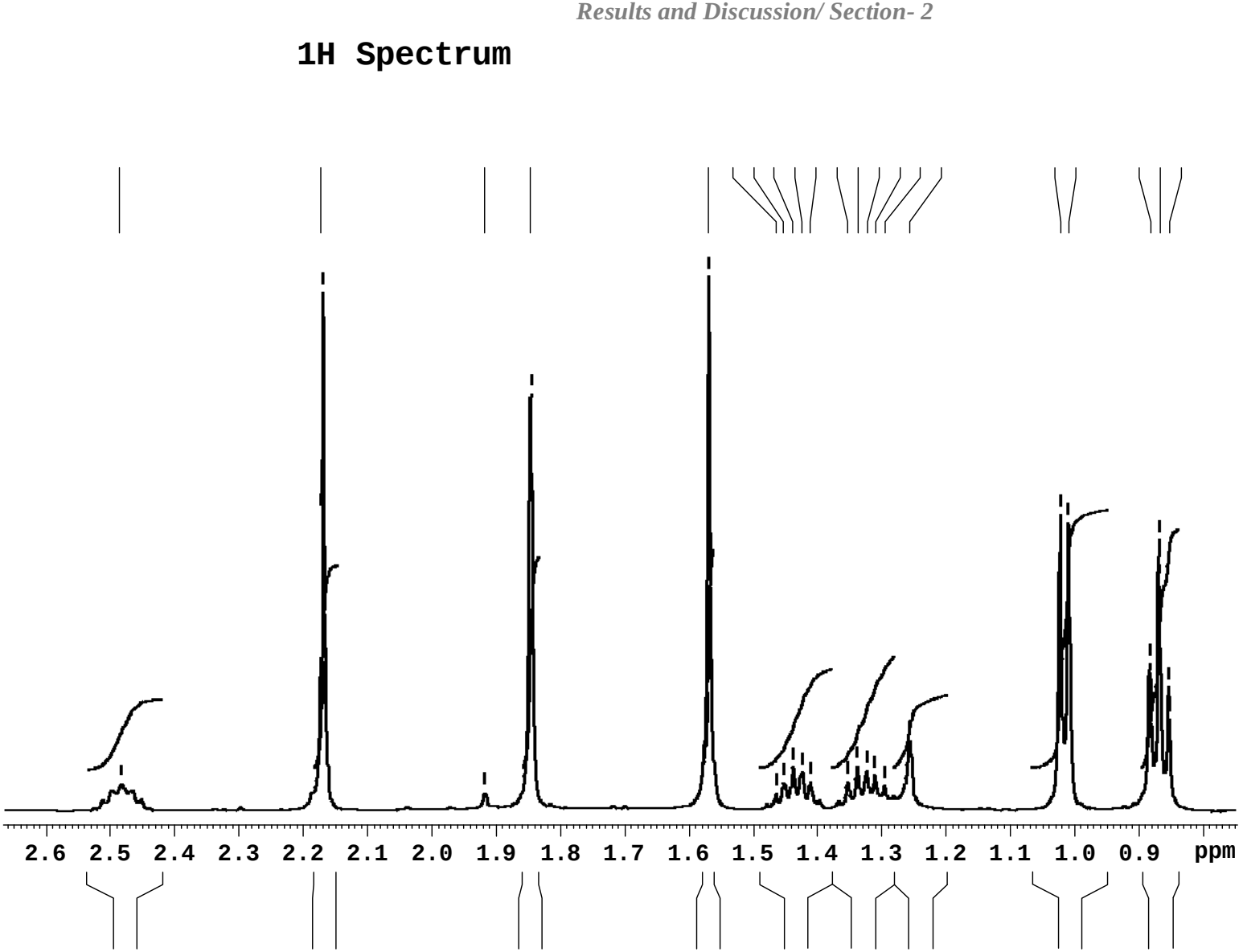


Figure 20. ^{1}H NMR spectrum of LOX inhibitor from *Penicillum frequentans* A-24 recorded in $CdCl_3$- d_6 (expansion of Figure 18)

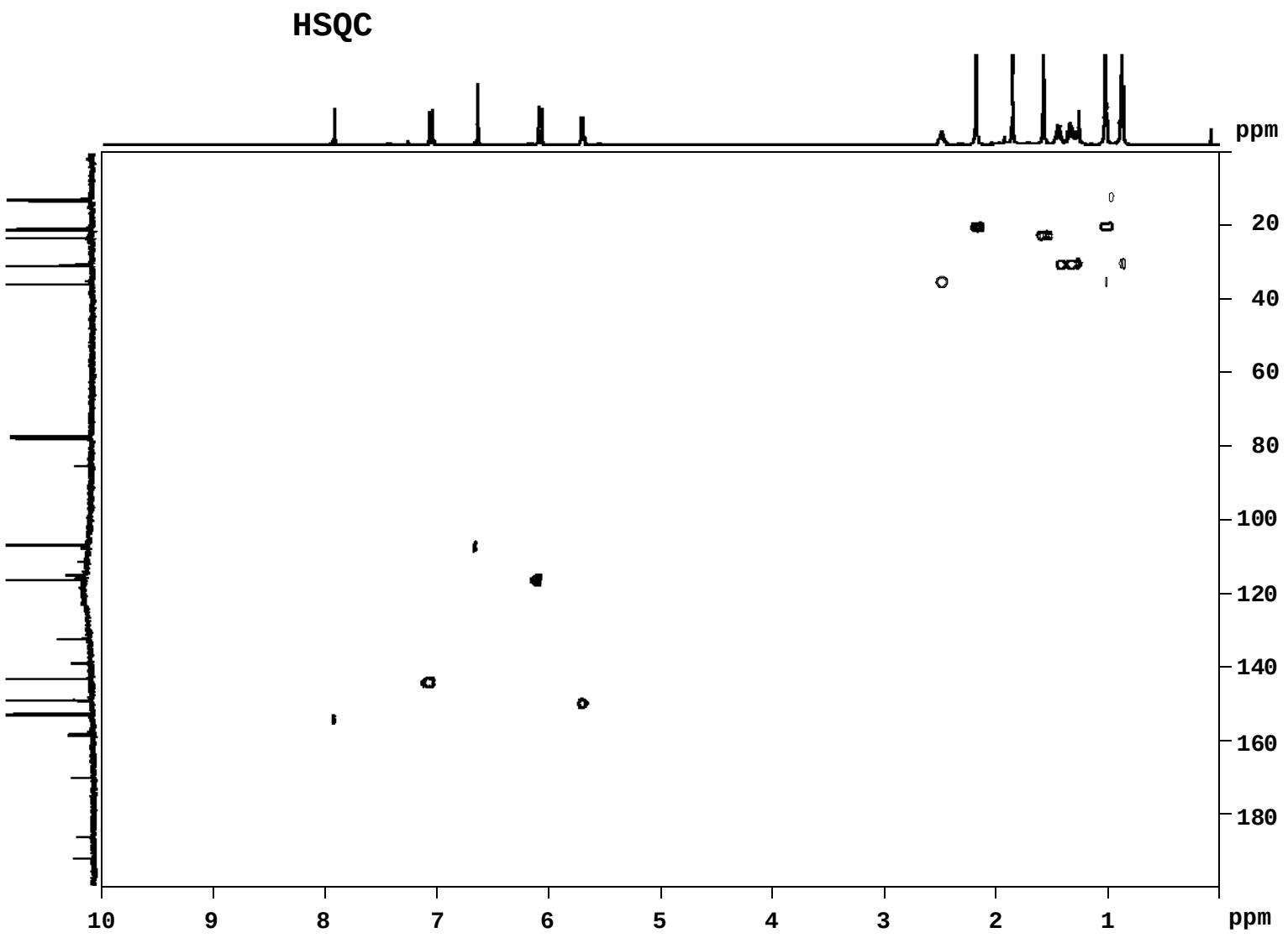


Figure 21. HSQC spectrum of LOX inhibitor from *Penicillum frequentans* A-24 recorded in $CdCl_3$- d_6

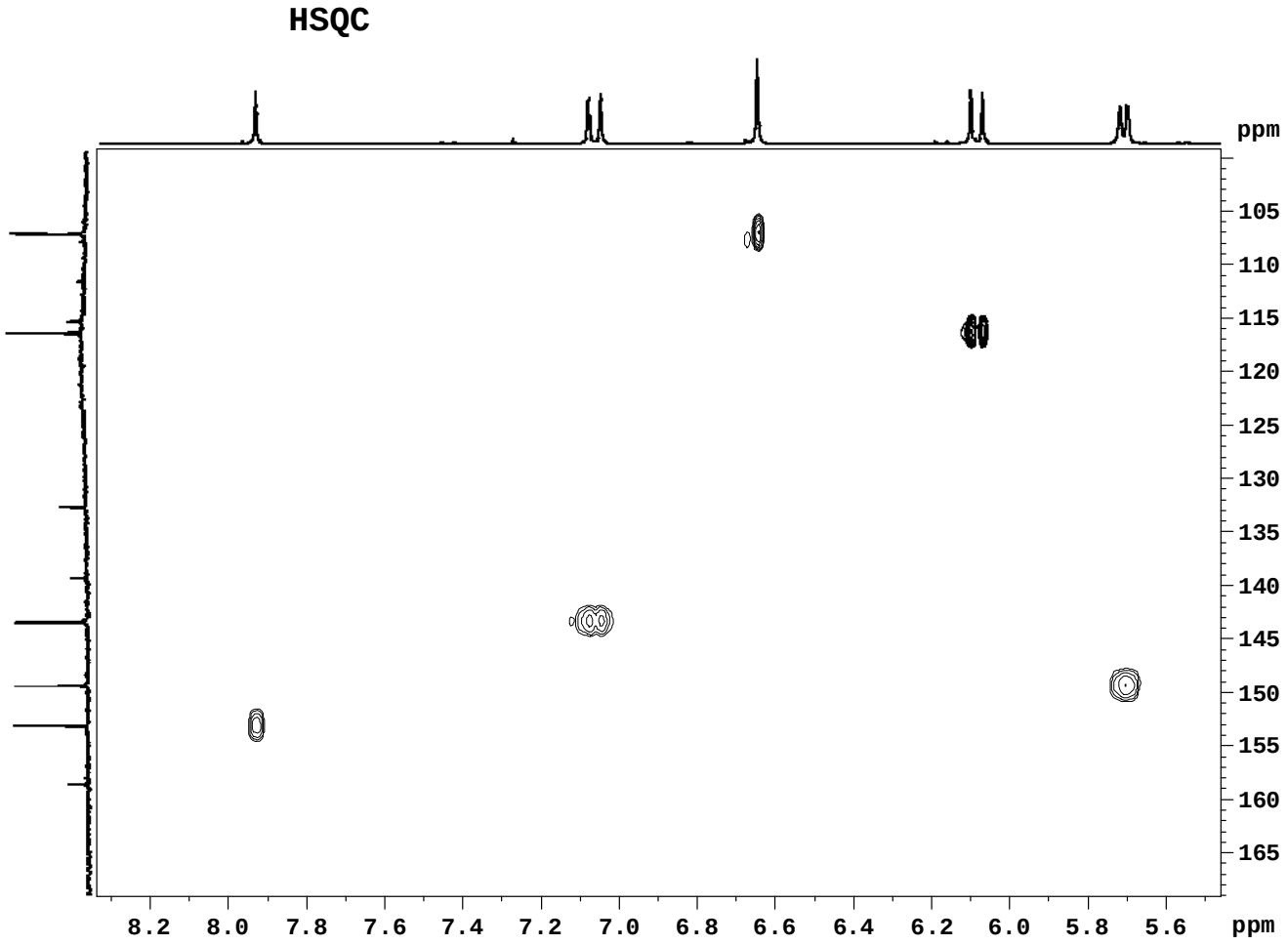


Figure 22. HSQC spectrum of LOX inhibitor from *Penicillum frequentans* A-24 recorded in $CdCl_3$- d_6 (expansion of Figure 21)

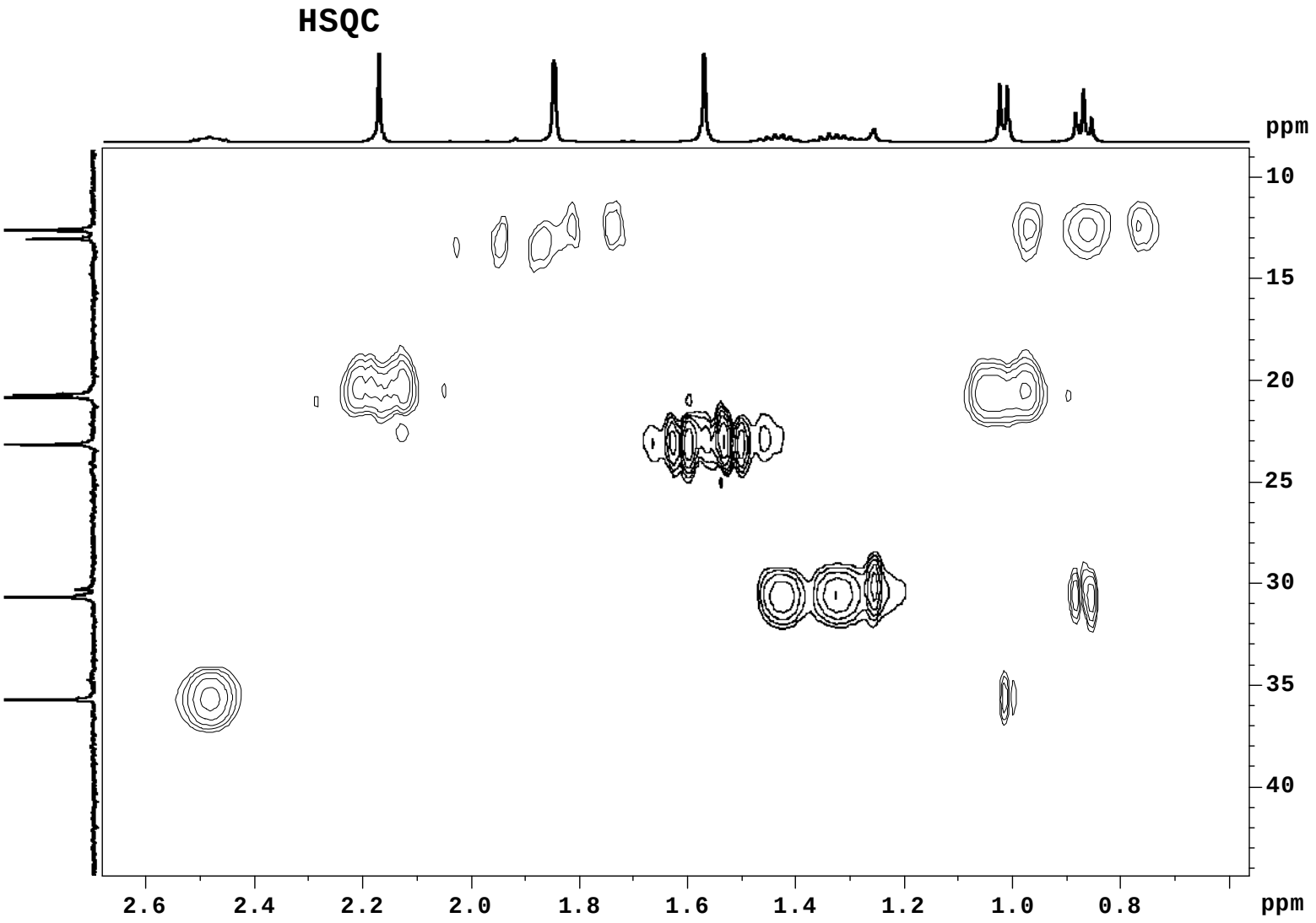


Figure 23. HSQC spectrum of LOX inhibitor from *Penicillum frequentans* A-24 recorded in $CdCl_3$- d_6 (Expansion of Figure 21)

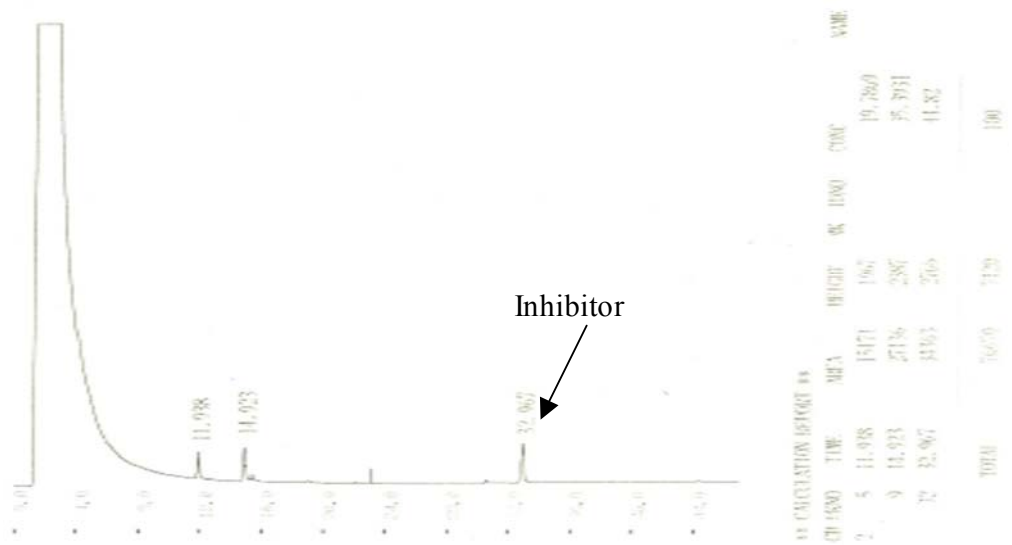


Figure 24. GC chromatogram of crude ethyl acetate extract of LOX inhibitor isolated from the fermented broth of *Penicillium frequentans* A-24

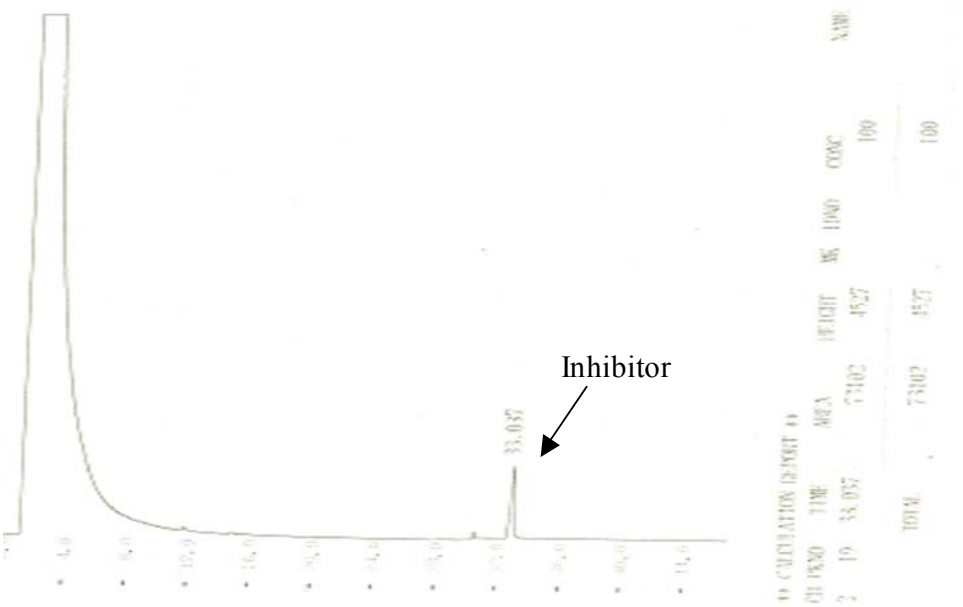


Figure 25. GC chromatogram of purified LOX inhibitor isolated from the fermented broth of *Penicillium frequentans* A-24

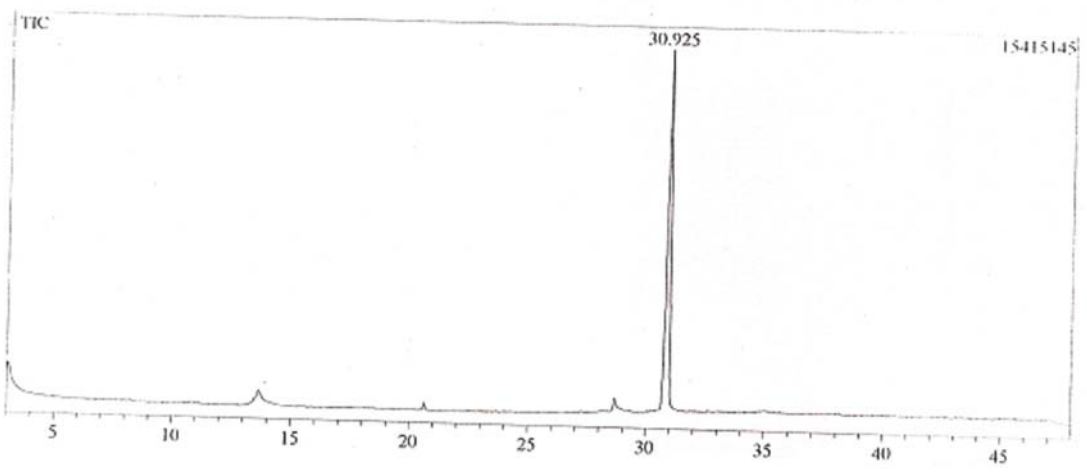


Figure: 26. GC-MS spectrum of LOX inhibitor from *Penicillium frequentans* A-24

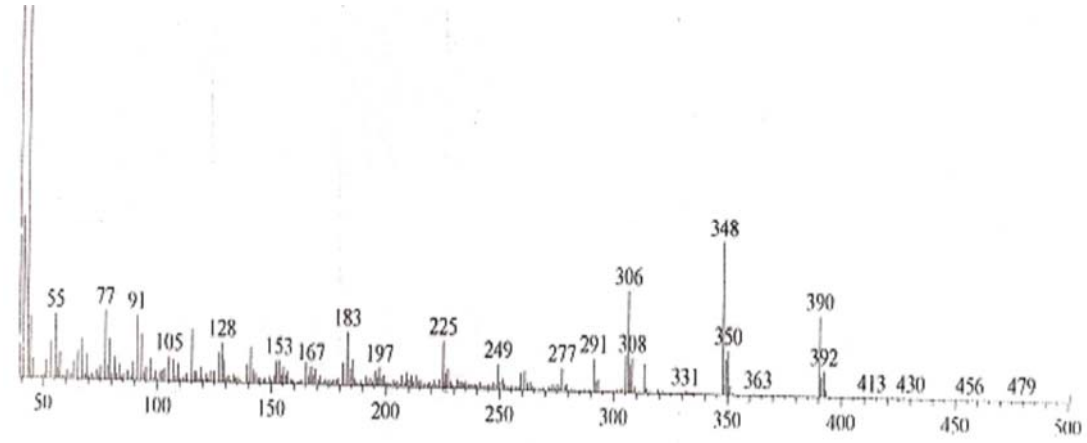


Figure 27. GC-MS fragmentation pattern of the major peak of LOX inhibitor from *Penicillium frequentans* A-24

From NMR spectra, GC-MS, spectral data and physico chemical properties the structure of the compound was determined as 6-H-2-Benzopyran-6,8(7H)-dione, 7-(acetyloxy)-5-chloro-3-[(1E,3E,5S)1-3,5-dimethyl-1,3 dimethyl-1,3-heptadienyl]-7- methyl, (7R)- also known as "Sclerotiorin". The molecular formula of the sclerotiorin was elucidated as $C_{21}H_{23}O_5Cl$. Molecular Weight: 390, (Figure 28).

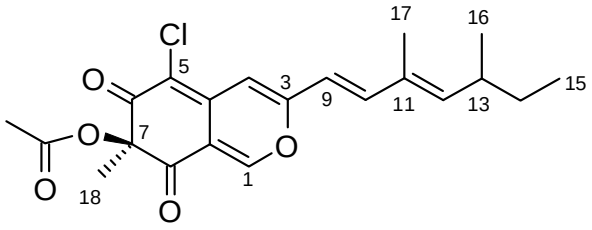


Figure 28. Structure of sclerotiorin

The assignments were confirmed by comparing with the structure as earlier reported (Holker et al., 1962;Whalley et al., 1976; Pairet et al., 1995).

3.2.7. Physico chemical properties of sclerotiorin:

Sclerotiorin is a yellow crystalline compound. It is highly soluble in Chloroform, ethyl acetate, DMSO, acetone, ethanol, methanol and insoluble in hexane, petroleum ether and in water. Sclerotiorin decomposes sharply at 205 ^{o}C, λ_{max} nm in methanol shows the (ε) values: 287 (9672) and 362 (23,732) (Figure 29). Sclerotiorin in methanol shows excitation maxima at 361nm and emission maxima at 559 nm (Figure 30). The IR spectra in $CHCl_3$ shows stretching frequencies 1738 cm^{-1}, 1720 cm^{-1}, 1666 cm^{-1} (broad), 1632 cm^{-1} (Figure 31). Sclerotiorin showed specific rotation $[\alpha]^{25}_{D}$ + 490^{o} (*c* = 1 in methanol).

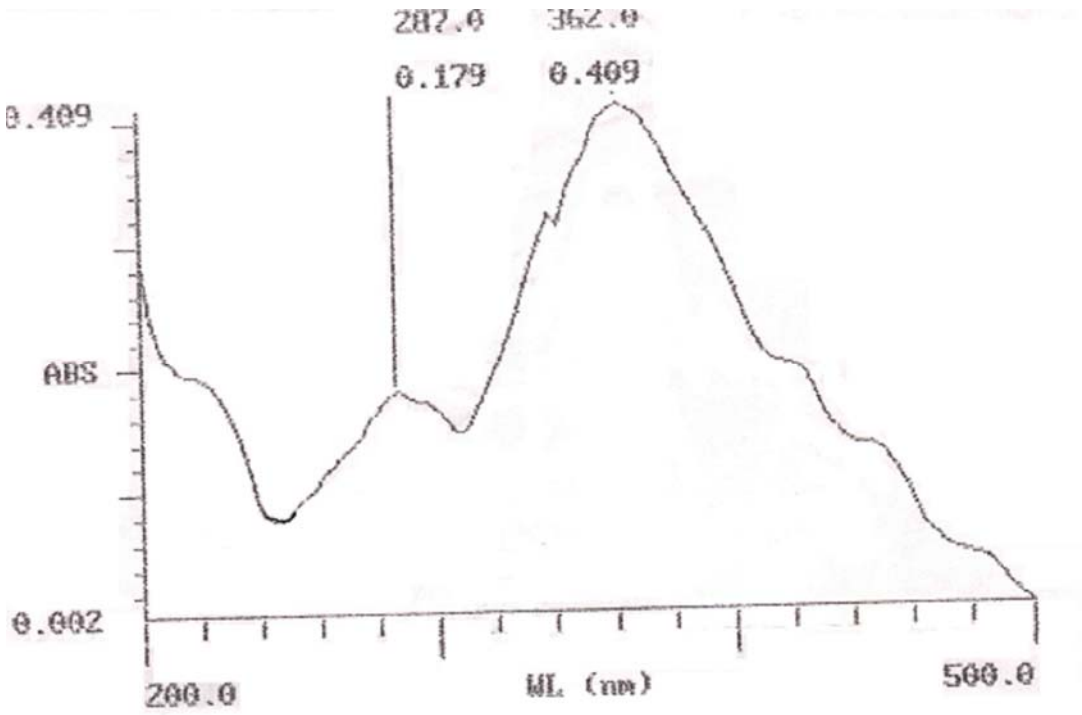


Figure 29. UV absorption spectrum of sclerotiorin isolated from fermented broth of *Penicillum frequentans* A-24

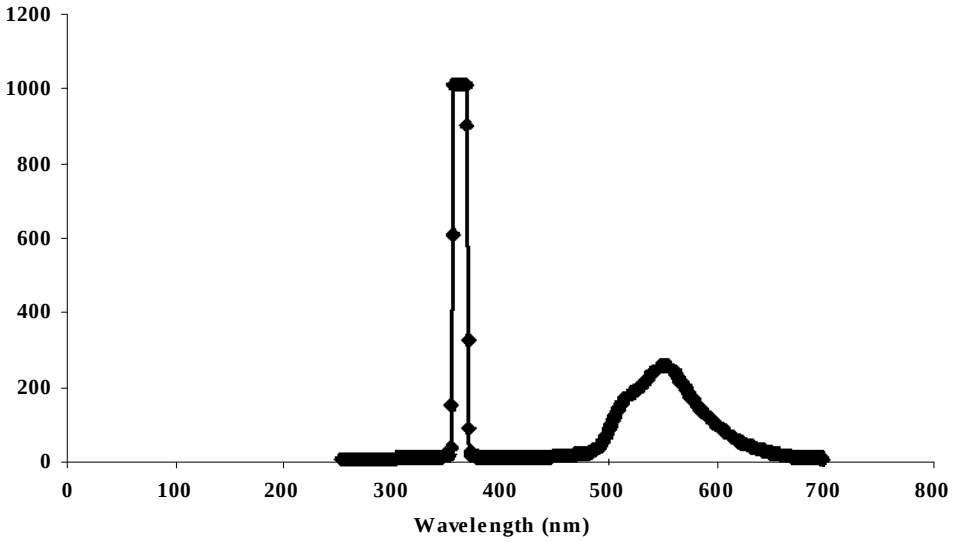


Figure 30. Emission spectrum of sclerotiorin isolated from fermented broth of *Penicillum frequentans* A-24.

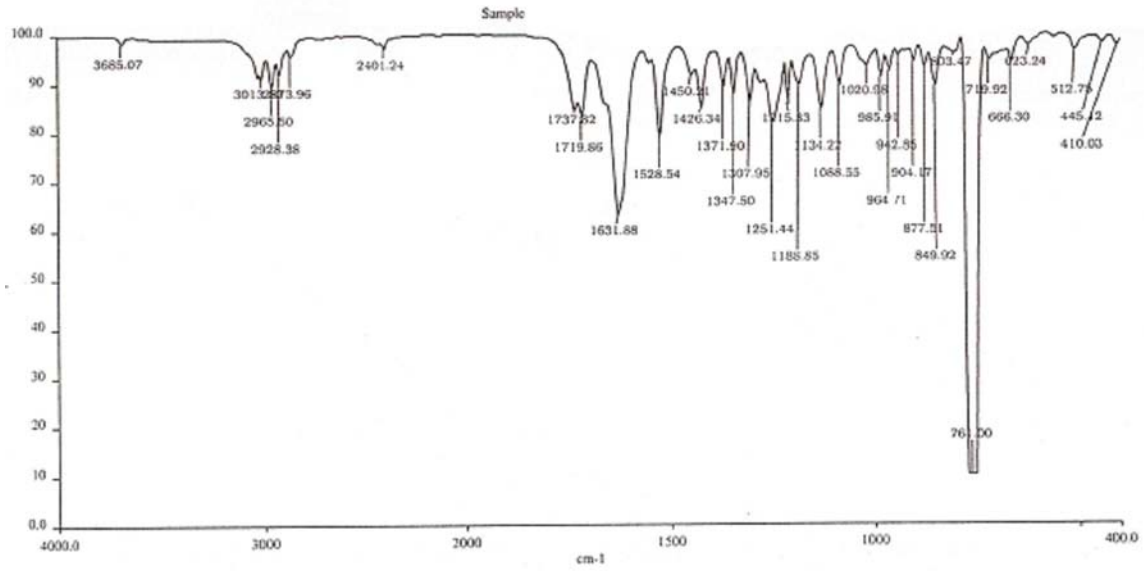


Figure 31. IR-spectrum of sclerotiorin isolated from fermented broth of *Penicillum frequentans* A-24

3.2.8. HPLC method development for the estimation of sclerotiorin:

Detection of sclerotiorin was developed by reverse phase HPLC on a C_{18} – 5 µm ODS column with isocratic and gradient elution of sclerotiorin at 362 nm. Ethyl acetate crude extracts of sclerotiorin were eluted with various solvent mixtures (Table 13). When sclerotiorin was eluted with 100% methanol or 100% acetonitrile, resolution was poor. Similar pattern was observed when an isocratic system of methanol: water 9:1, 7:3 and 1:1 was used. The results were satisfactory when mobile phase was shifted to acetonitrile: water 6.5:3.5.

Thus, acetonitrile/water gradient programme was used for sclerotiorin estimation. The Acetonitrile concentration was increased after an initial delay of 2 minutes from 0 to 100% over a period of 30 min time program with a flow rate of 0.5 ml/min at 362 nm. Sclerotiorin appeared with a RT value of 25 min and the resolution was satisfactory. Further the gradient programme was modified to get a better resolution. The Acetonitrile concentration was increased after an initial delay of 2 minutes from 60 to 100% over a

period of 30 min time program with a flow rate of 0.5 ml/min at 362 nm. Sclerotiorin showed a distinct peak with a RT value of 23 min (on a 30 cm column length) and 8.5 min (on a 15 cm column length) respectively (Figure 32). This mobile phase was used for further sclerotiorin estimations.

Table 13: HPLC method development for the estimation of sclerotiorin.

Mobile phase	Flow rate (mL/min)	Retention time (Minutes)	Number of impurity peaks in crude extract	Resolution
Isocratic elution				
Methanol (100%)	0.5	8.2	1	Poor
Acetonitrile (100%)	0.5	5.1	1	Poor
Methanol: Water (9:1)	0.5	6.2	1	Poor
Methanol: Water (7:3)	0.5	10.7	2	Poor
Methanol: Water (1:1)	0.5	15	2	Satisfactory
Acetonitrile: Water (6.5:3.5)	0.5	21	6	Satisfactory
Gradient elution				
Acetonitrile: water 0%-100% / 30min	0.5	25	6	Satisfactory
Acetonitrile: water 60%-100% / 30min	0.5	8.5	7-8	Good

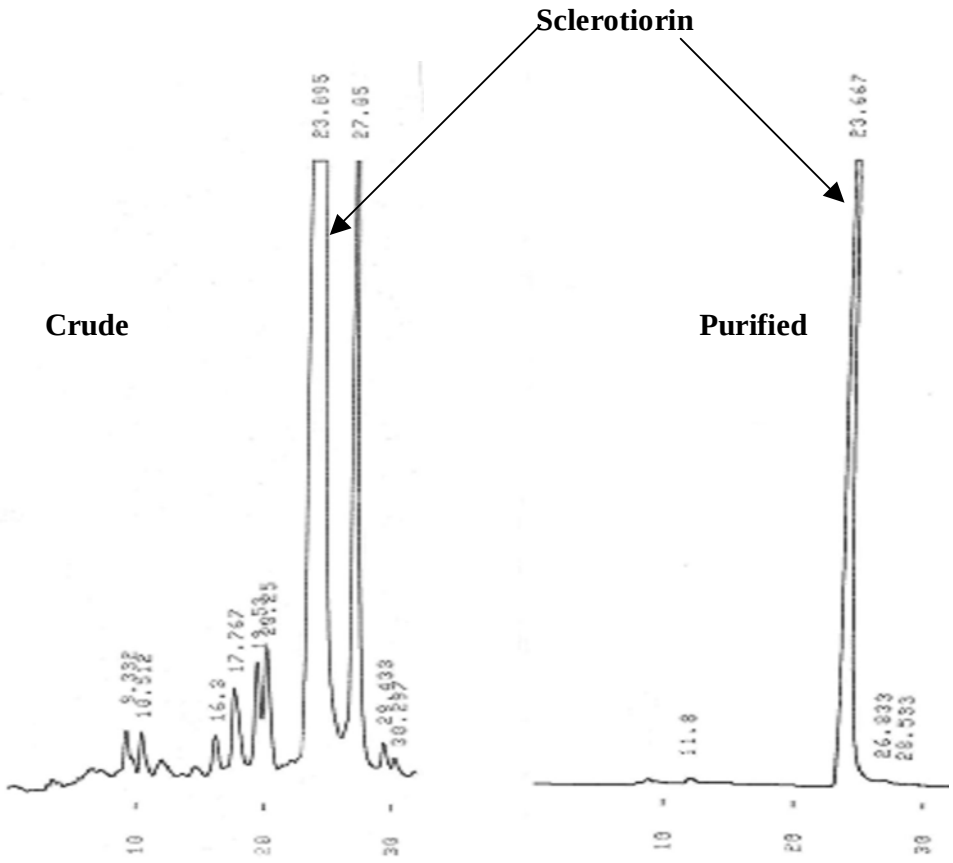


Figure 32. HPLC chromatogram of crude and purified sclerotiorin isolated from the fermented broth of *Penicillium frequentans* A-24

3.2.9. Sclerotiorin:

Sclerotiorin was first isolated in 1940 from laboratory cultures of *Penicillium sclerotiorum* Van Beyma, as chlorine containing fungal pigment by Curtin and Reilly and further assigned the name "sclerotiorin" by them with the molecular formula $C_{20}H_{20}O_5Cl$ (Curtin and Reilly 1940; Reilly et al., 1944). Later, sclerotiorin was obtained from five strains of *Penicillium multicolor* Grigorieva-Manilova and Poradielova (N.R.R.L. 764, N.R.R.L. 2058, N.R.R.L. 2059, N.R.R.L. 2060 and N.R.R.L. 2324). From the four of these strains sclerotiorin was isolated in considerably higher yield than had been obtained by Curtin and Reilly (1940). From the analysis of sclerotiorin and its derivatives the most probable empirical formula was designated as $C_{21}H_{23}O_5Cl$ (Birkinshaw, 1952). However the structural confirmation of sclerotiorin was further carried out and was included in to new structural class, characterized by a 6-oxoisochromane-ring system belonging to azaphilone class of compounds containing isochromane basic ring skeleton (Dean et al., 1959; Holker et al., 1962) In the year 1976 absolute configuration and stereochemistry of sclerotiorin was published with X-ray crystallographic evidences (Whalley et al., 1976).

Sclerotiorin was isolated as a pigmented secondary metabolite from *Penicillum* spp. Originally from *Penicillium sclerotiorum* Van Beyma (Curtin and Reilly 1940) *P. multicolor* (Birkinshaw, 1952), *P. implicatum* Biourge (Yamamoto and Nishikawa 1959) and *P. hirayamae* that produce (-) sclerotiorin (Udagawa, 1963). Some reports are from non *Penicillium* spp. like from *Talaromyces luteus* a structural analog (Satosh and Yamazaki 1989) and from cultured lichen mycobionts of *Pyrenula japonica* (Takenaka et al., 2000).

(-)Sclerotiorin, an antipode of (+)sclerotiorin was isolated from *Penicillium hirayamae* by Udagawa (1963), which just differed at C 7 stereochemistry and possessed all other character except showed specific rotation $[\alpha]^{26}_{D}$ - 480^{o} (C= 0.11 in ethanol). This compound was also called as 7-epi-sclerotiorin (Figure 33a) (Gregory and Turner 1963;Ellestad and Whalley 1965).

During the initial stages of the discovery of sclerotiorin much attention was paid to its chemistry and not to the biological activities (Eade et al., 1957;Birkinshaw and Chaplen 1958;Chong et al., 1971). Many nitrogen containing synthetic derivative compounds like sclerotioramine, N-methylsclerotioramine and aposclerotioramine, were synthesized by taking sclerotiorin as a starting material (Eade et al., 1957; Bell et al., 1964).

Halometabolite is defined as an organic halogen-containing molecule synthesized by a living organism from the metabolism of ionic halides. Some of the well-known halometabolites of microbial origin are Out of the halometabolites known, bromine derivatives are not produced with the same ease or abundance as their chloro-analogues. Iodo or fluoro metabolites have not been reported despite attempts by several investigators to obtain them from microorganisms.

Sclerotiorin is included under the list of novel halometabolites (Petty, 1961). Originally sclerotiorin ($C_{20}H_{20}O_5Cl$) was isolated as a chlorine containing halometabolite from *Penicillium sclerotiorum* by (Curtin and Reilly 1940), it was believed to have close resemblance with griseofulvin ($C_{17}H_{17}O_6Cl$) isolated by (Oxford et al., 1939). Birkinshaw re isolated sclerotiorin from *Penicillium multicolor* and assigned the corrected empirical formula ($C_{21}H_{23}O_5Cl$) (Birkinshaw, 1952).

3.2.10. Biological activities of sclerotiorin and structurally similar azaphilone compounds:

Azaphilones are defined as structurally diverse pigments, mostly secondary metabolites of fungal origin, and are highly oxygenated bicyclic quaternary rings showing various biological activities (Figure 33a,b) (Tabata et al., 1999;Kono et al., 2001). Sclerotiorin a member of azaphilones and its structurally similar azaphilone members are known for their various biological beneficiary activities.

From 1990, various biological activities of sclerotiorin and its structural analogs known as isochromophilones have been purified from the various cultures of *Penicillium*

spp (Omura et al., 1993; Arai et al., 1995). Sclerotiorin and other structurally similar azaphilones were reported as chalamydospore like cell inducing substances (Natsume et al., 1988). Two new structural analogs called TL–1 (which closely resembles 7-epi-sclerotiorin) and TL -2 isolated from *Talaromyces luteus* were found inhibitory to monamine oxidase (MAO) (Satosh and Yamazaki 1989; Fujimoto et al., 1990). Sclerotiorin was discovered to be a Phospholipase A2 and lipase inhibitor and a patent has been filed (Nakamura et al., 1990; Negishi et al., 2000).

Isochromophilone I-II, were isolated from the fermented broths of *Penicillium multicolor* F-2338 along with sclerotiorin as novel HIV gpl20- CD4 receptor antagonists (Omura et al., 1993; Matsuzaki et al., 1995). Sclerotiorin and other azaphilones produced from the fermented broths of *Penicillium sclerotiorum* X 11853 were found to show endothelin ET_A and ET_B receptor antagonistic activity (Pairet et al., 1995). Isochromophilones III-VI from *Penicillium multicolor* FO-3216, were found inhibitory to acyl-CoA cholesterol acyltrasferase (ACAT) (Arai *et al*. 1995). Sclerotiorin with other azaphilones were reported as anti-atherosclerosis agents by inhibiting cholesteryl ester transfer protein (CETP) (Tomoda et al., 1999).8-O-Methylsclerotioramine, Sclerotiorin and Isochromophilones VI produced from *Penicillium multicolor* F-1753 were found to be inhibitory and antagonistic to Grb2-Shc domain binding (Nam et al., 2000a,b). Isochromophilones IX isolated from the *Penicillium* sp is reported as GABA (γ-amino butyric acid) containing isochromophilone antibiotic compound against methicillin resistant *Staphylococcus aureus* (Michael et al., 2003).

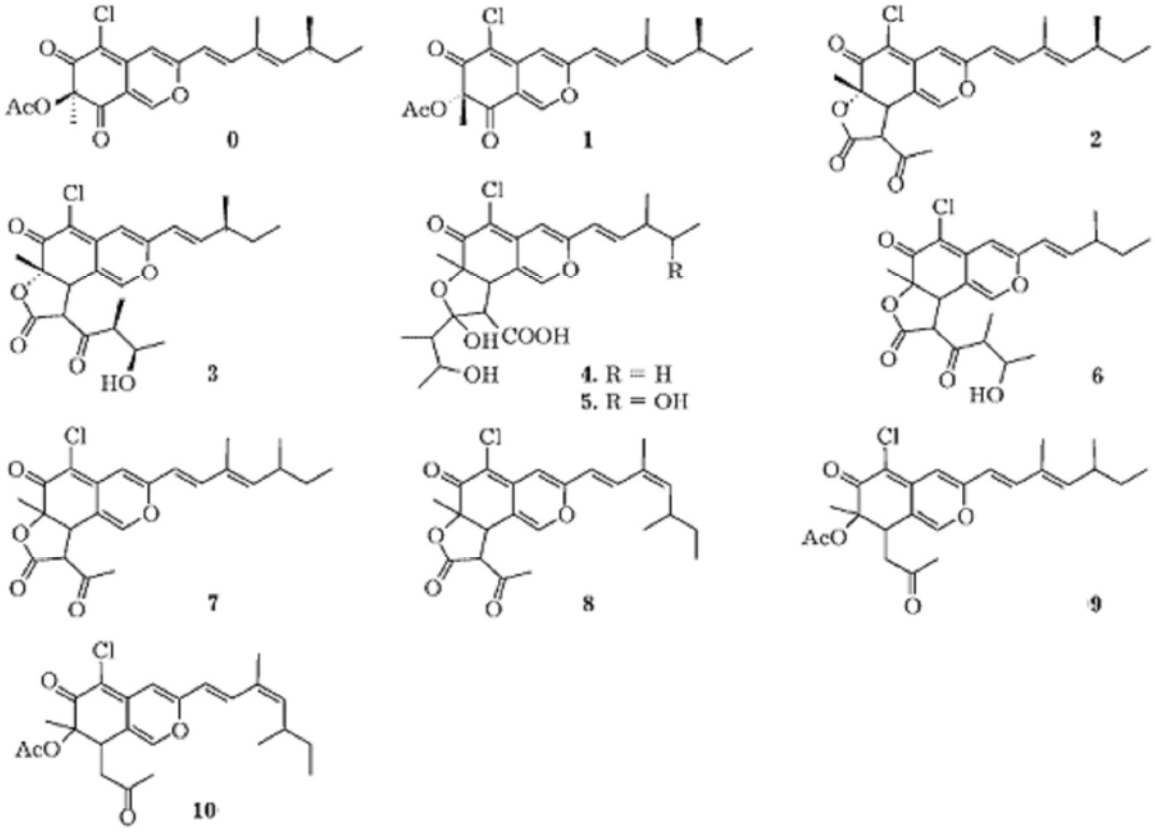


Figure 33a. Sclerotiorin and structurally similar halogenated azaphilones

(Figure details 0=sclerotiorin, 1=7-epi sclerotiorin, 2=Rubrorotiorin, 3-6=azaphilone A-D and 7-10=Isochromophilones I-IV)

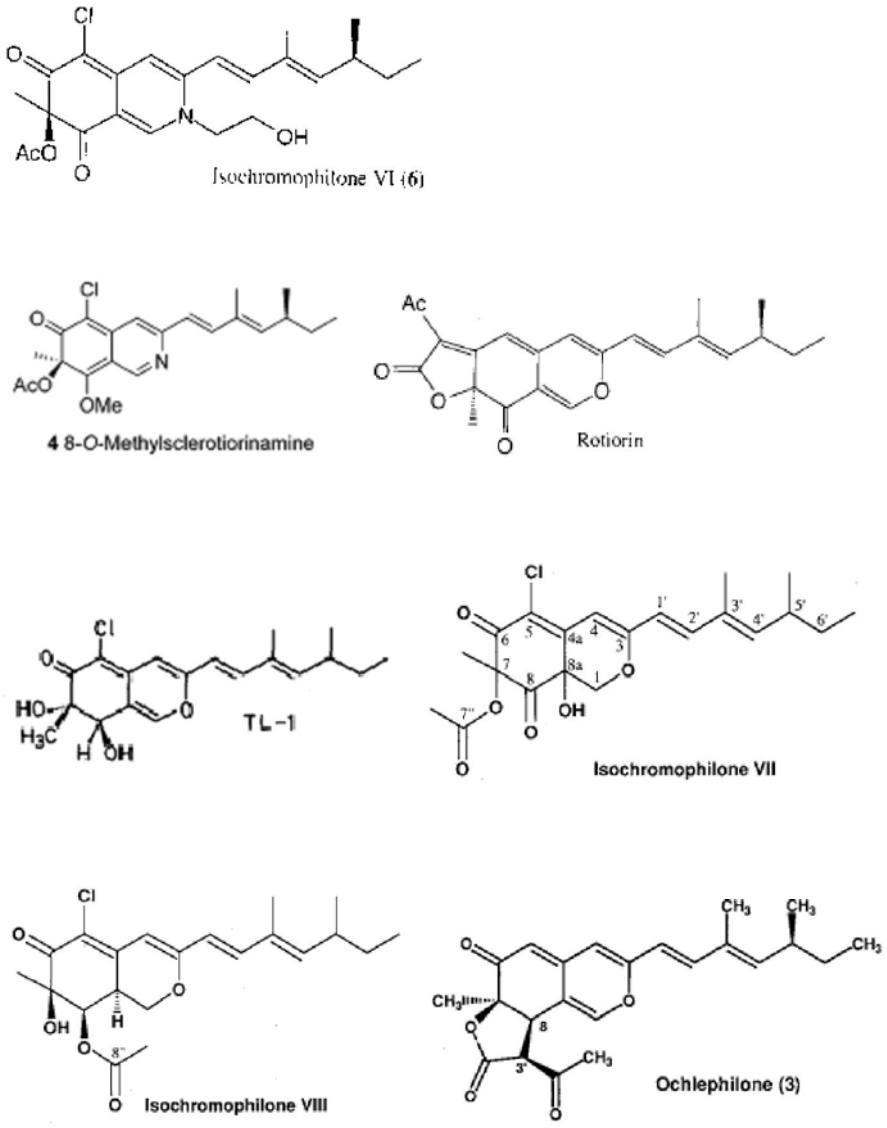


Figure 33b. Sclerotiorin and structurally similar halogenated azaphilones

SECTION–3

BIOLOGICAL ACTIVITIES OF SCLEROTIORIN

3.3. Inhibition studies of sclerotiorin on lipoxygenases:

3.3.1. Effect of Sclerotiorin on the activity of LOX:

Sclerotiorin demonstrated a dose response inhibition against LOX. As the concentration of the inhibitor increased, the residual enzyme activity decreased rapidly (Figure 34). The inhibitor concentration leading to 50% loss of enzyme activity, half maximal inhibitory value or IC_{50} was calculated as 4.2 μM. Comparative evaluation of sclerotiorin with other LOX inhibitors such as resveratrol (Pinto et al., 1999), curcumin (Began et al., 1998), nigerloxin (Rao et al., 2002b) and asperenone (Rao et al., 2002a) was carried out. Sclerotiorin showed highest inhibition against lipoxygenase when compared with other known LOX inhibitors (Table 14).

3.3.2. Kinetic studies of sclerotiorin against LOX:

3.3.3. Determination of the reversibility of the enzyme-sclerotiorin interaction:

In the presence of different concentrations of sclerotiorin the enzyme activity was dependent on the enzyme concentration. The plots of the residual enzyme activity versus the concentrations of enzyme at different concentrations of sclerotiorin gave a family of straight lines (Figure 35) that passed through the origin, indicating that the inhibition of the enzyme by sclerotiorin was reversible (Chen and Kubo, 2002).

3.3.4. Lineweaver–Burke plot:

Under the conditions employed in the present investigation, the oxidation reaction of linoleic acid by soybean LOX follows Michaelis-Menten kinetics (Axelrod et al., 1981; Began et al., 1998). The kinetic parameters for LOX obtained from Lineweaver-Burk plot show that K_M is equal to 33 μM and a V_{max} is equal to 100 μmol/min/mg protein (Figure 36). The results illustrated in Figure 36 show that increasing the concentration of sclerotiorin resulted in a family of lines intercepting on 1/V axis in a parallel manner, with the increasing concentration of the inhibitor, decreasing values in maximum velocity (V_{max}) and Michaelis-Menton constant (K_M) were recorded. The data demonstrated that the inhibitory effect of sclerotiorin was uncompetitive type (Figure 36). The inhibition kinetics and constants values are as shown in Table 15. The equilibrium constant for inhibitor binding, *Ki,* value was

determined as 9 µM by plotting a secondary plot *Ki* = Michaelis-Menton constant (K_M values obtained from the LB plot) versus concentration of sclerotiorin 2, 4.2 and 10 µM respectively (Figure 37).

Table 14: Comparison of sclerotiorin with other LOX inhibitors

Inhibitors	Inhibitor source	LOX IC_{50} µM	Inhibition	References
Sclerotiorin	*Penicillium frequentans* CFTRI A-24	4.2	Uncompetitive	This study
Resveratrol	Red wine	15	Competitive	Pinto et al., 1999
Curcumin	*Curcuma longa*	8.6	Competitive	Began et al., 1998
Nigerloxin	*Aspergillus niger* CFR-W-105	79	Mixed	Rao et al., 2002a
Asperenone	*Aspergillus niger* CFTRI 1105	300	Mixed	Rao et al., 2002b

Table 15: Kinetics and inhibition constants of soybean LOX by sclerotiorin

Kinetic constants	Concentration
IC_{50}	4.2 µM
K_M	33.0 µM
K_{cat}	50µmol/min/mg protein
Inhibition type	Uncompetitive
K_i	9.0 µM

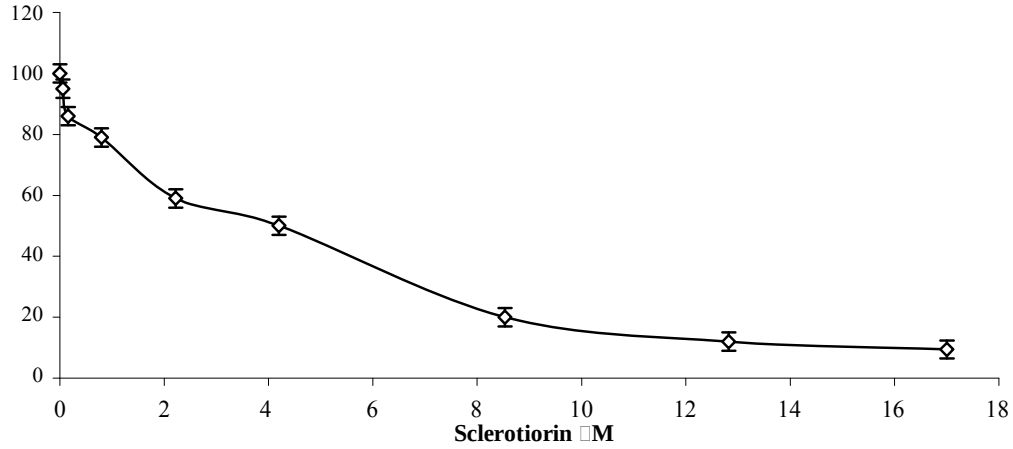


Figure 34. Dose dependent inhibition of LOX activity by sclerotiorin

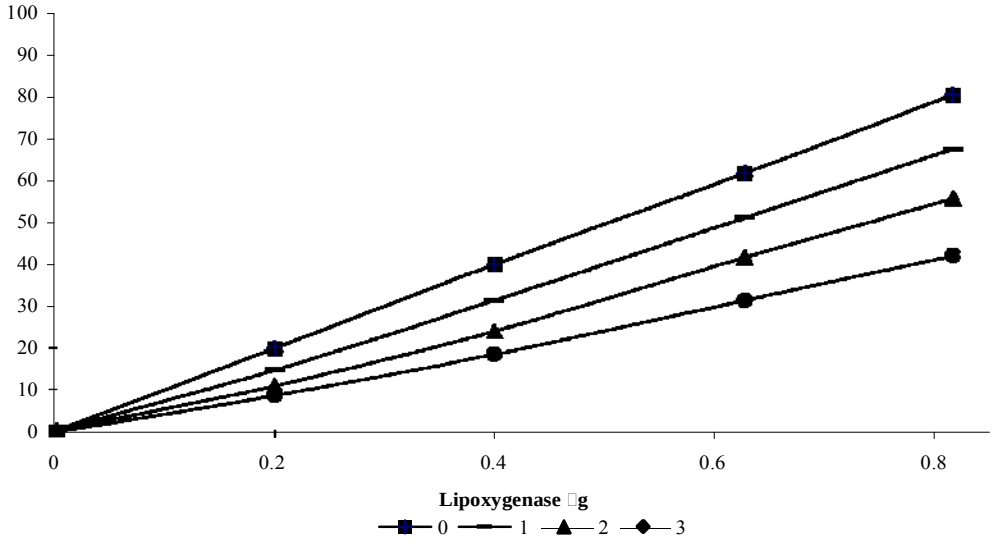


Figure 35. Reversible binding of sclerotiorin to LOX

(Figure legends: concentration of sclerotiorin 0=Control (without sclerotiorin), 1=10 µM, 2=5 µM, 3=2 µM)

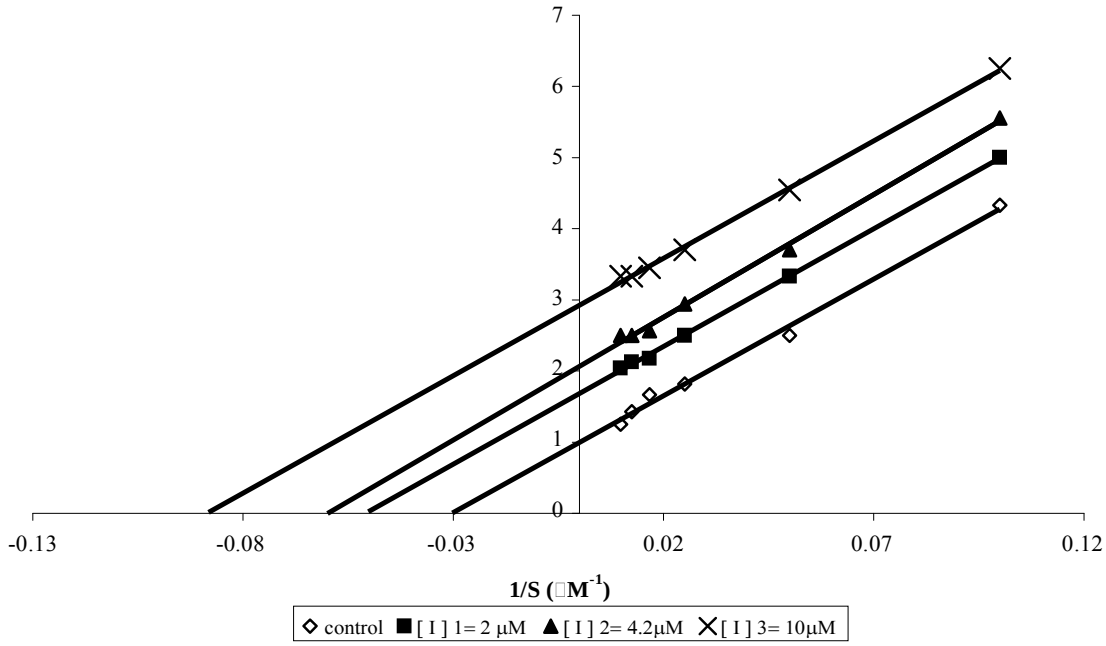


Figure 36. Double reciprocal plot of LOX in presence of sclerotiorin

(Figure legends: concentration of sclerotiorin= [I], □ =Control (without sclerotiorin), ■[I]=2 µM, ▲[I]=4.2 µM, X[I]=10 µM)

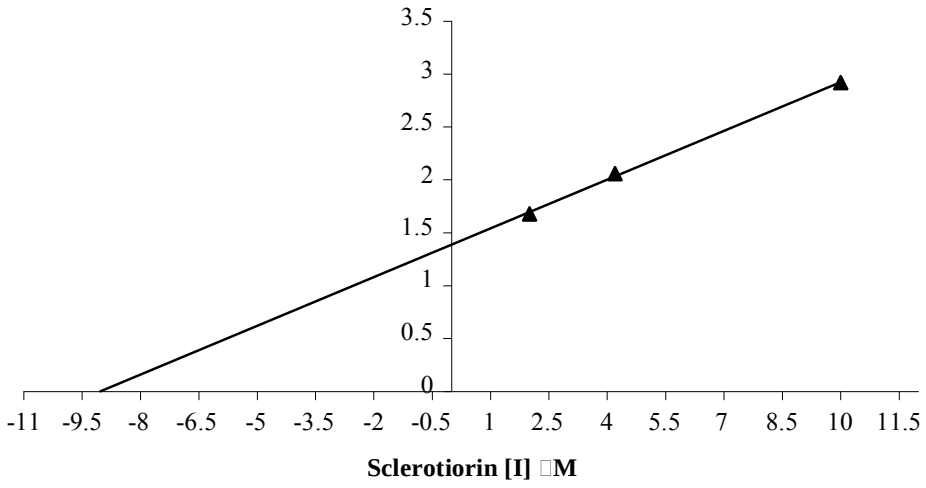


Figure 37. Secondary plot to determine Ki constant of sclerotiorin

3.3.5. Antioxidant property of sclerotiorin:

Oxidation of lipids is a significant factor in foods rich with fats, which brings about biochemical changes leading to loss of flavor, colour and nutritive values (Grechkin, 1998) The factors inducing such a process are both enzymatic and non-enzymatic. It is against this background that LOX inhibition by sclerotiorin was investigated along with antioxidant potential. As lipoxygenase enzyme generates peroxide free radicals, one of the modes of action of a lipoxygenase inhibitor could be due to antioxidant nature of the compound. Further, many of the natural antioxidants are known to inhibit lipoxygenase by interacting with the radicals generated in the lipid peroxidation process (Yasumoto et al., 1970). Hence, the total antioxidant potential of sclerotiorin was studied first using the phosphomolybdate method and then other methods.

3.3.6. Phosphomolybdate method:

The total antioxidant potential of sclerotiorin was studied using the phosphomolybdate method, which is based on the reduction of Mo (VI) to Mo (V). The formation of green phosphate complex Mo (V) at acidic pH indicative of antioxidant activity, which is measured in molar equivalent linearity intervals (Prieto et al., 1999). Sclerotiorin showed the formation of Mo (V) complex at 695 nm with the increasing concentration showing a molar equivalent linearity range at 100 µM to 1000 µM (Table 16). This was compared to a standard synthetic antioxidant, Butylated Hydroperoxy Toluene (BHT), which showed molar equivalent linearity at 695 nm 200 µM-2000 µM. Sclerotiorin showed redox potential which is twice as high as molar equivalent with respect to standard antioxidant BHT.

3.3.7. 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) method:

After establishing the redox antioxidant potential of sclerotiorin, our next studies were on its free radical quenching ability, determined by the DPPH method (Blots, 1958), which is a well-known chemical model system to evaluate the antioxidant property of compounds. Sclerotiorin showed decolorization of DPPH in a dose dependent manner and the half maximal ED_{50} (Effective Dose) value of the inhibitor

required to reduce the absorbance by 50% was found to be 0.12 µM (Figure 38). These results showed that sclerotiorin has a potent scavenging ability in comparison with BHT, which showed an ED_{50} of 35 µM (Table 16).

Table 16: Comparison of antioxidant potential of sclerotiorin to standard BHT

Assays	**Half maximal Value**	**Sclerotiorin, µM**	**BHT, µM**
DPPH	ED_{50}	0.12	35.0
Fe^{2+}- ascorbate induced lipid peroxidation	PD_{50}	64.0	6.0
Phospho-molybdate Total antioxidant potential	Molar equivalent	100-1000	200-2000
LOX inhibition	IC_{50}	4.2	270

3.3.8. Liposome model TBARS method:

Free radicals such as superoxide anions ($O_2^{\cdot -}$) hydroxyl radical ($^{\cdot}OH$), malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) trigger the process of Lipid peroxidation (LPO), a well-known phenomenon occurring in both plant and animal systems. Malondialdehyde (MDA) is a major end product of the LPO process and taken as a key index molecule (Summerfield and Tappel, 1983).

A liposome emulsion model lipid peroxidation was induced by ferrous ascorbate system and the malondialdehyde (MDA) was formed from the peroxidation was reacted with thiobarbituric acid (TBA) to give a red coloured species, which was read at 535nm (Buege and Aust, 1978). The results indicated that sclerotiorin showed protection against LPO in a dose dependent manner (Figure 39) with the half maximal Protection Dose (PD_{50}) value of 64 µM, as against synthetic BHT, which showed 6 µM (Table 16).

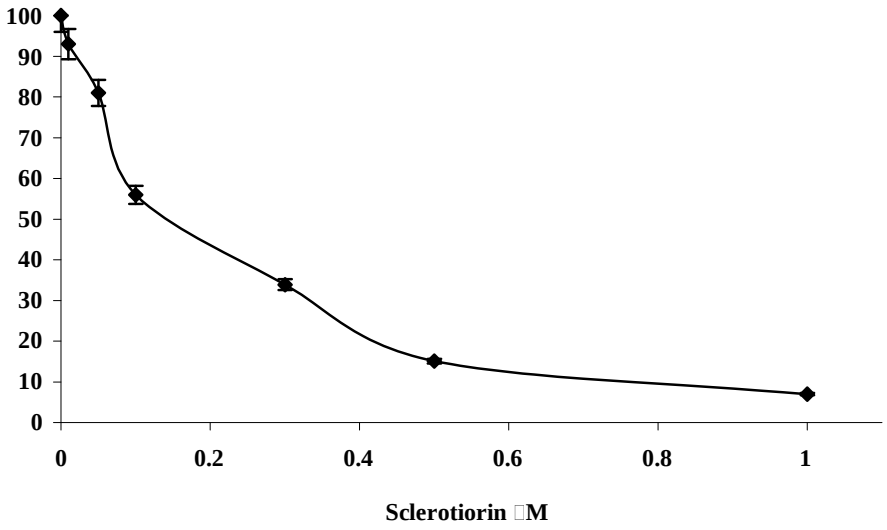


Figure 38. DPPH radical scavenging activity by sclerotiorin

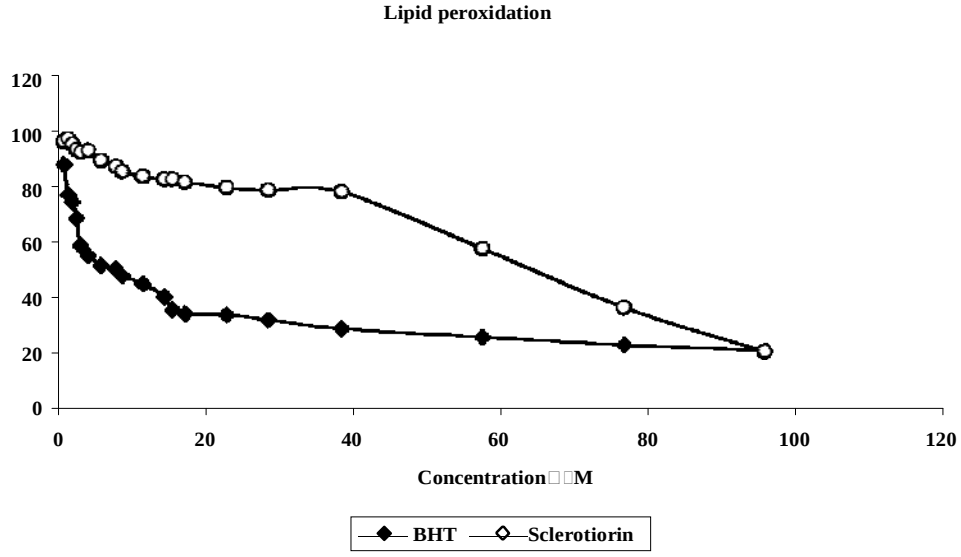


Figure 39. Lipid peroxidation (LPO) inhibition by sclerotiorin and BHT

3.3.9. Metal binding assay method:

Biological membranes are rich with unsaturated fatty acids and are surrounded by metal containing fluids. These inherent metal ions, like Fe^{2+}/Cu^{+}, are known to initiate LPO (Horton and Fairhurst 1987). Sclerotiorin was tested for its metal binding ability.

The assay uses ferrozine reagent to react with metal ions such as ferrous species by competing for the formation of a Ferrozine- Fe^{2+} coloured complex at an absorbance maxima 562 nm (Carter, 1971). The results showed the formation of Ferrozine-Fe^{2+} complex complete even in the presence of an increasing concentration of sclerotiorin from 100 µM till 500 µM. This suggested that sclerotiorin does not possess metal chelating property, unlike EDTA used as control, which blocked the ferrozine-Fe^{2+} complex completely even at very low concentration of less than 20 µM.

3.3.10. Mechanism of action of sclerotiorin on LOX:

There are three modes of lipoxygenase inhibition: (a) Inhibition by chelating iron metal ion in catalytic site (Skrzypczak et al., 2000), (b) Removal of oxygen from the system (Guindon et al., 1987), (c) preventing interaction with free radical formation (Van-der-Zee et al., 1989).

The experimental results suggest that sclerotiorin might be inhibiting LOX activity by trapping radical intermediates that are formed by enzyme substrate catalysis. This hypothesis is further supported by its uncompetitive nature of inhibition, because lipid radicals can be generated at the active site only when there is an enzyme substrate complex. All uncompetitive inhibitors bind exclusively to ES complex (Enzyme Substrate) with or no affinity to the free enzyme itself. Further the radical scavenging ability and protection shown in a non-enzymatic LPO model, suggests that lipoxygenase inhibition of sclerotiorin is due to its antioxidant nature by trapping or quenching the fatty acid radical species and not by Fe^{2+} or Fe^{3+} metal ion chelation.

3.3.11. Effect of sclerotiorin on 5-lipoxygenase:

In mammals, the products of lipoxygenase-catalyzed reactions are responsible for variety of human disorders, such as atherosclerosis, allergy, inflammation, asthma, and hypersensitivity (Steinburg, 1999; Prigge et al., 1997). Zileuton, a well-known 5-LOX inhibitor and other CysLT1 receptor antagonists like montelukast, zafirlukast and pranlukast have shown significant improvement in reversing the process of asthma (Bell et al., 1992).

As sclerotiorin inhibited soybean LOX in an uncompetitive manner with IC_{50} of 4.2 μM, we studied sclerotiorin activity against human PMNL 5-Lipoxygenase also. The results showed that the effect of sclerotiorin demonstrated a dose dependent inhibition of 5- LOX. As the concentration of the inhibitor increased, the residual enzyme activity decreased rapidly as shown in Figure 40. IC_{50} was calculated as 16 μM and comparative evaluation of sclerotiorin with other 5-LOX inhibitors was carried out NDGA, Eugenol and phenidone showed 30, 46 and 65 μM respectively.

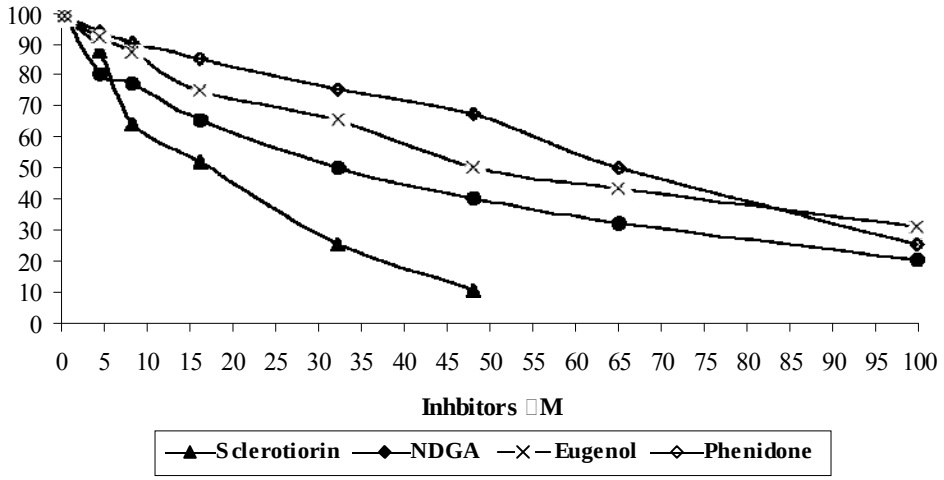


Figure 40. Dose dependent inhibition of sclerotiorin against 5-LO

3.3.12. Other biological properties of sclerotiorin:

As sclerotiorin inhibited soybean LOX and 5-LOX, it was interesting to study other biological activities. Sclerotiorin up to 300 μM did not show any inhibition against other mammalian enzymes such as esterases (rat brain acetylcholine esterase) (Ellman et

al., 1961), porcine pancreatic lipase (Weibel et al., 1987), peptidases (angiotensin converting enzyme) and oxido-reductases (polyphenol oxidase).

3.3.13. Effect of Sclerotiorin on platelet aggregation:

Some of the soybean and mammalian lipoxygenase inhibitors such as baicalein, quercetin, caffeic acid, esculetin and curcumin have shown to possess potent platelet aggregation inhibition activity (Bertz et al., 1981; Tang and Eisenbrand, 1992; Ammon et al., 1993; Sekiya and Okuda, 1982; Sekiya et al., 1982). Since sclerotiorin inhibited LOX and as azaphilone compounds have shown various receptor antagonistic properties (Matsuzaki et al., 1995a,b; Pairet et al., 1995), we examined if this compound could also inhibit platelet aggregation.

Sclerotiorin showed significant inhibition of human platelet aggregation in dose dependent manner in an ADP induced platelet aggregation with an IC_{50} valve of 250 µM (Figure 41) and standard aspirin showed 17 µM. Inhibition shown by sclerotiorin is unfavorably high when compared to other reported inhibitors from microbial origins like PI-200 and PI-201 with IC_{50} value of 0.38 mM and 0.71 mM respectively, isolated from *Streptomyces* sp A 7498 (Kishimura *et al.* 1992). Asperenone, a lipoxygenase and a platelet aggregation inhibitor showed an IC_{50} value of 0.23 mM (Rao *et al.* 2002a).

3.3.14. Effect of sclerotiorin on rat eye lens aldose reductase:

Aldose reductase (EC 1. 1. 1. 21) an NADPH-dependent oxidoreductase catalyzes the conversion of glucose to sugar alcohol and promotes accumulation of sorbitol in various tissues by polyol pathway under the condition of hyperglycemia such as diabetes mellitus. These elevated intracellular sorbitol levels lead to many diabetic complications such as retinopathy, cataract, neuropathy and nephropathy (Kador 1988; Gabbay, 1973; Kador et al., 1990). Inhibitors against aldose reductase can reverse this biochemical change and may delay or even prevent this complication. Hence inhibitors against aldose reductase have a pharmacological application (Kador *et al.* 1985).

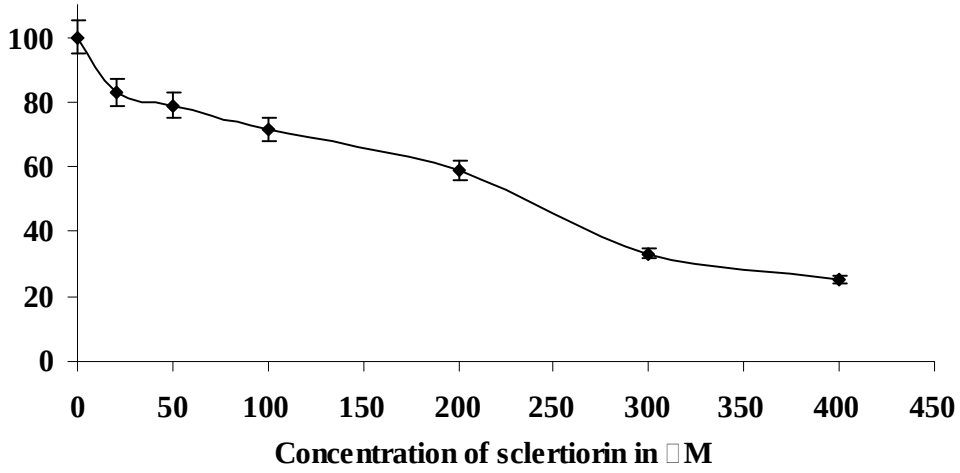


Figure 41: Dose dependent inhibition of sclerotiorin on ADP induced human platelet aggregation

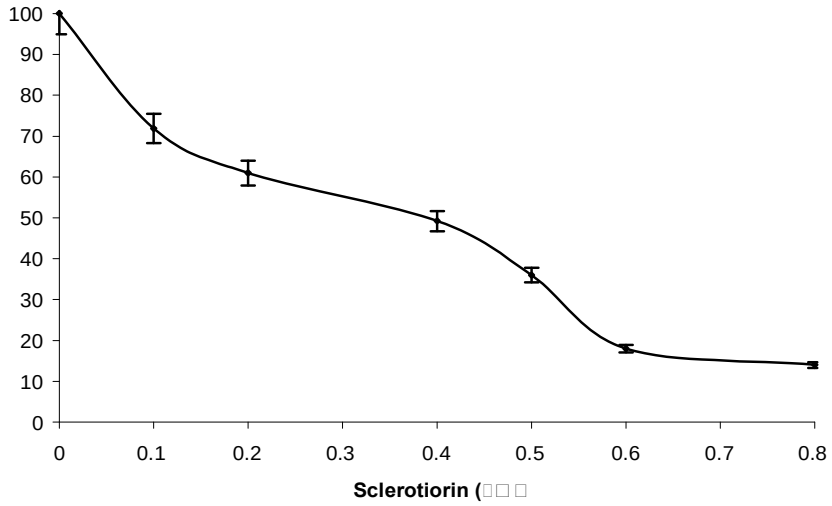


Figure 42. Dose dependent inhibition of rat eye lens aldose reductase by sclerotiorin

Sclerotiorin showed a dose-dependent inhibition against rat eye lens aldose reductase and had a half maximal value IC_{50} of 0.4 μM (Figure 42). In comparison with reported aldose reductase inhibitors of microbial origin such as YUA001, produced by *Corynebacterium* sp YUA-25, which has an IC_{50} of 1.8 mM (Bahn et al., 1998), and is moderate when compared to thiazocin A, produced by *Actinosynnema* sp c-304, which has an IC_{50} of 0.45 μM (Ozasa et al., 1991). Sclerotiorin compares well with other fungal inhibitors from our laboratory (Rao *et al.* 2002b, 2003) (Table 17).

Table 17: Comparison of sclerotiorin with other fungal aldose reductase inhibitors isolated at CFTRI

Inhibitors	Inhibitor source	Aldose reductase IC_{50} (μM)	Reference
Sclerotiorin	*Penicillium frequentans* CFTRI-A-24	0.4	This study
Nigerloxin	*Aspergillus niger* CFR-W-105	69	Rao et al. (2002b)
Asperaldin	*Aspergillus niger* CFTRI 1046	27	Rao et al. (2003)

3.3.15. Antimicrobial property of sclerotiorin:

As azaphilone compounds have been tested for broad-spectrum antimicrobial activities (Jongrungruangchok, 2004) and as halogenated compounds are good antibacterial agents, sclerotiorin was also tested against both Gram-positive and Gram-negative bacteria. Antibacterial activity was tested using agar well diffusion method (Owais *et al.* 2005). Sclerotiorin at 150 µg inhibited Gram-positive bacteria and was less effective against Gram-negative cultures (Table 18). Among the Gram-positive cultures, it showed potent inhibition against *Bacillus* spp.

Table 18: Antibacterial activity of sclerotiorin

Bacterial culture	Origin	Zone of inhibition (in mm)
Micrococcus luteus	NCIB 196	10
Bacillus cereus	F 4810	16
Bacillus subtilis	NCIB 3610	17
Staphylococcus aureus	CFTRI-CI-01	Nil
Klebsiella pneumoniae	CFTRI-CI-12	10
Escherichia coli	NCIB 9132	10
Salmonella typhi	CFTRI-CI-18	9
Enterobacter aerogenes	CFTRI-CI-10	Nil
Yersinia enterocolitis	CFTRI-CI-19	Nil
Listeria monocytogenes	CFTRI-CI-21	10
DMSO (solvent control)		Nil

Sclerotiorin was tested against some of plant and animal pathogenic fungi using agar well diffusion method (Wilson et al., 2005). Sclerotiorin at different concentrations dose dependently inhibited test fungal cultures (Table 19). Among the cultures tested, it showed potent inhibition against *Candida, Fusarium and Penicillium.*

Table 19: Antifungal activity of sclerotiorin

Fungal Strains		**Zone of Inhibition in mm**		
Species	**Origin**	**10µg**	**50µg**	**100µg**
Aspergillus niger	*CFTRI-1105*	02	05	10
Fusarium moniliforme	M3125	03	12	16
Alternaria alternata	lab isolate	04	08	12
Penicillium sp	lab isolate	04	10	17
Candida albicans	NCIM-3074	06	07	15
Candida tropicalis	NCIM-3118	06	12	18

Isochromophilone III, V and VI activity were reported to be active against *Staphylococcus aureus* FDA 209P, *Bacteroides fragilis* ATCC 23745 and *Pyricularia oryzae* KF 180 at 50 µg, but not against *Mycobacterium smegmatis* ATCC607, *Escherichia coti* NIHJ, and *Pseudomonas aeruginosa* P3 (Arai et al., 1995).

In another finding on antimicrobial activity of azaphilones with special reference to pencolide, sclerotiorin and isochromophilone VI isolated from the culture broths of *Penicillum sclerotiorum* were active towards *Candida albicans, Streptomyces pyogenes, Staphylococcus aureus, Salmonella typhimurium* and *Escherichia coli* (Lucas et al., 2007; Michael et al., 2003).

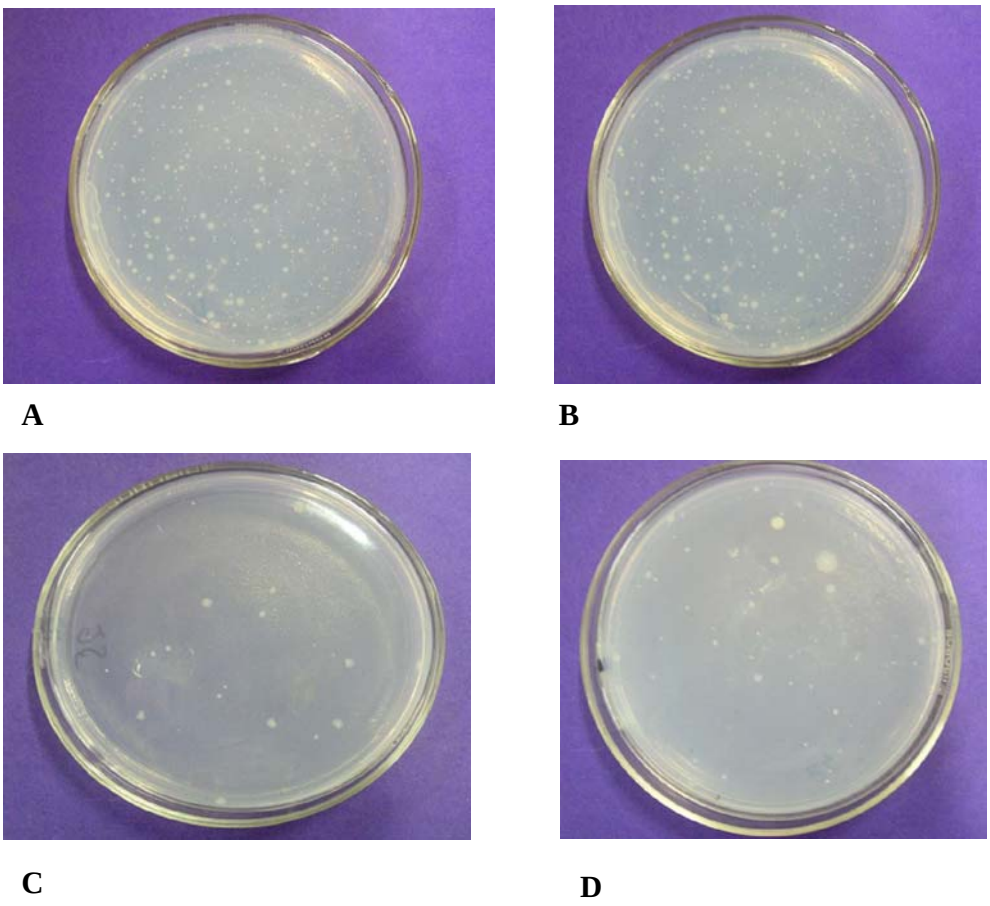
3.3.16. Mutagenicity Ames test:

The Ames test is a biological assay to assess the mutagenic potential of chemical compounds (mutagens). As cancer is often linked to DNA damage, this test serves as a quick and reliable assay to estimate or screen for the compounds that are carcinogenic in nature. Ames test detects chemicals compounds, which can induce mutation in test strains of *Salmonella typhimurium* (TA 1353, TA 97a, TA 100 and TA 98), which lack (*his*$^-$) genes. The compounds that bring reverse mutation in these (*his*$^-$) strains and restores the capability of the bacteria to synthesize an essential amino acid Histidine (*his*$^+$), in an amino acid deficient media are considered as carcinogens (Ames et al., 1975).

Before starting the assay, DMSO and inhibitory action sclerotiorin against all *Salmonella typhimurium* strains was confirmed. Sclerotiorin up to 75 µg per plate showed no inhibitory effect. Sclerotiorin mutagenicity against all the four strains TA 1353, TA 97a, TA 100 and TA 98 were shown negative. There was no increase in the revertant colonies in any of the concentration of sclerotiorin tested as compare to DMSO control (Figure 43).

Positive control plates (without S9 mixture) were incorporated with standard mutagens, sodium azide for TA 100 and TA 1535, 4NQNO for 97a and 2-nitroflurene for TA 98. For plates (with S9 mixture) standard mutagens 2-aminoanthracene for TA 100, TA 1535 and TA 98, and 2-amino fluorine for TA 97a were used as shown in Table 20. Negative control plates were maintained with DMSO for each bacterial strain.

Sclerotiorin mutagenicity against all four-tester strains TA 1535, TA 97a, TA 100 and TA 98 were found negative at all concentration levels (Table 20) and the results suggest that sclerotiorin is non mutagenic in nature and is a safe compound.



A **B**

C **D**

Figure 43. Ames mutagenicity test with *Salmonella typhimurium* TA 100

Plate A-B positive control with sodium azide (A+S9, B-S9); Plate C- negative control with DMSO; Plate-D sclerotiorin (75 µg / plate)

Table 20: Mutagenic activity of sclerotiorin at various doses in the strains of *Salmonella typhimurium* with and without S9 mixture.

Dose µg/plate	**Revertants / plate (mean ± S.D.)**							
	TA 1535		**TA 97a**		**TA 100**		**TA 98**	
	- S9	+S9	- S9	+S9	- S9	+S9	- S9	+S9
DMSO	21 ± 03.2	22 ± 09.1	24 ± 08.0	29 ± 08.6	29 ± 08.8	33 ± 09.5	29 ± 03.3	27 ± 06.4
25	32± 03.5	22 ± 04.3	23 ± 03.5	19 ± 02.5	38 ± 03.9	34 ± 13.0	30 ± 07.3	22 ± 07.0
50	21 ± 07.9	24 ± 08.0	36 ± 04.9	18 ± 04.7	32 ± 04.9	24 ± 10.1	27 ± 12.3	29 ± 04.1
75	26 ± 08.1	22 ± 01.5	26 ± 08.1	22 ± 02.3	27 ± 05.2	41 ± 02.5	34 ± 09.2	34 ± 06.6
[b]Control	713 ± 33.4	350±37.2	587 ± 52.2	795±35.3	872 ± 26.1	984±13.0	506 ± 41.7	359±32.1

[b]Control = Positive controls for (-S9): NaN_3 (1.2 µg/plate) for TA 100 and (1 µg/plate)TA 1535; 2NF (1 µg/plate) for TA 98, 4-NQNO (1µg/plate) for 97a: Positive controls: for (+S9): 2AAn (2.5 µg/plate) for TA 1535, (1 µg/plate) ,(0.75 µg/plate) for TA 98; 2-AF (10 µg/plate) for TA 97a.

3.3.17. Anticancer property of sclerotiorin:

5-LO protein has been reported in cancer cell lines of animal or human origin including brain (Boado et al., 1992), breast (Przylipiak et al., 1998; Avis et al., 2001), colon (Wachtershauser et al., 2000), esophageal mucosa (Zhi et al., 2003; Hoque et al., 2005), lung (Avis et al., 1996), kidney. Currently pharmacological agents that specifically inhibit the LO-mediators are of concern in clinical chemoprevention studies.

Literature on anticancer property of azaphilones is scanty. Isochromophilone III, V and VI have shown cytotoxicity against B-16 melanoma cells (Arai et al., 1995). Some of the azaphilones have shown tumor promotion inhibition in mice study (Yasukawa et al., 1994) and in complication of leukemia (Takahashi et al., 1998).

3.3.17.1. Cytotoxicity of sclerotiorin against Hep3B cells:

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form dark blue formazan crystals (Mosmann, 1983). These crystals are impermeable to cell membranes, resulting in their accumulation within healthy cells. These crystals can be solublized using a suitable solvent. The number of surviving cells is directly proportional to the level of the formazan product created. The effect of sclerotiorin on the viability of Hep3B (human derived hepatocarcinoma) cell line is depicted in Figure 44.

The percentage decolorisation was plotted against the concentration of the sample (R^2= 0.9405), and the amount of sample necessary to decrease 50% of the absorbance of MTT was calculated and expressed as EC_{50}. The results showed an EC_{50} of sclerotiorin at 40 ppm. EC_{50} value was calculated from the regression equation (R^2= 0.9405). Viability was expressed as percentage of control and data were expressed as average ± SD of three independent experiments.

3.3.17.2. Antiproliferative effect of sclerotiorin:

Cell proliferation was inhibited strongly in a dose dependent manner (Figure 45). Sclerotiorin was relatively non-toxic to Hep3B cells up to 15 ppm and at 20 ppm it showed upto 30% toxicity after exposure for 72 hours. At concentrations of 25 ppm and above, a drastic reduction in cell viability (greater than 60%) was observed indicating a very high antiproliferative action of the compound on Hep3B cells.

It is possible that inhibition of cell proliferation by sclerotiorin could be due to inhibition of the growth of the cells and/or by induction of apoptosis. Therefore, further investigation was carried out to find out if apoptosis could be a reason for the antiproliferative effect of sclerotiorin in the hepatocellular carcinoma cells.

3.3.17.3. Induction of apoptosis by sclerotiorin in Hep3B cells:

Suppression of growth and induction of apoptosis has been observed by many chemotherapeutic agents in transformed tumour cells. Induction of apoptosis in tumour cells is an efficient target for drug development and has become a major focus in the study of cancer therapy (Chodon et al, 2007).

Evidence for induction of apoptosis by sclerotiorin was sought by looking for the condensation of nuclear material and formation of apoptotic bodies. The progression of cell death was followed by treating the Hep3B cells with 40 ppm of sclerotiorin at different time intervals of 30-120 minutes followed by washing with PBS (pH 7.4) and staining with an equal mixture of ethidium bromide and acridine orange (Parks et al., 1979).

The morphological assessment of apoptosis was evidenced by fluorescent microscopic examination. Fluorescent nuclei were screened for normal morphology (unaltered chromatin) and apoptotic nuclei comprising those with fragmented (scattered) and condensed chromatin were observed (Figure 46).

It was observed that the cell death progresses via nuclear condensation followed by formation of apoptotic bodies in a time dependent manner and sclerotiorin induced apoptosis in these cancerous cell lines in less than 2 hours of incubation.

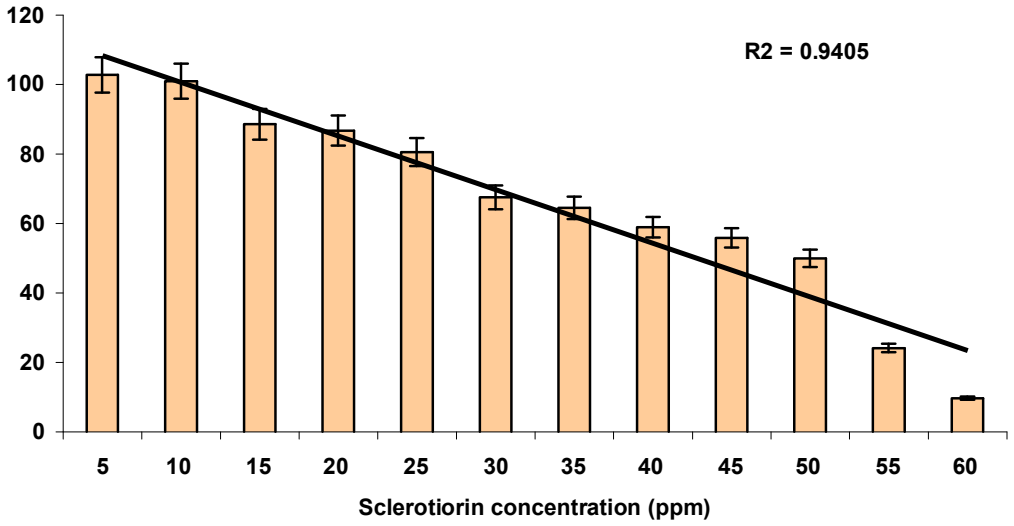


Figure 44. Effect of the sclerotiorin on the viability of Hep3B cells

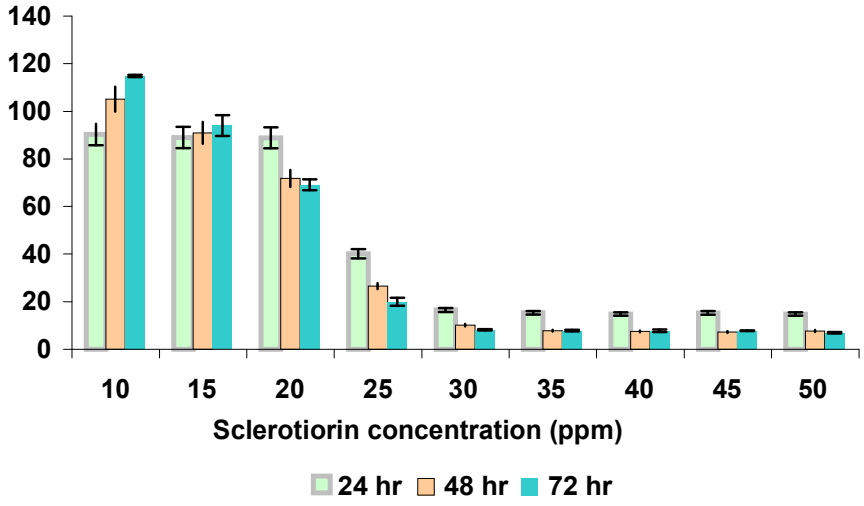


Figure 45. Antiproliferative effect of sclerotiorin on Hep3B cells

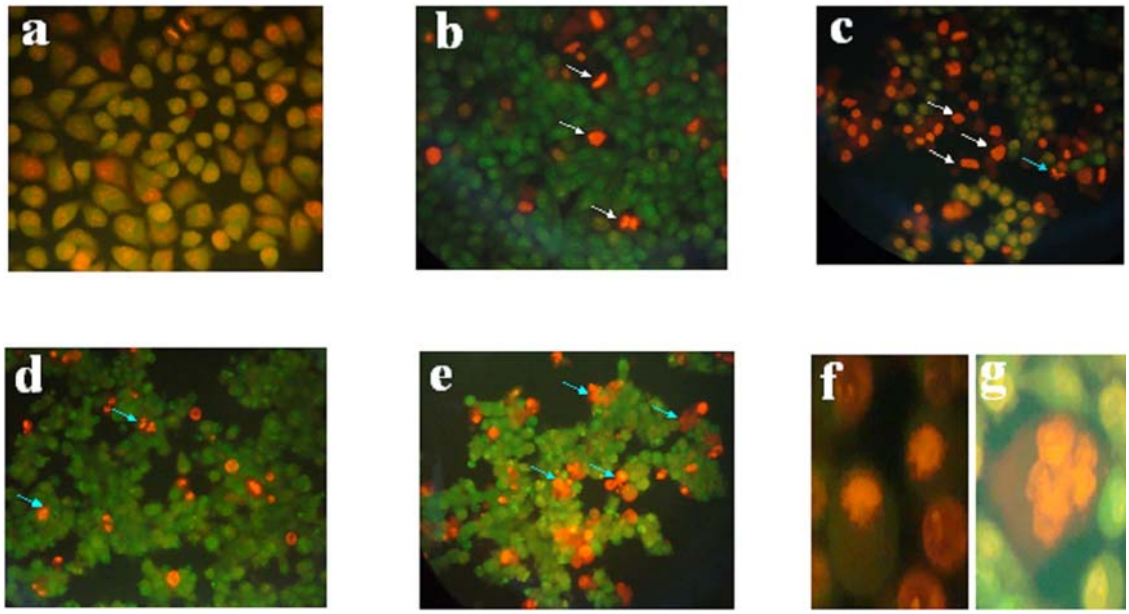


Figure 46. Morphological assessment of apoptosis of Hep3B cells treated with sclerotiorin by fluorescent microscopic

Fluorescent microscopic pictures of Hep3B cells treated with 40 ppm of sclerotiorin, stained with an equal mixture of ethidium bromide and acridine orange and observed at40x magnification using a fluorescence microscope. (a) Zero minute (b) 30 minutes (c) 60 minutes (d) 90 minutes (e) 120 minutes. (f) and (g) indicate enlarged views of formation of condensed chromatin and apoptotic borespectively. The figures are representative of 3 independent experiments done in triplicates White arrows indicate condensed chromatin while the blue arrows indicate apoptotic bodies.

SECTION–4

MEDIA OPTIMIZATION

3.4. Media optimization for sclerotiorin production:

One of the most common features in the study of a fermentation process is development of the media that is optimal for the desired output of interest, whether this be maximal biomass yield, yield of metabolic product or enzyme levels within the cell. Many of the approaches for this purpose reported in literature involve the "One factor at a time" approach, also called as the classical method of optimization. This involves a single dimensional search, which requires changing one variable while fixing the others at a certain level. Optimization studies in the present investigation were carried out by the classical single dimensional approach.

Literature survey showed that, sclerotiorin producing cultures were mostly isolated from wild. *Penicillum sclerotiorum* was first isolated from air by Prof. Boedijn in 1935 in Buitenzorg, Java, Indonesia (Curtin and Reilly 1940). Five strains of *Penicillum multicolor* G-M and P, used by Birkinshaw (1952) were obtained from Dr. KB. Raper of the Northern Regional Research Laboratory. More details are mentioned in "A Manual of the Penicillia" (Raper and Thom, 1949). *Penicillum hirayamae* was isolated from milled rice by (Udagawa, 1963) and *P. implicatum* Biourge was isolated from soil (Raper and Thom, 1949). *Penicillum sclerotiorum* X-11853 was isolated from the foam of a tropical forest stream (Pairet et al., 1995) and *Penicillum multicolor* F-1753 was isolated from soil (Nam et al., 2000a,b). Table 21 highlights many sclerotiorin and other azaphilone producing *Penicillium* spp.

Penicillium frequentans CFTRI A-24 produced sclerotiorin on potato dextrose broth (PDB), which contains only potato starch and dextrose as sole nutrients. As the yields of this inhibitor were low (0.10 to 0.15 g/L), experiments were conducted to optimize the nutrient sources such as carbon, nitrogen and mineral salts on sclerotiorin production. Therefore the current study was carried out to optimize physical and nutritional parameters for the optimum production of sclerotiorin and recovery.

Table 21: List of sclerotiorin and other azaphilone producing cultures

Culture name	Metabolites	Sclerotiorin Yields and Method of purification	Origin	Reference
P. sclerotiorum Van Beyma, CBS 287	Sclerotiorin, Sclerotiose and Rotiorin	0.1g/ g biomass Mycelium	Air	Curtin and Reilly 1940
P. multicolor G-M and P NRRL-764, 2058, 2059, 2060 and 2324	Sclerotiorin, Pincolide, Sclerotioramine	0.15 g/ g biomass Mycelium	Soil	Birkinshaw, 1952
P. hirayamae CMI 78255	(-) Sclerotiorin	Mycelium	Milled rice	Udagawa, 1963
P. implicatum Biourge,CBS-184, NRRL 2061	Sclerotiorin	Mycelium	Soil	Yamamoto, 1959
P. multicolor FO-2338, FERM P-13405	Sclerotiorin, Isochromophilone I and II	2.5 g/L Chromatography	Soil	Matsuzaki et al., 1995a; Tomoda et al., 1999
P. Sclerotiorum X-11853 CBS 123.94	Sclerotiorin,5Chloroisorotiorin, TL-1, TL-2, Ochrephilone, 5-deacetylsclerotiorin. 5-decloro analogs.	0.1 g/L Preparative HPLC	Foam of a tropical forest stream	Pairet et al., 1995
P. multicolor FO-3216 FERM P-14152	Isochromophilone III, IV, V and IV	-	-	Arai et al., 1995
P. multicolor F 1753	8-O-Methylsclerotiorinamine and Isochromophilone IV	39 mg/L, column chromatography	Soil	Nam et al., 2000a,b
Penicilium sp Accession no-MINAP9902	Isochromophilone VI and IX	-	-	Michael et al., 2003
Penicilium sp FO-4164	Sclerotiorin, Isochromophilone VI, VII and VIII, Ochrephilone,	380 mg/L, Prep HPLC	Soil	Yang et al., 1995
P. sclerotiorum BCRC	Sclerotiorin	5.16 mg/g biomass, Column chromatography	-	Weng et al., 2004

CBS-Central Bureau voor Schimmel cultures; **NRRL**-Northern Regional Research Laboratory; **CMI**-Commonwealth Mycological Institute; **FO**- National Institute of Bioscience and Human Technology; **FERM**- Agency of Industrial Science and Technology; **X**-Xenova Culture Collection; **BCRC**- Bio resources Collection and Research Center at Food Industry Research and Development Institute.

3.4.1. Media selection for sclerotiorin production:

Two approaches were adopted. One, the popularly used media for fungal growth was screened for sclerotiorin production. Second, media reported for the sclerotiorin and similar compounds were checked.

First experiments were carried out to see the production of sclerotiorin on other fungal media such as sucrose broth, modified sucrose broth (replaced with commercial sugar and salt), Czapek-dox broth, glycerol broth and malt extract broth were tested. PDB was used as control (Table 22)

Table 22: Production of sclerotiorin on general fungal media

Media	Crude wt (g/L)	Sclerotiorin (g/L)
Sucrose broth	0.95	0.328
Modified sucrose broth	1.03	0.296
Czapex-dox broth	0.79	<0.005
Glycerol broth	1.30	0.070
Malt extract broth	1.20	0.023
PDB	0.60	0.155

It was observed that, sucrose broth as well as modified sucrose broth gave the highest yield than the control PDB. Sclerotiorin production was not observed on Czapex-dox broth, which is an organic nitrogen free media and contains mineral salts with sucrose as a sole carbon. The possible other reason could also be the neutral pH of the media. Glycerol and malt extract broth did not show sclerotiorin with better yields either.

Sclerotiorin production was also tested on some of the other reported media, which were used for the production of sclerotiorin and other structurally similar isochromophilones (compositions as shown in section 2.4.3). Results of these experiments are as shown in Table-23.

Table 23. Production of sclerotiorin on various Production media used for the production of azaphilones

Media	Crude weight (g/L)	Sclerotiorin (g/L)	Reference
Production media –I	1.02	0.364	Matsuzaki et al., 1995a
Production media –II	1.38	0.673	Nam et al., 2000a
Production media –III	2.0	0.953	Arai et al., 1995
Production media –IV	1.27	0.330	Arai et al., 1995
PDB control	0.58	0.165	

Production media-I (Matsuzaki et al., 1995a), which contains sucrose and glucose along with organic nitrogen produced sclerotiorin with better yields than sucrose broth alone. Production media-II and III (Nam et al., 2000a), which contains glucose and starch as carbon source respectively, produced sclerotiorin with better yields of the entire media tested. Production media-IV (Arai et al., 1995), which contains glucose and glycerol with organic nitrogen has produced low levels of sclerotiorin, which is comparable with that of glycerol broth.

The results showed that sclerotiorin yields to be much better on reported production media than on general fungal media. Production media-II and III were the best of al media, which produced 0.673 and 0.953 g/L sclerotiorin respectively. Further, all production media had various combinations of organic nitrogen sources such as yeast extract, peptone, meat extract, soybean meal and corn steep liquor, and salts such as KH_2PO_4, $MgSO_4$ and NaCl/KCl. These results encouraged us to go further to look for a media, which could produce sclerotiorin with yields better than Production media III (0.95 g/L). Hence Production media III was selected for further optimization study.

3.4.2. Estimation of sclerotiorin in HPLC:

Different concentrations of pure sclerotiorin ranging from 5 to 25 µg were used to construct a standard curve by HPLC by plotting peak area versus sclerotiorin concentration (Figure 47). Based on the straight line equation derived from the standard curve sclerotiorin from unknown samples were estimated.

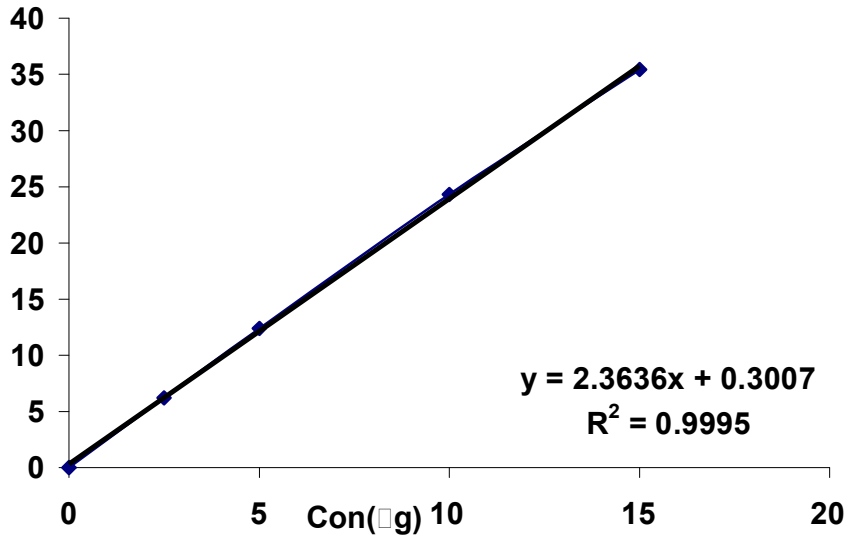


Figure 47. HPLC standard curve of sclerotiorin.

3.4.3. Optimization of physical parameters:

3.4.3.1. Time course fermentation for sclerotiorin production:

A time course of sclerotiorin production on submerged fermentation was carried out with Production media III. The production of sclerotiorin started after 48 hours, giving a maximum yield after 5 days. Biomass increased steadily till a maximum of 18 g/L (dry weight) at 96 hr after which there was no further increase. Maximum sclerotiorin of 0.93 g/L was observed at 120 hr after this inhibitor levels decreased (Figure 48). Based on this experiments, the time of fermentation was fixed at 5 days.

3.4.3.2. Effect of temperature on production of sclerotiorin:

Effect of incubation temperature on production of sclerotiorin was carried out with Production media III at different temperatures. Further increase of temperature till 40 ^{o}C and above decreased the production of sclerotiorin. Low temperatures did not favour the sclerotiorin production. Sclerotiorin production was higher at temperatures

between 26 to 30 ^{o}C. Sclerotiorin reached a maximum of 0.9 g/L at 30 ^{o}C (Figure 49). Subsequent optimization studies were carried out at this temperature.

3.4.3.3. Effect of initial pH on production of sclerotiorin:

Effect of initial pH on production of sclerotiorin was carried out with production media III, by changing the initial pH of production media from 2.8 to 9.0. It was seen that at low initial pH values of 2.8 there was no growth and subsequently no sclerotiorin production. Sclerotiorin formation was observed in flasks that had initial pH range from 4.0 to 6.0. above 7.0 did not to produce sclerotiorin. Maximum sclerotiorin yield of 0.9 g/L was observed at initial pH 6.0 (Figure 50). Therefore further subsequent optimization studies were carried out with initial pH 6.0.

3.4.3.4. Effect of aeration on production of sclerotiorin:

The effect of aeration on the production of sclerotiorin was studied at shake flask levels by varying the ratio of volume of media to flask volume from 0.1 to 0.4. This was studied by keeping the flask volume constant and varying media from 50 ml to 200 ml. This was carried out on Production media III, with initial pH 6, incubation at 30 ^{o}C and 5 days fermentation. Sclerotiorin production increased gradually as the volume: flask ratio increased (Figure 51). Sclerotiorin yield was maximum 0.9 g/L when the volume: flask ratio was 0.2 and at ratio above this sclerotiorin yields dropped.

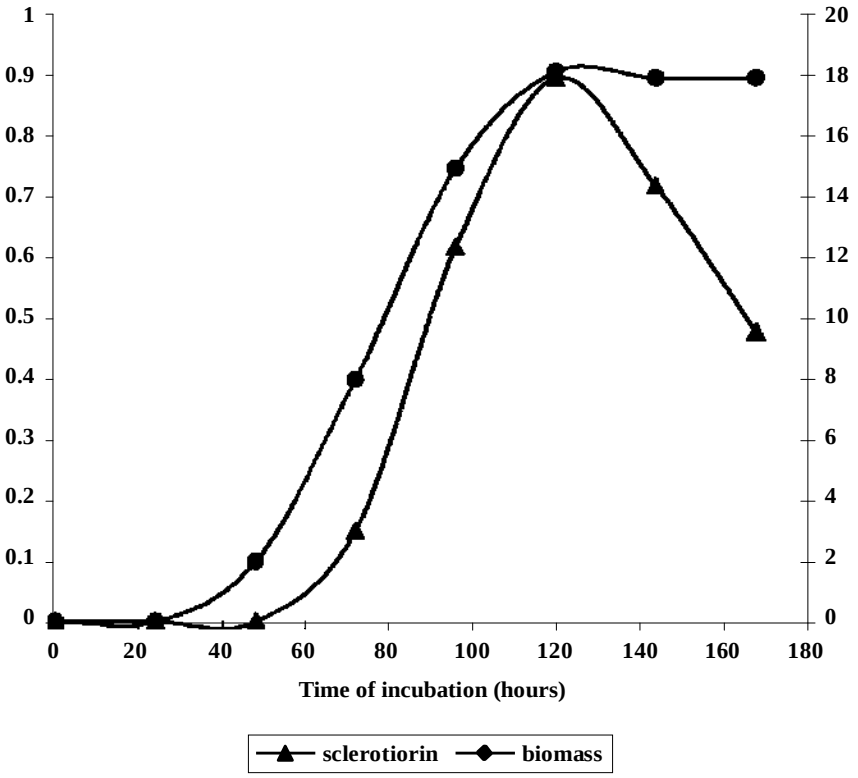


Figure 48. Time course fermentation of sclerotiorin production

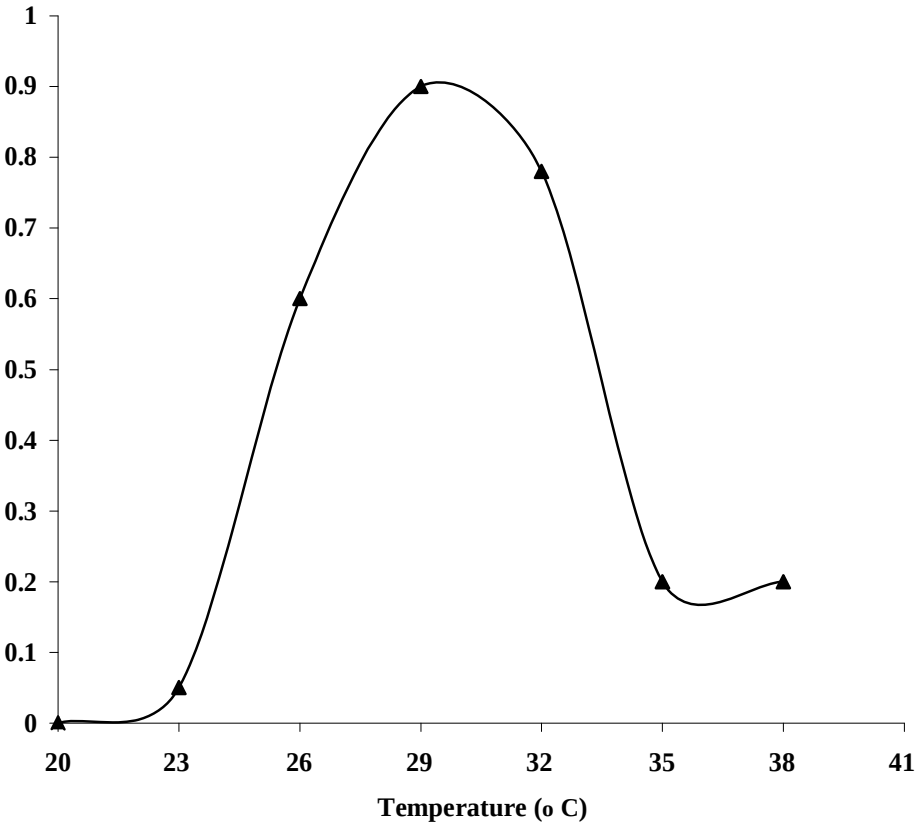


Figure 49. Effect of temperature on sclerotiorin production

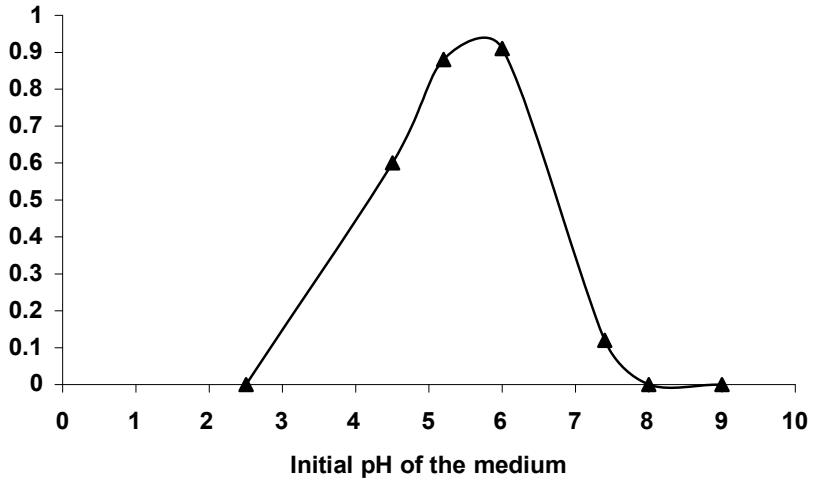


Figure 50. Effect of initial pH on sclerotiorin production

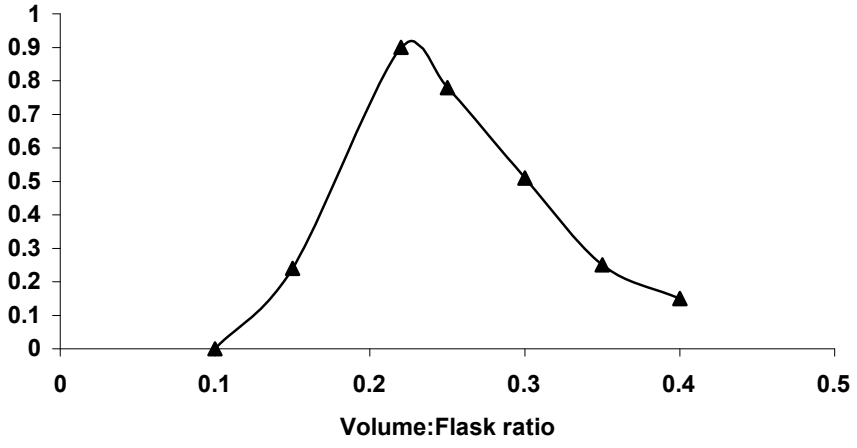


Figure 51. Effect of aeration on production sclerotiorin

3.4.4. Optimization of nutritional parameters for the production of sclerotiorin:

3.4.4.1. Effect of different carbon sources on sclerotiorin production:

Penicillium has a characteristic radial outward growth from the site of inoculum and sclerotiorin production is typically characterized by pigmentation on the reverse side of the culture plate. This concept was adopted for screening of various carbon sources for the production of sclerotiorin. The radial outward growth was used as an indicative measure for the growth of the organism. The intensity of pigmentation on the reverse side of the plate was qualitatively visualized as an indicative measure of sclerotiorin produced by the culture (Figure 52). 5 µl from spore suspension was point inoculated at the center of each plate containing different carbon sources. The radial size of the colony and pigmentation on the reverse side of the plates were recorded at every 24 hr (Table 24 and 25).

Various carbohydrates were incorporated at 2% (w/v) instead of starch from the Production media III. All other ingredients were kept constant potato dextrose agar (PDA) and Production media III were used as control plate.

Table 24: Radial growth measurement (in cm) of *P. frequentans* on different carbon media

Carbon (2% w/v)	24hr	48hr	72hr	96hr	120hr	144hr
Glucose	-	1.7	2.4	3.2	3.7	4.5
Sucrose	-	1.7	2.3	2.8	3.0	3.6
Fructose	-	1.8	2.3	2.8	3.1	3.6
Galactose	-	1.1	1.6	2.1	2.6	3.1
Mannose	-	1.5	2.0	2.6	3.6	4
Maltose	-	1.1	1.6	2.1	2.7	3.2
Xylose	-	2.1	2.3	3.1	3.2	3.5
Cellobiose	-	1.5	2,2	2.6	3.1	3.6
Lactose	-	1.1	1.6	2.1	2.6	3.4
PDA	-	1.5	2.0	2.6	3.6	4
Production Media III	-	2.1	2.4	3.1	3.8	4.8

Table 25. Pigmentation observed on the reverse side of the plates on different carbon media

Carbon (2% w/v)	24hr	48hr	72hr	96hr	120hr	144hr
Glucose	-	+	++	++++	++++	5+
Sucrose	-	++	+++	+++	++++	++++
Fructose	-	+	++	+++	+++	++++
Galactose	-	+	+	++	+++	+++
Mannose	-	+	++	++	+++	+++
Maltose	-	+	++	++	+++	+++
Xylose	-	+	+	++	+++	++++
Cellobiose	-	-ve	+	++	+++	+++
Lactose	-	-ve	+	+	++	++
PDA	-	+	+	++	+++	+++
Production Media III	-	++	+++	++++	++++	5+

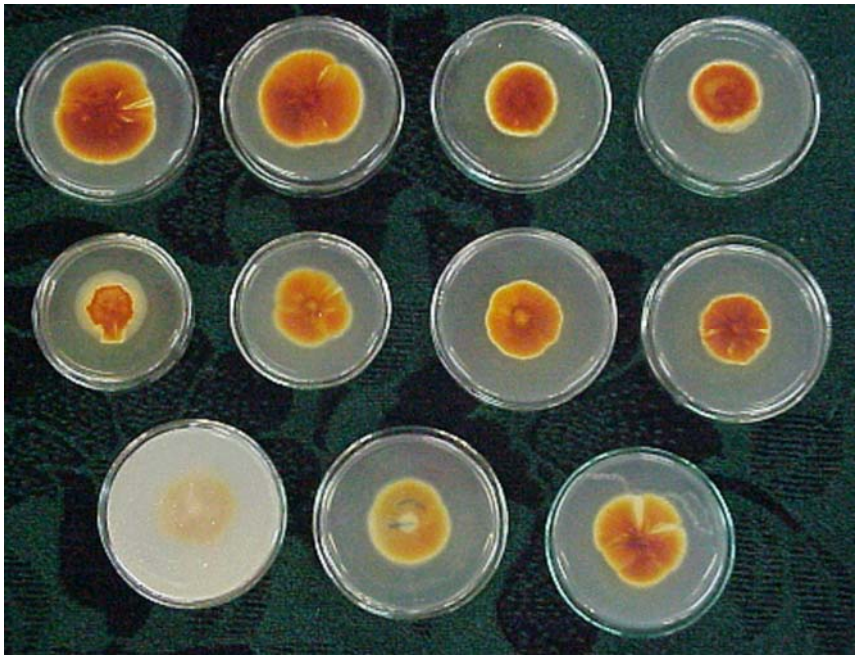


Figure 52. Radial outgrowth and pigmentation on reverse side of the plates Description as (from left to right);

Starch (Production Media III), Glucose, Sucrose, Fructose

Galactose, Mannose, Maltose, Xylose

Cellobiose, Lactose, PDA

The results clearly indicated that among the carbon sources tested, glucose showed highest radial outward growth of 4.5 cm and also showed highest level of pigmentation. Glucose plate visualization was very close to Production media III. Though sucrose and fructose also had fairly higher levels of pigmentation, but was not up to the extent of glucose. Therefore these results were validated in shake flask experiments (Table 26). PDB and Production media III were used as control.

Table 26. Validation of rapid qualitative carbon selection test

Carbon (2% w/v)	Crude weight (g/L)	Sclerotiorin (g/L)
Glucose	1.32	0.68
Sucrose	0.98	0.33
Fructose	0.97	0.36
Galactose	0.52	0.24
Mannose	0.58	0.15
Maltose	0.62	0.18
Xylose	0.48	0.29
Cellobiose	0.63	0.28
Lactose	0.33	0.09
Starch	2.1	0.93
Production Media III (control)		
PDB (control)	0.53	0.155

Starch (control; Production media III) showed the maximum yields (0.93 g/L) than glucose (0.68 g/L), sucrose (0.33 g/L), and fructose (0.36 g/L) (Table 26). Glucose showed maximum yields of sclerotiorin production when compared to all other sugars.

3.4.4.2. Effect of different nitrogen sources on sclerotiorin production

Various organic and inorganic nitrogen sources were incorporated at 0.4% in place of yeast extract from the Production media III (Table 27). All other ingredients than nitrogen were kept constant. PDA and Production media III was used as control plate.

Table 27. Effect of different nitrogen supplements on sclerotiorin production

Nitrogen supplement (0.2% w/v)	Sclerotiorin yield (g/L)
Production media III (control)	0.94
PDB (control)	0.16
Organic nitrogen sources	
Peptone	1.09
Yeast extract	0.94
Malt extract	0.68
Beef extract	0.54
Soybean meal	0.33
Corn steep liquor	0.36
Inorganic nitrogen sources	
Ammonium chloride	0.12
Ammonium nitrite	0.11
Ammonium sulfate	0.12
Sodium nitrate	0.18
Diammonium hydrogen Ortho phosphate	0.02
Urea	0.07

The results indicated that nitrogen replacements of both organic and inorganic forms did not bring much improvement in the yields when compared to Production media III only peptone, showed slightly higher yields (1.09 g/L). However the organic nitrogen contents showed better yields when compared to inorganic nitrogen sources.

All the ammonium salts incorporated media had very low sclerotiorin levels and had turned red in color. In an earlier investigation, Sclerotioramine ($C_{21}H_{24}O_4NCl$) an amino product of sclerotiorin has been reported to be a natural product isolated from cultures fluids of *Penicillium multicolor,* which had been incubated for a longer than normal time period. However, as sclerotiorin is known to be very susceptible to amination and as *P. multicolor* is a known producer of ammonia in old cultures sclerotioramine was more likely a result of the action of ammonia on sclerotiorin rather than a direct product of the organism (Eade et al., 1957).

3.4.4.3. Effect of inorganic salts for sclerotiorin production:

Production media III contains KH_2PO_4 and $MgSO_4$ as inorganic nitrogen constituents in it. The individual effect of these salts was evaluated by removing one salt at a time.

Table 28. Effect of removal of inorganic salts on sclerotiorin production

Production media III with out	Sclerotiorin yield (g/L)
KH_2PO_4	0.53
$MgSO_4$	0.62
Production media III	0.93
PDB control	0.15

Results showed that removing of inorganic salts KH_2PO_4 and $MgSO_4$ reduced the sclerotiorin yields from 0.9 g/L to 0.53 and 0.62 respectively (Table 28). The results indicated that both KH_2PO_4 and $MgSO_4$ were required for the production of sclerotiorin.

Hence the next experiments were conducted by varying the individual concentrations from 0.1 to 1.5 g/L. The optimum concentration of KH_2PO_4 and $MgSO_4$ were found to be 1 and 0.5 g/L respectively. The results showed that at these concentrations highest sclerotiorin values of 0.92 and 0.93 g/L respectively were observed (Figure 53).

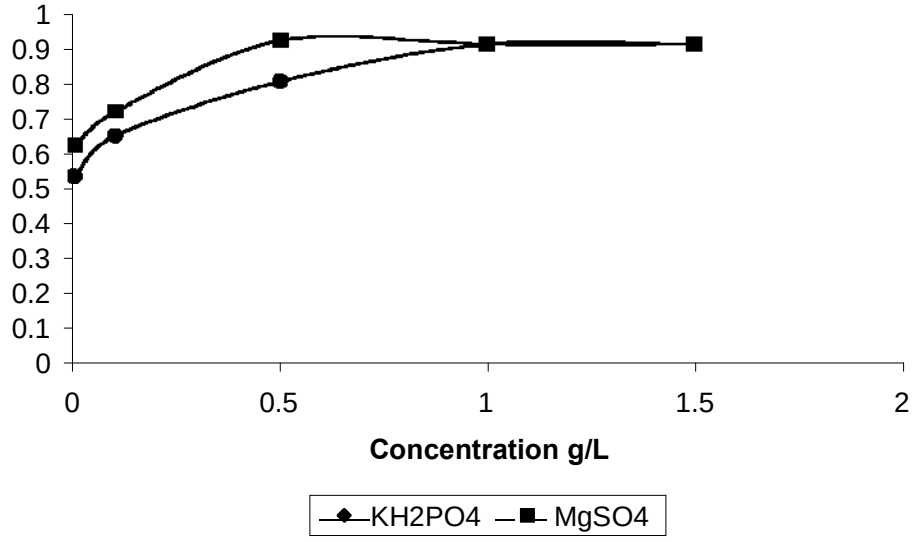


Figure 53. Effect of inorganic salts KH_2PO_4 and $MgSO_4$ on sclerotiorin production.

Literature reports on sclerotiorin production have suggested that, halides and nitrates do not affect the production of sclerotiorin but do so on production of other metabolites like sclerotiose, rotiorin and pincolide (Albericci et al., 1942;Jackman et al., 1958;Birkinshaw et al., 1963).

Growth of *P. sclerotiorum* on media containing KBr and KI in place of the usual KCl did not yield halide derivatives analogous to sclerotiorin, Increased chloride concentration had no effect on the production of sclerotiorin and only affected the chlorine content of the other products (Reilly and Curtin, 1943). The KCl metabolism of *Penicillium sclerotiorum* is an inverse function of the nitrate concentration of the culture media. The production of sclerotiose was almost inversely proportional to the nitrate content of the media (Reilly et al., 1944).

However there is an ambiguity whether halides are essential in halometabolite biosynthesis. Some of the study support that halides are needed for the production of halometabolite. Griseofulvin, was obtained when *Penicillium brefeldianum* was grown on Czapek-Dox, a chloride-containing media; but instead of griseofulvin an unrelated and non halogenated substance, fulvic acid, was obtained when this species was grown on a Raulin-Thom media which does not contain deliberately added chloride (Dean et al., 1957).

In order to see the effect of chloride content on sclerotiorin production different inorganic salts such as sodium chloride, potassium chloride, magnesium chloride and calcium chloride were incorporated at a concentration of 0.1% (w/v) to production media III the results indicated that there was no effect of any of these salts on sclerotiorin yields (Table 29)

Table 29. Effect of chlorides on sclerotiorin production

Production media III With (0.05%)	**Sclerotiorin yield (g/L)**
Sodium chloride	0.99
Potassium chloride	0.92
Magnesium chloride	0.92
Calcium chloride	0.91
Production media III	0.96
PDB control	0.15

3.4.5. Final media design for the production of sclerotiorin from *P. frequentans*:

From the results shown in Table-26 and Table-27, it was observed that glucose gave the best yield of 0.68 g/L and peptone showed highest yields of sclerotiorin production (1.09 g/L).

These results indicated that if glucose, starch and peptone are incorporated together in to Production media III, yields of sclerotiorin could be enhanced. Hence the next experiments were conducted by incorporating varying concentration of individual

components to Production media III. Glucose concentration was varied from 5 to 50 g/ L (Figure 54) and peptone from 1 to 10 g/L (Figure 55). The results indicated that sclerotiorin yields from an initial 0.95 g/L was enhanced to 1.74 g/L by the incorporation of optimum glucose concentration at 20 g/L. Concentrations above 20 g/L had no effect on sclerotiorin production. Similarly peptone increased the yield from an initial 0.95 g/L to 1.15 g/L at optimum peptone concentration of 5 g/L.

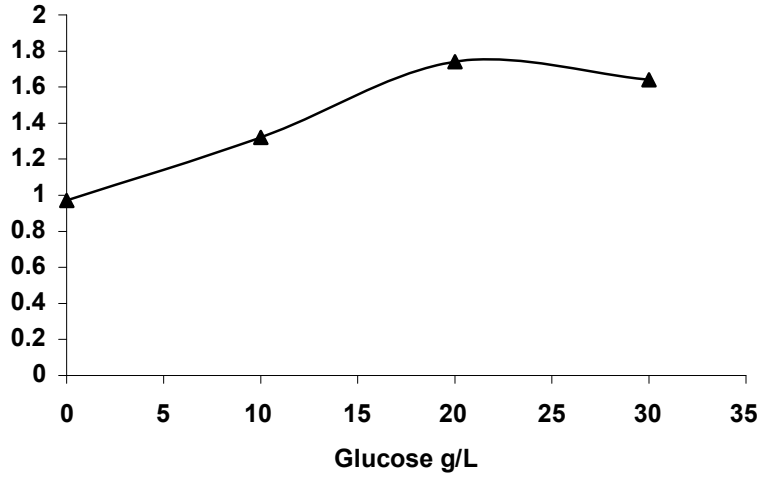


Figure 54. Effect of glucose addition to production media III

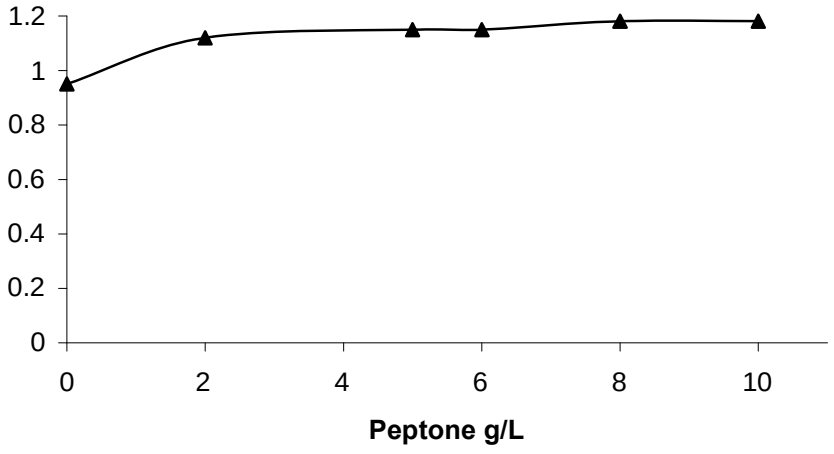


Figure 55. Effect of peptone addition to Production media III

3.4.6. Final optimized media for the sclerotiorin production:

Since both glucose and peptone incorporations increased sclerotiorin yields these two components were added to Production media III at their optimum concentrations. Based on these results sclerotiorin production from *P. frequentans* was optimized with the following media ingredients.

Final medium for the production sclerotiorin

Starch	20 gm
Glucose	20 gm
Yeast extract	4 gm
Peptone	5 gm
KH_2PO_4	1.0 gm
$MgSO_4$	0.5 gm
NaCl	0.5 gm
Agar	1 gm
Distilled water	1000 ml
pH	6.0

3.4.7. Optimization of down stream process conditions for sclerotiorin recovery:

In the next phase of optimization studies downstream processing techniques for isolation and purification of sclerotiorin from the fermented broths of *P. frequentans* were carried out. All experiments were carried out on Production media III.

3.4.7.1. Choice of the extraction solvent:

The effect of different aqueous and organic solvents was studied on the extraction of sclerotiorin from both biomass and fermented broth. Both polar and non-polar solvents were tried for the effective recovery of sclerotiorin. Among the organic solvents tried, ethyl acetate gave maximum recovery of sclerotiorin from whole broth followed by chloroform, Acetone, diethyl ether and methanol. From biomass extraction,

acetone gave maximum recovery of sclerotiorin followed by methanol, ethyl acetate, ethanol and chloroform (Table 30).

Table 30: Selection of extraction solvent for effective recovery of sclerotiorin

Solvents	Sclerotiorin extracted from whole broth (g/L)	Sclerotiorin extracted from dry biomass g/ g biomass
Ethyl acetate	0.96	0.039
Chloroform	0.83	0.029
Acetone	0.81	0.045
Methanol	0.59	0.040
Diethyl Ether	0.73	0.018
Ethanol	0.4	0.038

Different extraction conditions were tested for the maximum recovery of sclerotiorin (Table 31). Whole broth extraction after the end of fermentation gave the maximum yields than only broth (supernatant) extraction. Acidic pH extraction of biomass gave more sclerotiorin, but whole broth extraction after the end of fermentation gave better levels of sclerotiorin than only biomass extraction. This could be because the broth after fermentation was acidic.

Table 31: Different extraction conditions for sclerotiorin recovery

Parameters	Sclerotiorin content g/L
Whole broth extraction	0.94
Only broth extraction	0.12
Wet biomass extraction	0.65
Dry biomass extraction	0.78
Acidic pH biomass extraction	0.89
Neutral pH biomass extraction	0.45
Alkaline pH biomass extraction	0.22

3.4.7.2. Effect of pH on the extraction of sclerotiorin:

To determine if the pH of fermented broth affected the extractability of sclerotiorin, the pH of fermented broth was varied from 2.5 to 8.0 using dilute hydrochloric acid or dilute alkali (Figure 56). Sclerotiorin extraction was maximum at acidic pH range between 2.5 to 4.0 reaching a maximum of 0.92 to 0.93 g/L. A further increase in pH from 5.0 to 8.0 and above, the sclerotiorin extractability declined. These results indicated that, the pH of the fermented broth in the range of 2.5 to 4.0 was found to be the ideal pH for the extractability of sclerotiorin.

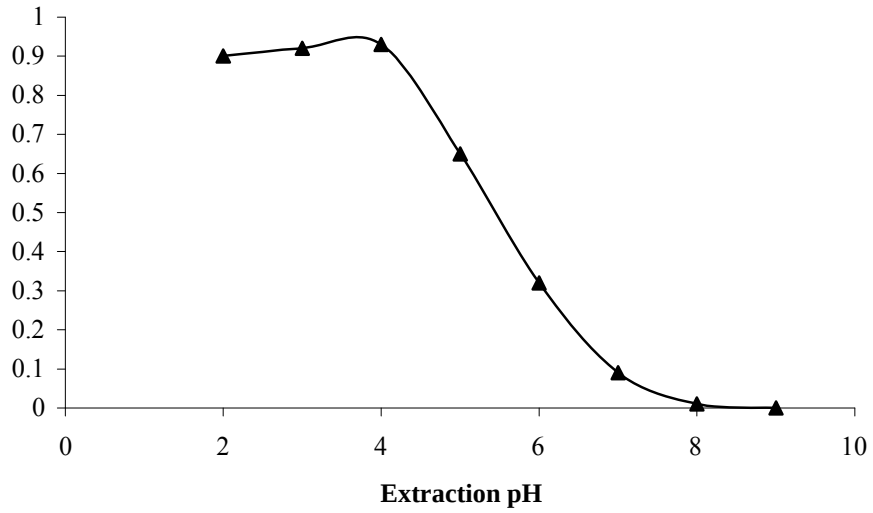


Figure 56. Effect of pH during extraction of sclerotiorin from the fermented broth

3.4.7.3. Ratio of fermented broth to solvent:

The effect of fermented broth to solvent ratio on extraction efficiency of sclerotiorin was followed by varying the amount of ethyl acetate added to fermented broth (Figure 57). When the broth to solvent ratio was increased from 1:0.5 to 1:2.5 there was an initial low extraction of sclerotiorin, which increased dramatically from 0.56 to 0.96 g/L. Broth to solvent ratio above 1:1.5 did not increase sclerotiorin extraction. These results indicate that broth to ethyl acetate ratio of 1:1.5 to be ideal for efficient sclerotiorin extraction.

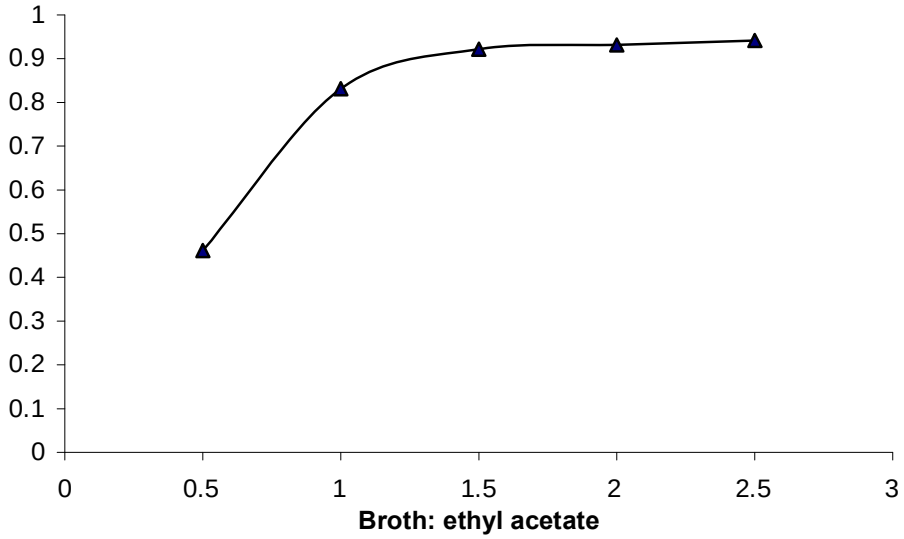


Figure 57. Effect of broth to ethyl acetate ratio on sclerotiorin extraction.

3.4.7.4. Effect of static and agitated conditions during extraction:

The extent of recovery of sclerotiorin was studied at broth to ethyl acetate ratio of 1:1.5(v/v) in conical flasks under static and agitated conditions at 30 ^{o}C. The agitation of the fermented broth with the solvent was carried out by incubating the flasks on a rotary shaker at 250 rpm. Under static conditions, a maximum of 0.22 g/L was extracted after 90 min of extraction. Beyond this time, sclerotiorin concentration in the extract remained constant (Figure 58). Further, it was observed that under agitated conditions, the rate of extraction of sclerotiorin increased gradually till 120 min (0.96 g/L). As compared to static extraction, for a fixed time, the agitated mode of extraction gave much higher yields.

3.4.7.5. Repeated extraction of biomass:

Repeated extraction is generally applied to remove the traces of solute that is left on the leached solids after the first extraction. This technique was generally used for extraction of enzymes such as amylases or pectinases from the fermented bran of *Aspergillus* sp (Singh et al., 1999; Singh and Soni, 2001). The advantage of this method is to improve product recovery.

After the whole broth extraction the remaining biomass (vacuum dried) was extracted with acetone on a rotary shaker (1:20, w/v) incubated at 30 ^{o}C for 120 minutes. At the end of one extraction, the solvent was removed and fresh solvent was added to the same broth. Four such cycles were carried out to study the efficiency of repeated extraction, which was a function of the number of cycles needed to achieve maximum. The results indicated that sclerotiorin was leached out of the biomass completely with four cycles of acetone extractions. A total sclerotiorin of 0.026 g /g biomass was achieved (Table 32).

Table 32: Repeated extraction of biomass for sclerotiorin

Extraction cycles	Sclerotiorin (g/g biomass)
I	0.015
II	0.008
III	0.002
IV	0.001
Total	0.026

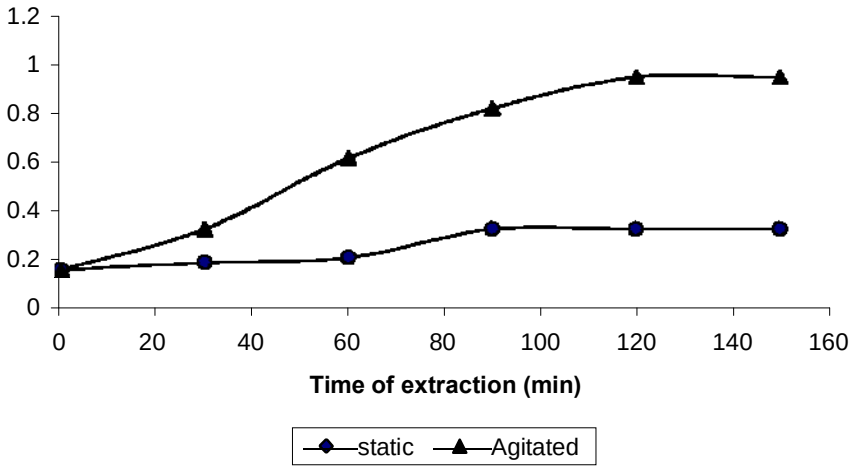


Figure 58. Effect of the extraction time of sclerotiorin on static and agitated conditions

3.4.8. Validation of the optimized medium:

Validation of the optimized media was done on both in shake flask as well as on fermentor.

3.4.8.1. Shake flask study:

Production of sclerotiorin was carried out on a 3 L optimized media. Each 500 ml Erlenmeyer flasks were dispersed with 100 ml of medium. Fermentation was carried out for 5 days with 200 rpm at 30 ^{o}C. After the end of 5 days 150 ml of ethylacetate was added to each flask to extract the crude compound. The results indicated that biomass achieved a maximum level of 22.45 g/L at 96 hours (dry weight) and further there was no increase of biomass weight. Sclerotiorin production achieved a maximum of 2.01 g/L at 120 hours. Production of sclerotiorin started at 48 hours of fermentation. Initial pH 6.0 was dropped to 4.9 with in 24 hours and later to 3.2 till end of fermentation. The gradual drop in the pH was an indication of the organism's growth (Figure 59).

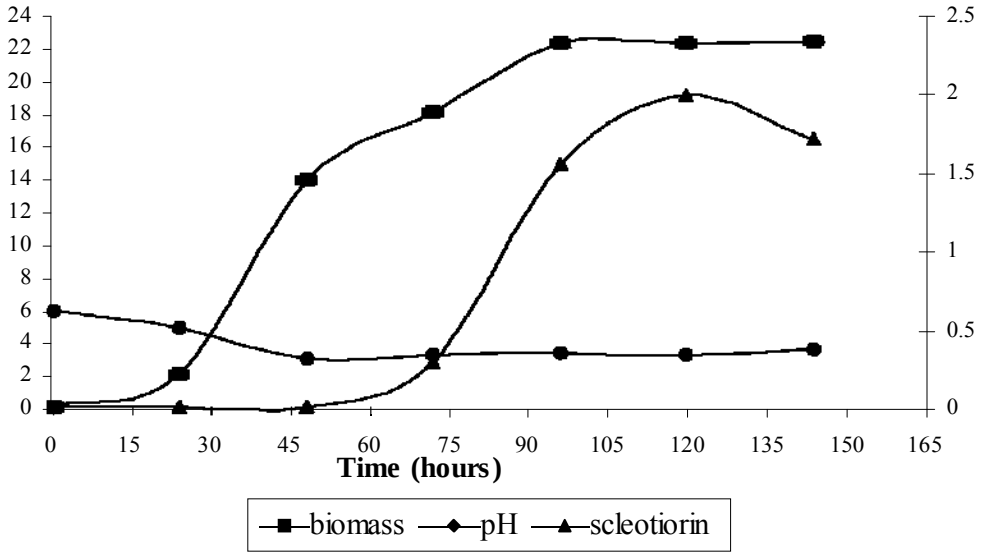


Figure 59. Production of sclerotiorin from *P. frequentans* with optimum conditions in shake flask

Sclerotiorin yields as well as biomass weights were increased on final production media, when the results were compared with that of initial PDB (0.15 g/L sclerotiorin) and Production media III (0.95 g/L sclerotiorin). There was a significant enhancement in sclerotiorin yields on the final optimized media (2.01 g/L) an increase of 13.4 fold over PDB media and 2.1 fold increase over Production media III (Table 33).

3.4.8.2. Fermentor study:

Production of sclerotiorin was carried out on a 10 L fermentor (NBS, USA). 6L of optimized media was inoculated with 2L of 2 day old seed culture media (containing same media compositions), to bring the final volume to 8 L. Aeration was maintained at 1 vvm with 250 rpm at 30 ^{o}C. pH was monitored manually at 3.2 after 24 hr and the initial DO was set at 100%. The results indicated that, the maximum biomass 28.6 g/L was achieved at 72 hours and a maximum sclerotiorin yield of 2.56 g/L was found at 120 hours. pH was dropped to 3.2 in less than 24 hr of fermentation and there after it was monitored manually at 3.2 till the end of fermentation. The fall in pH pattern indicated the utilization of carbohydrate by organism. The fall of DO pattern also indicated the growth of the organism. Sclerotiorin formation was maximum after the complete exertion of carbohydrate 120 hr (Figure 60 and 61).

When the fermentor results were compared with that of shake flask results the yields in terms of sclerotiorin as well as biomass weight were much higher. The probable reason for better productivity over shake flask could be due to the regulation of pH, temperature, DO (Table 34).

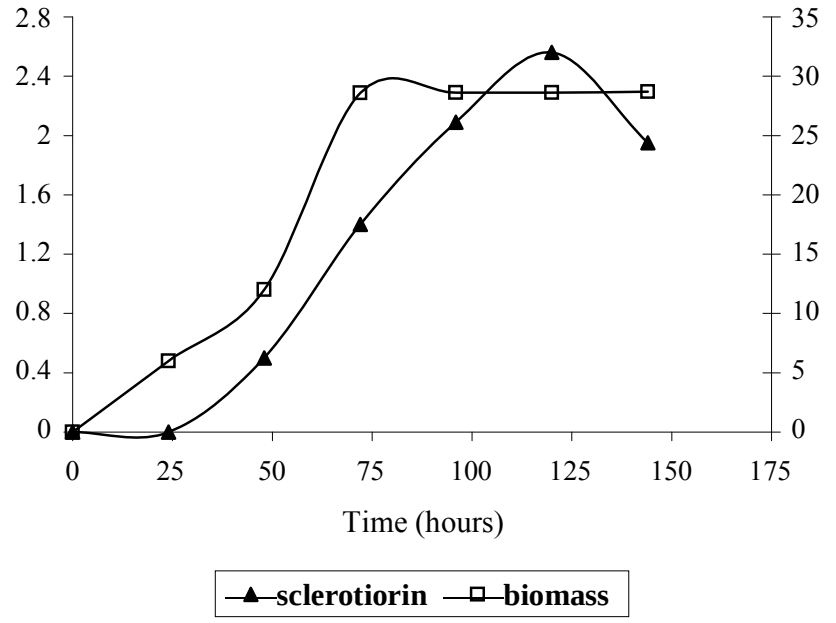


Figure 60. Sclerotiorin and biomass production in a fermentor

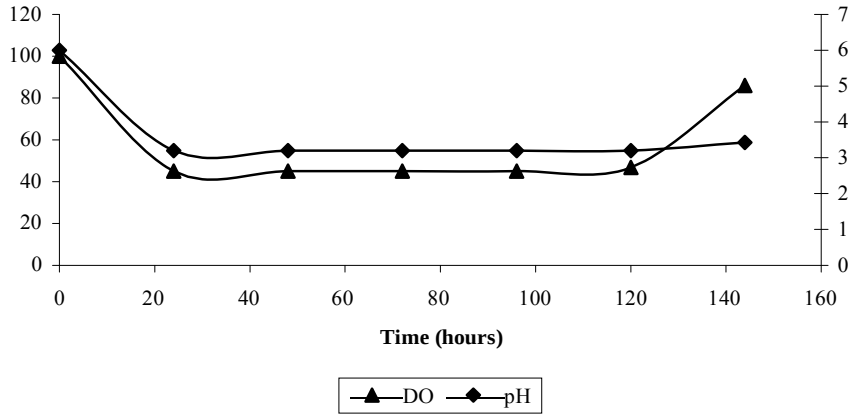


Figure 61. Dissolved oxygen and pH trends during *P. frequentans* fermentation

Table 33: Comparison of biomass and sclerotiorin yields before and after media optimization studies on shake flask

Parameters	PDB	Production media III	Optimized media
Biomass (g/L)	10	18.2	22.45
Sclerotiorin (g/L)	0.15	0.95	2.01
Time taken to reach maximum sclerotiorin concentration (hours)	120	120	120

Table 34: Comparison of shake flask and fermentor studies

Parameters	Shake flask fermentation	Fermentor
Biomass (g/L)	22.45	28.6
Sclerotiorin (g/L)	2.01	2.56
Time taken to reach maximum sclerotiorin concentration (hours)	120	120

SECTION–5

SAFETY STUDIES, *IN VITRO* AND *IN VIVO* EVALUATION OF INHIBITOR

3.5. Antioxidant potential of sclerotiorin from *Penicillium frequentans* A-24:

Antioxidants reduce or delay the rancidity of fats and oils in foods; synthetic antioxidants such as BHA and BHT are effectively used as food additives to protect from rancidity. Apart from their beneficial effect many of these synthetic commercial antioxidants have limitations associated with their use because of their possible toxic effect. These compounds may cause liver swelling and adversely influence liver enzymes (Halladay et al., 1980)**.** Hence there is a need for antioxidants from natural sources as alternatives.

Sclerotiorin effectively inhibited soybean LOX as an uncompetitive, reversible inhibitor with an IC_{50} value 4.2 μM by its antioxidant mechanism and also 5-LOX with an IC_{50} value 16 μM. sclerotiorin exhibited potent free radical scavenging property in DPPH assay system with IC_{50} value of 0.12 μM. Sclerotiorin inhibited lipid peroxidation in Fe^{2+}- ascorbate induced lipid peroxidation with a IC_{50} value 64 μM. Sclerotiorin showed lipid peroxidation inhibition in both enzymatic and non-enzymatic systems.

With this background, in the current study we evaluated the possible antioxidant effect of sclerotiorin in both *in vitro* and *in vivo* models**.**

3.5.1. *In vitro* antioxidant potency of sclerotiorin:

In vitro antioxidant activity of sclerotiorin was tested on four oil model systems in both emulsions as well as in alcohol suspension models. Linoleic acid (a standard fatty acid) and groundnut oil were used as test oil samples. The antioxidant effects were compared with that of BHT and Vitamin-E. An initial experiment showed that sclerotiorin at 50-300 ppm and above was imparting intense color to the medium, which is mostly not desirable in antioxidant applications. Therefore sclerotiorin concentration was fixed to 50 ppm The rate of oxidation in the experimental samples were measured by recording the absorption at 234 nm of the conjugated dienes generated at regular intervals.

3.5.1.1. Linoleic acid emulsion model:

Results indicated that in linoleic acid emulsion model the presence of sclerotiorin and vitamin-E delayed the onset of linoleic acid oxidation by 3 days respectively at a concentration of 50 µg/ml (50 ppm) and showed 30% protection at the end of 13 days, whereas the control samples reached the maximum absorbance. BHT delayed the onset of oxidation even after 14 days of incubation and showed 60% protection at a similar concentration (Cillard et al., 1980)(Figure 62 A).

3.5.1.2. Linoleic acid alcohol suspension model:

In alcohol suspension model, sclerotiorin and Vitamin-E delayed the onset of linoleic acid oxidation by 3 days at a concentration of 50 µg/ml. Sclerotiorin showed 37% protection as compared to Vitamin-E which showed 33% protection at the end of 21st day, whereas BHT did not show the onset of oxidation even after 21st days of incubation and showed 77% protection at the end of 21st days of incubation (Yagi 1984) (Figure 62 B).

3.5.1.3. Groundnut oil emulsion model:

In the emulsion model the rate of oxidation was delayed by 3 days by both sclerotiorin and vitamin-E. At the end of 24 days of incubation, Vitamin-E and sclerotiorin both showed 44% inhibition at a concentration of 50 µg/ml. BHT showed no initiation in oxidation and showed a protection of 76% at the end of 24 days, where the control showed a maximum absorbance (Cillard et al., 1980)(Figure 63 A).

3.5.1.4. Groundnut oil suspension model:

In groundnut oil-alcohol suspension model, Vitamin-E and sclerotiorin delayed the onset of linoleic acid oxidation by 3 days and 2 days respectively at a concentration of 50 µg/ml. Both vitamin-E and sclerotiorin showed 30 and 20% protection respectively at the end of 25th day. BHT did not show the onset of oxidation even after 25 days of incubation and showed 60% protection at the end of 25 days of incubations (Yagi 1984) (Figure 63 B).

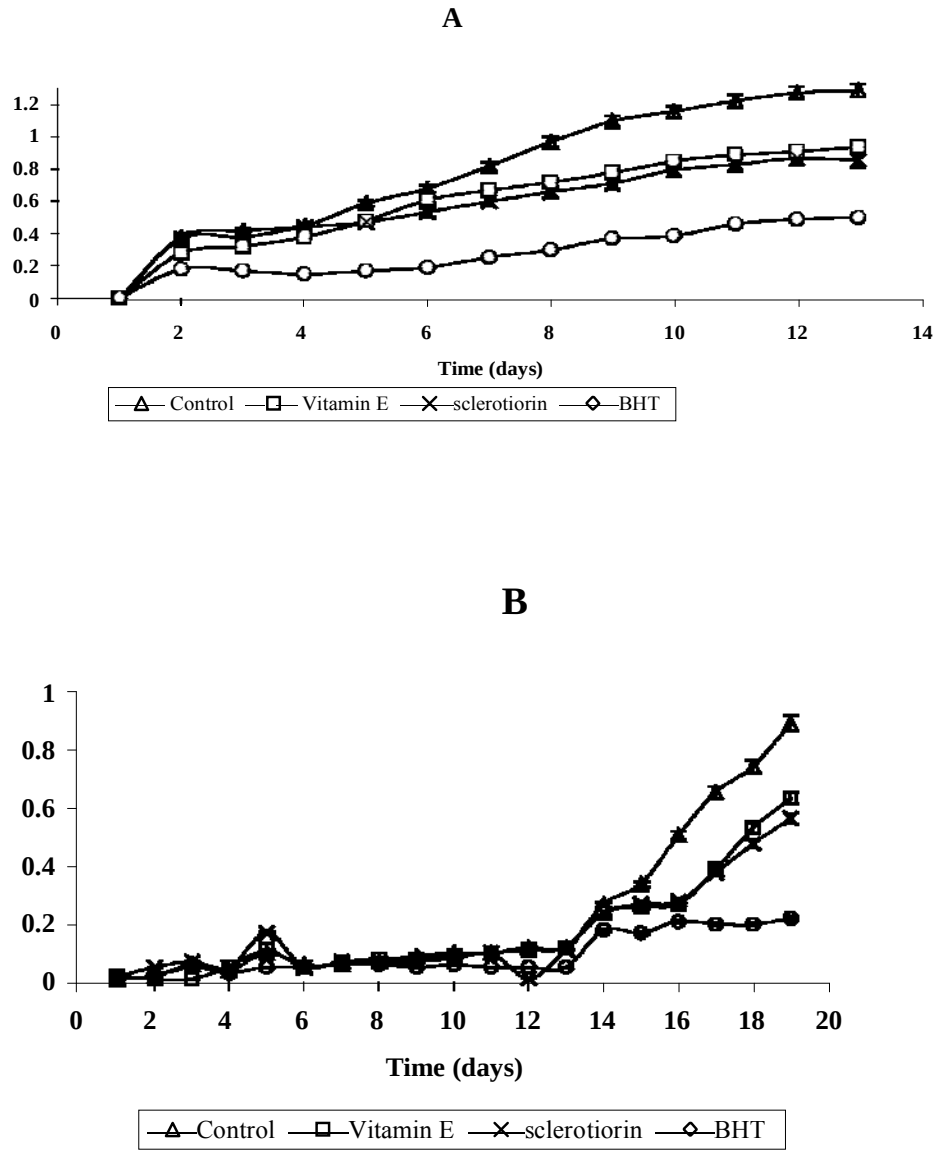
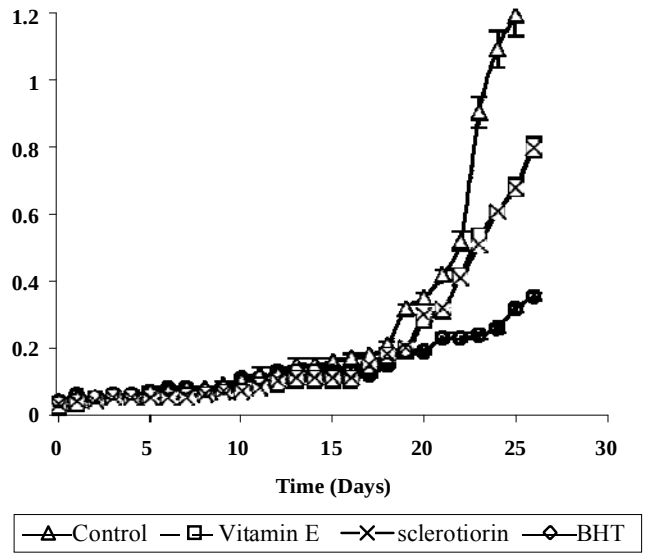


Figure 62. Conjugated diene formation in (A) linoleic acid emulsion (B) linoleic acid suspension

A



B

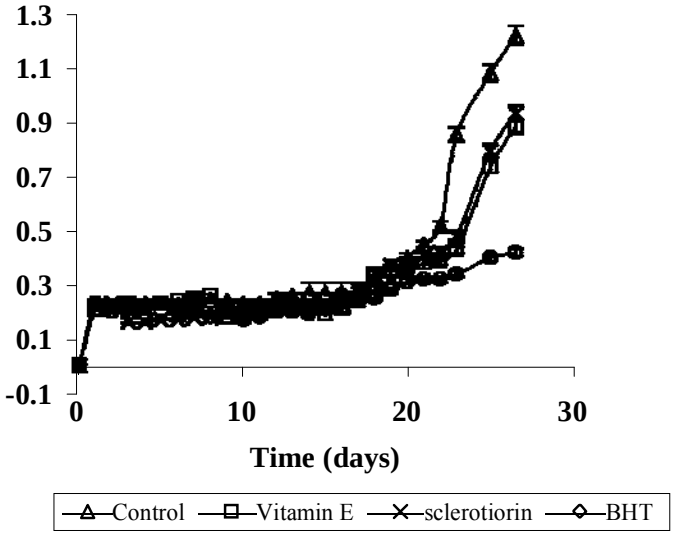


Figure 63. Conjugated diene formation in (A) groundnut oil emulsion (B) groundnut oil suspension

3.5.1.5. Estimation of Antioxidant potential of sclerotiorin with Rancimat method:

Oxidation of fats is enhanced by oxygen and temperature leading to the formation of free volatile acids, which affect consistency and the taste of a product or in other words bring rancidity. This process can be estimated rapidly with the help of Rancimat, which exposes the sample to elevated temperatures while pumping air into it. To study the antioxidant potential of sclerotiorin a commercial Saffola kardi oil was used.

Sclerotiorin antioxidant effect was estimated by incubating the compound at 120 ^{o}C with Saffola kardi oil (virgin) at different time intervals. The ability of compound to withstand or delay the onset of oxidation was studied on a Rancimat. Three concentrations (50,100 and 300 ppm) were tested. The effect was compared with standard Tertiary Butyl Hydroquinone (TBHQ) at similar concentrations (Table 35).

Table 35: Sclerotiorin antioxidant activity in comparison with standard TBHQ

Concentration of antioxidant	**Virgin Oil**		**TBHQ**		**Sclerotiorin**	
Virgin Oil	1.26h	1.12h	N.A	N.A	N.A	N.A
50 ppm	N.A		1.54 h	1.60 h	1.34 h	1.20 h
100 ppm	N.A		2.79 h	2.42 h	1.47 h	1.26 h
300 ppm	N.A		4.86 h	4.60 h	1.43 h	1.50 h

The Table shows that with increasing concentrations of TBHQ, the ability to withstand rancidity was more, whereas increasing sclerotiorin concentration hardly had any effect up to 300 ppm. Efficacy of 50 ppm TBHQ is more or less equivalent to 300 ppm of sclerotiorin. Unfortunately sclerotiorin at 50 ppm imparted golden yellow colour to the oil, while TBHQ at even 300 ppm did not impact the natural color of the oil.

3.5.2. *In vivo* antioxidant potency of sclerotiorin:

The antioxidant effect of orally administered sclerotiorin in carbon tetrachloride (CCl_4) induced oxidative stress was studied in experimental mice. Carbon tetrachloride

mediated hepatotoxicity was also examined in this study for a possible hepatoprotective effect by sclerotiorin.

3.5.2.1. Sclerotiorin effect on serum and hepatic antioxidant molecules:

The results indicated that, hepatic reduced glutathione concentration was significantly depleted as a result of carbon tetrachloride administration, was restored by sclerotiorin treatment, especially at 25mg/kg and 50mg/kg dose. Circulatory glutathione level was similarly beneficially modulated by sclerotiorin administration. The effect of the test compound at 25mg/kg and 50mg/kg was even better than the beneficial effect exerted by exogenous vitamin-E at similar dose. Hepatic total thiols content was similarly increased by administration of sclerotiorin at 25mg/kg and 50mg/kg dose (Table 36 and 37).

3.5.2.2. Sclerotiorin effect on serum and hepatic antioxidant enzymes:

Results indicated that among the antioxidant enzymes, activities of catalase and glutathione peroxidase in liver were significantly elevated by sclerotiorin dosage (25 mg/kg). The hepatic antioxidant enzymes in sclerotiorin administered mice are presented in (Table 38).

3.5.2.3. Sclerotiorin effect on plasma non-specific enzymes:

Activities of aspartate amino-transferase (SGPT), alanine aminotransferase (SGOT) and alkaline phosphatase were measured in serum samples of experimental mice groups (Table 39). As expected, carbon tetrachloride administration resulted in enormous increase in the activities of aminotransferases in serum. Sclerotiorin pretreatment did not offer any protection against such an increase in these plasma non-specific enzymes caused by carbon tetrachloride. However, there was a significant countering of the increase in serum alkaline phosphatase activity by the test compound. As reported by other workers, the present study also elicited a significant increase in the activities of serum alanine amino-transferase(SGOT), aspartate aminotransferase (SGPT) and alkaline phosphatase.

Table 36: Serum antioxidant molecules in sclerotiorin administered mice

Parameters	Normal Control	Solvent Control	Sclerotiorin 10mg/kg	Sclerotiorin 25mg/kg	Sclerotiorin 50mg/kg	Vitamin-E 25mg/kg
Lipid peroxides (µM/mg protein)	0.039 ± 0.5	0.166 ± 0.95	0.158 ± 0.36	0.145 ± 1.74	0.138 ±1.51	0.141 ± 1.2
Vitamin C (µg/ml)	24.4 ±7.58	30.6 ± 13.92	35.2 ± 20.11	32 .0 ± 7.54	34.1 ± 13.13	30.41 ± 12.97
GSH (µg/ml)	20.0 ± 5.40	17.7 ± 7.03	18.4 ± 17.55	24.7 ± 14.75*	34.4 ± 23.35**	21.62 ± 16.39
Total thiols (mM/mg protein)	2.57 ± 7.44	3.93 ±12.71	3.77 ± 16.49*	3.21 ± 18.59	3.56 ± 15.04	2.61 ± 24.00**

Values represented mean ± SEM of 12 animals in each group**.** Solvent control group was compared with the normal control**;** Experimental groups were compared with solvent control. Significantly different **(**$^{**}P < 0.01$), ($^{*}P < 0.05$)

Table 37: Liver antioxidant molecules sclerotiorin administered mice

Parameters	Normal Control	Solvent Control	Sclerotiorin 10mg/kg	Sclerotiorin 25mg/kg	Sclerotiorin 50mg/kg	Vitamin-E 25mg/kg
Lipid peroxides (µM/mg protein)	0.053 ±0.32	0.234 ± 0.84	0.160 ± 0.85	0.152 ± 0.56	0.133 ± 0.65	0.153 ± 0.35
Vitamin C (µg/ mg protein)	0.189 ±. 009	0.06 ± 0.009	0.012 ± 0.001	0.04 ± 0.008	0.016 ± 0.001**	0.026 ± 0.002**
GSH (µg/mg protein)	0.292 ± 0.006	0.214 ± 0.017	0.214 ± 0.013	0.384 ± 0.074*	0.366 ± 0.003*	0.268 ± 0.003
Total thiols (µM/mg protein)	2.01 ± 0.014	2.03 ± 0.017	1.98 ± 0.008	3.55 ± 0.054**	3.53 ± 0.013**	2.67 ± 0.014

Values represented mean ± SEM of 12 animals in each group**.** Solvent control group was compared with the normal control**;** Experimental groups were compared with solvent control. Significantly different **(**$^{**}P < 0.01$), ($^{*}P < 0.05$)

Table 38: Activities of hepatic antioxidant enzymes in sclerotiorin administered mice

Enzyme	Normal Control	Solvent Control	Sclerotiorin 10 mg/kg	Sclerotiorin 25 mg/kg	Sclerotiorin 50 mg/kg	Vitamin-E 25mg/kg
Catalase U/mg Protein	14.17 ± 0.66	13.90 ± 0.681	12.27 ± 0.66	17.88 ± 0.79**	18.56 ± 0.79**	19.62 ± 0.22**
SOD U/mg Protein	2.26 ± 0.12	3.60 ± 0.13	3.29 ± 0.12	3.80 ± 0.25	3.68 ± 0.24	3.14 ± 0.09
GPx U/mg Protein	10.61 ± 0.60	11.24 ± 0.64	11.95 ± 0.82	13.12 ± 0.84	11.38 ± 0.735	7.00 ± 0.42**

Values represented mean ± SEM of 12 animals in each group, solvent control group was compared with the normal control**;** Experimental groups were compared with solvent control. Significantly different **(**$^{**}P < 0.01$), ($^{*}P < 0.05$)

Table 39: Activities of plasma non-specific enzymes in mice administered sclerotiorin

Parameters	Normal Control	Solvent Control	Sclerotiorin 10mg/kg	Sclerotiorin 25mg/kg	Sclerotiorin 50mg/kg	Vitamin-E 25mg/kg
SGOT IU/L	42.1 ± 10.7	318.4 ± 26.5	308 ± 21.2	298.5 ± 10.1	305.2 ± 17.6	313.1 ± 16.4
SGPT IU/L	32.36 ± 3.4	288.8 ± 13.3	279.7 ± 21.2	266.3 ± 18.5	251.7 ± 8.0	265.0 ± 13.3
Alkaline Phosphatase U/L	246.7 ± 6.5	346.4 ± 18.8	305.2 ± 16.7	306.7 ± 17.4	336.8 ± 15.0	291.9 ± 7.3

Values represented mean ± SEM of 12 animals in each group, solvent control group was compared with the normal control**;** Experimental groups were compared with solvent control. Significantly different **(**$^{**}P < 0.01$), ($^{*}P < 0.05$)

Hepatic reduced glutathione concentration, which was significantly depleted as a result of carbon tetrachloride administration, was restored by sclerotiorin treatment, especially at 25 mg/kg and 50 mg/kg dose (Figure 64). Circulatory glutathione level was similarly beneficially modulated by sclerotiorin administration (Figure 65). The effect of the test compound at 25 mg/kg was even better than the beneficial effect exerted by exogenous vitamin-E at similar dose (Figure 64 and 65). Hepatic total thiols content was similarly increased by administration of sclerotiorin at 25 mg/kg and 50 mg/kg dose (Table 37). Among the antioxidant enzymes, activities of catalase and glutathione peroxidase in liver were significantly elevated by sclerotiorin dosage 25 mg/kg (Table 38).

Carbon tetrachloride mediated hepatotoxicity was also examined in this study for a possible hepatoprotective effect by the test compound sclerotiorin. A significant increase in the activities of serum SGOT, SGPT and alkaline phosphatase was observed as a result of carbon tetrachloride administration.

Pretreatment with sclerotiorin however did not seem to exert any significant protection against hepatic damage caused by carbon tetrachloride, as suggested by the biochemical parameters examined in this investigation.

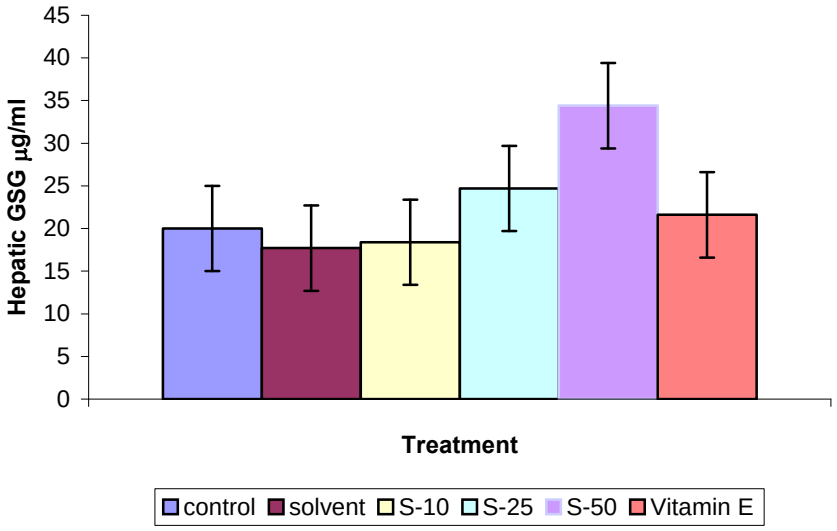


Figure 64. Activity of hepatic antioxidant enzyme glutathione (GSH)

Activities of hepatic antioxidant enzymes glutathione in sclerotiorin administered mice (S= Sclerotiorin dose mg/kg weight). Values represented mean ± SEM of 12 animals in each group, solvent control group was compared with the normal control**;** Experimental groups were compared with solvent control.

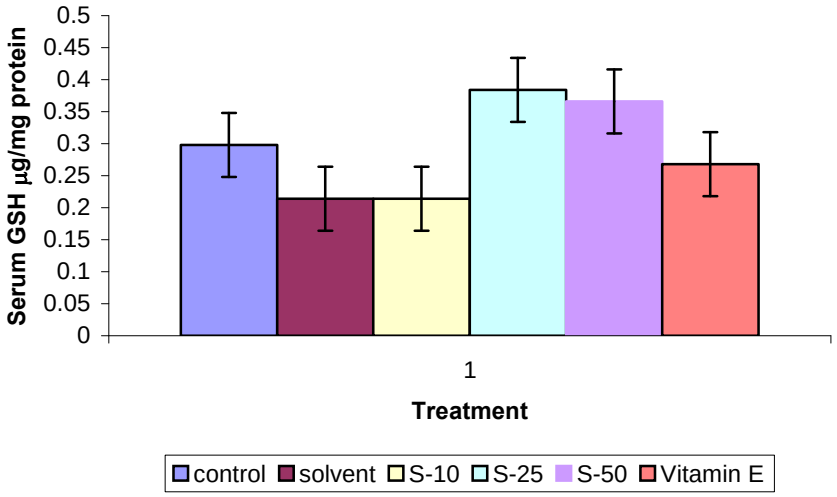


Figure 65. Activity of serum antioxidant enzyme glutathione (GSH)

Activities of serum antioxidant enzymes glutathione in sclerotiorin administered mice (S= Sclerotiorin dose mg/kg weight). Values represented mean ± SEM of 12 animals in each group, solvent control group was compared with the normal control**;** Experimental groups were compared with solvent control

SECTION–6

SUMMARY AND HIGHLIGHTS

A total of 74 forest soil isolates were subjected to screening of LOX inhibition, out of which 6 cultures were subjected to a secondary screening. Of these 6 isolate CFTRI-A-24, which gave a consistent inhibition was characterized and identified as *Penicillum frequentans*.

The inhibitor was produced in large scale from *Penicillum frequentans* CFTRI-A-24 on potato dextrose broth submerged fermentation medium. At the end of the fermentation the broth was extracted with ethyl acetate. The inhibitor was purified from the crude extract using column and thin layer chromatography techniques. From ^{1}H NMR spectra, GC-MS, and IR spectral data the structure of the inhibitor was determined as 6-H-2-Benzopyran-6,8(7H)-dione, 7-(acetyloxy)-5-chloro-3-[(1E,3E,5S)1-3,5-dimethyl-1,3 dimethyl-1,3-heptadienyl]-7- methyl, (7R)- also known as "Sclerotiorin". The molecular formula of the sclerotiorin was elucidated as $C_{21}H_{23}O_5Cl$ and a molecular weight of 390. Physicochemical properties of the inhibitor were determined. It has a melting point of 205 ^{o}C,λ_{max} nm in methanol shows the (ε) values: 287 (9672) and 362 (23,732), specific rotation $[\alpha]^{25}_{D}$ + 490^{o} (c = 1 in methanol) and is highly soluble in Chloroform, ethyl acetate, DMSO, acetone, ethanol, methanol and insoluble in hexane, petroleum ether and water.

Analytical methods for the rapid detection of sclerotiorin were developed using GC and HPLC. For GC a BP-1 capillary column was used with the temperature program: 120 ^{o}C/ 10 ^{o}C/min/ 180 ^{o}C/ 4 ^{o}C/min, 280 $^{\circ}C$/ 15 min; Injection 250 ^{o}C; (FID) at 280 ^{o}C, flow 2 ml/min N_2, Sclerotiorin was detected at 33 min. For HPLC a C_{18} column (5 µm, 250 mm x 4.6 mm, Shimpack CLC-ODS) was used with the gradient program: acetonitrile: water gradient from 60% to 100% for a period of 30 min, flow 0.5 ml/min. Sclerotiorin was detected at 362 nm at 23 and 8.5 min with a 30 cm and 15 cm column length respectively

Sclerotiorin effectively inhibited soybean 15-lipoxygenase in an uncompetitive reversible manner with an IC_{50} value 4.2 µM. By its antioxidant mechanism sclerotiorin trapped the radical intermediates formed during the enzyme reaction. Further, it inhibited

5-lipoxygenase from human PMNLs with an IC_{50} value 16 μM. The compound exhibited potent free radical scavenging property in DPPH assay system with ED_{50} value of 0.12 μM. Sclerotiorin inhibited lipid peroxidation in Fe^{2+}- ascorbate induced lipid peroxidation assay with IC_{50} value 64 μM.

Apart from these activities sclerotiorin inhibited rat eye lens Aldose Reductase (IC_{50}-0.4μM) and human platelet aggregation (IC_{50} 250μM). As an antimicrobial agent sclerotiorin was effective against *Bacillus* spp and *Candida* spp. Mutagenic property of sclerotiorin was tested using Ames test, with standard tester strains *Salmonella typhimurium* TA 1535, TA 97a, TA 98 and TA 100 and was found to be non mutagenic in nature. Anticancerous property of sclerotiorin was tested on Hep3B (human derived hepatocarcinoma), which showed cytotoxic activity with an EC_{50} of 0.2 ppm. Sclerotiorin was also found to be antiproliferative in nature and induced apoptosis in cancerous cell lines in less than 2 hours of incubation.

Both carbon and nitrogen sources influenced sclerotiorin production. A combination of carbon (starch and glucose), nitrogen (yeast extract and peptone) enhanced the yields. Among the salts $MgSO_4$ and KH_2PO_4 were effective in increasing the yields. Sclerotiorin production was maximum when the production medium was supplemented with (g/L) glucose 20, starch 20, yeast extract 4, peptone 5, $MgSO_4$ 0.5, KH_2PO_4 1, NaCl 0.5, agar 1 and pH 6.0. A highest yield of sclerotiorin was achieved at 120 hours at 30 ^oC with the initial pH 6.0 at 200 rpm. These optimized conditions yielded sclerotiorin at 2.01 g/L, which was 13.4 fold higher than PDB medium

Downstream process optimization was carried out for the effective recovery of the inhibitor. Extraction of the fermented broth using fermentation broth to ethyl acetate ratio of 1:1.5 v/v for two hours, under acidic pH range 2.5-4 with agitated conditions resulted in maximum inhibitor recovery. With the optimum physico-chemical and downstream parameters sclerotiorin yields were found to be 2 g/L.

A process was also developed to selectively precipitate the inhibitor directly from the fermented crude. Crude compound from the fermented broth extractions were dissolved with MeOH (1:60, w/v) and gently heated. To this distilled water was added with gentle stirring and was incubated on a rotary shaker at 100 rpm at room temperature for 3 hours. After 3 hours the turbid liquid was filtered on a Whatman filter paper No-1. Upon drying the filter paper at 50 ^{o}C, yellow amorphous sclerotiorin was obtained which was 96% pure by GC. This method was which is much more effective and faster in terms of inhibitor isolation in a larger scale.

Sclerotiorin was evaluated for antioxidant activity both *in vitro* and *in vivo*. *In vitro* antioxidant property of sclerotiorin was evidenced in linoleic acid suspension and emulsion, as well as in groundnut oil emulsion and suspension oil models. The compound showed antioxidant effect comparable to Vitamin-E at (50 ppm) in the *in vitro* systems. Concentrations above this were imparting color to the medium, which is not desirable in antioxidant applications

In vivo antioxidant effect of orally administered sclerotiorin in carbon tetrachloride (CCl_4) induced oxidative stress was studied in experimental mice. Vitamin-E was used as a control. Among the antioxidant molecules in serum and liver, glutathione concentration (GSH), which was significantly depleted as a result of carbon tetrachloride administration, was restored by sclerotiorin treatment, especially at 25 mg/kg and 50 mg/kg dose. Hepatic total thiols content was similarly increased by administration of sclerotiorin at 25 mg/kg and 50 mg/kg dose. Among the antioxidant enzymes, activities of catalase and glutathione peroxidase in liver were significantly elevated by sclerotiorin dosage 25 mg/kg.

Carbon tetrachloride mediated hepatotoxicity was also examined. Pretreatment with sclerotiorin however did not seem to exert any significant protection against hepatic damage caused by carbon tetrachloride, as suggested by the biochemical parameters examined in this investigation.

Highlights:

The work reported in this thesis begins with the isolation of fungus *Penicillium frequentans* CFTRI-A-24, which produced a potent lipoxygenase inhibitor. The inhibitor was identified as "Sclerotiorin", which showed several other biologically beneficiary activities like antioxidant, antimicrobial, aldose reductase inhibition, platelet aggregation inhibition, antimutagenic and anticancerous properties. These significant findings have not been reported. A medium was designed with optimum physical and chemical parameters for the efficient production of sclerotiorin with better yields. Carbon tetrachloride induced hepatotoxicity and oxidative stress was examined in a mice model. Pretreatment with sclerotiorin did not exert any significant protection against hepatic damage or antioxidant *in vivo*.

Recommendations for further work:

- Strain improvement of *Penicillium frequentans* CFTRI A-24 to produce sclerotiorin in larger quantities
- Comparison of *Penicillium frequentans* CFTRI A-24 with other sclerotiorin producing cultures like *P. sclerotiorum, P. multicolor*
- Checking the culture for the production of other analogs of sclerotiorin (isochromophilones I-IX)
- Sclerotiorin has a characteristic absorption and emission spectra which can be exploited for protein interaction studies
- Sclerotiorin can be explored further in the areas of diabetes complications since it has shown potent inhibitions against aldose reductase
- Sclerotiorin has shown anticancer property and also lipoxygenase inhibition. A detailed study along with 5-LO enzyme activity levels in cancerous cells can be conducted

REFERENCES

Abe N, Nemoto A, Tsuchiya Y, Hojo H and Hirota A. Studies on the 1, 1-diphenyl-2-picrylhydrazyl radical scavenging mechanism for a 2-pyrone compound. *Biosci. Biotechnol. Biochem.* 64 (2000) 306-313.

Abe N. Mechanism of radical scavenging by microbial antioxidants. *Kagaku to Seibutsu.* 38 (2000) 5-7.

Aebi H. Catalase *in vitro. Meth. Enzymol*. 105 (1984) 121-126.

Aharony D and Stein RL. Kinetic mechanism of guinea pig neutrophil 5–lipoxygenase. *J. Biol. Chem*. 261 (1896) 11512-11519.

Ainsworth GC and Bisby GR. A dictionary of the fungi. Imperial mycological institute, (1945) 431 pp.

Albericci VJ, Curtin TP and Reilly D. Sclerotiose-a Polysaccharide Metabolic Product of *Penicillium Sclerotiorum* van Beyma. *Biochem. J.* 37 (1942) 243-246.

Alcaraz MJ and Ferrandiz ML. Modification of arachidonic acid metabolism by flavonoids. *J. Ethnopharmacol.* 21 (1987) 209-229.

Alcaraz MJ, Ferrandiz ML and Villar A. flavonoid inhibition of soybean lipoxygenase. *Pharmazie*. 41 (1986) 299-300.

Amagata T, Whitman S, Johnson T, Stessmann CC, Carroll J, Loo C, Clardy J, Lobkovsky E, Crews P and Holman TR. Sponge derived terpenoids with selectivity towards human 15-lipoxygenase versus human 12-lipoxygenase. *J. Nat. Prod.* 66 (2003) 230-235.

Ames BN, Mc-cann J and Yamasaki E. Methods for detecting carcinogens and mutagens with the *Salmonella*, mammalian-microsome mutagenicity test. *Mutation Research.* 31 (1975) 347-363.

Ammon HP, Safayhi H, Mack T and Sabieraj J. Mechanism of anti-inflammatory action of curcumin and boswelic acid. *J. Ethnopharmacol*. 38 (1993) 113-119.

Anderson KM, Alrefai WA, Bonomi PA, Anderson CA, Dudeja P and Harris JE. A genomic response of H-358 bronchiolar carcinoma cells to MK 886, an inhibitor of 5-lipoxygenase, assessed with a cDNA array. *Anticancer. Res.* 20 (2000) 2433-2439.

Angelo St. AJ and Ory RL. Lipoxygenase inhibition by naturally occurring monoenoic fatty acids. *Lipids.* 19 (1984) 34-37.

Arai N, Shiomi K, Tomoda H, Tabata N, Yang DJ, Masuma R, Kawakubo T and Omura S. Isochromophilones III-VI, inhibitors of acyl–CoA: cholesterol acyltransferase produced by *Penicillium multicolor* FO-3216. *J. Antibiot.* 48 (1995) 696-702.

Argentieri DC, Ritchie DM, Ferro MP, Kirchner T, Wachter MP, Anderson DW, Rosenthale ME and Capetola RJ. Tepoxalin: a dual cycloxygenase/ 5-lipoxygenase inhibitor of arachidonic acid metabolism with potent anti-inflammatory activity and a favorable gastrointestinal profile. *J. Pharmacol. Exp. Ther*. 271 (1994) 1399-408.

Avis I, Hong SH, Martinez A, Moody T, Choi YH and Trepel J. Five-lipoxygenase inhibitors can mediate apoptosis in human breast cancer cell lines through complex eicosanoid interactions. *FASEB. J.* 15 (2001) 2007-2009.

Avis I, Martinez A, Tauler J, Zudaire E, Mayburd A and Abu-Ghazaleh R. Inhibitors of the arachidonic acid pathway and peroxisome proliferator-activated receptor ligands have superadditive effects on lung cancer growth inhibition. *Cancer. Res.* 65 (2005) 4181-4190.

Avis IM, Jett M, Boyle T, Vos MD, Moody T and Treston AM. Growth control of lung cancer by interruption of 5-lipoxygenase mediated growth factor signaling. *J. Clin. Invest.* 97 (1996) 806-813.

Axelrod B. Lipoxygenases. Whitaker JR (ed). Food related enzymes. *Adv. Chem.* American chemical society, Washington. 136 (1974) 324-349.

Axelrod B, Cheesbrough TM and Laakso S. Lipoxygenase from Soybeans. *Meth. Enzymol.* 71 (1981) 441-451.

Bahn Y, Park J, Bai D, Takase S and Yu J. A novel aldose reductase inhibitor isolated from alkalophilic *Corynebacterium* sp YUA25. *J. Antibiotcs.* 51 (1998) 902-907.

Beauchamp C and Fridovich I. Superoxide dismutase: improved assay and an assay applicable to acrylamide gel. *Anal. Biochem.* 44 (1971) 276-287.

Began G, Sudarshan E and Rao AGA. Inhibition of lipoxygenase-1 by phosphatidylcholine micelles-bound curcumin. *Lipids.* 33 (1998) 1223-1228.

Bell AA, Chatterjea JN, Holker JSE, Staunton J and Whalley WB. The chemistry of fungi, the synthesis of aposclerotamnic acid. *J. Chem. Soc.* (1964) 4307-4310.

Bell RL, Young PR, Albert D, Lanni C, Summers JB and Brooks DW The discovery and development of zileuton: an orally active 5-lipoxygenase inhibitor. *Int. J. Immunopharmacol.* 14 (1992) 505-510.

Bennet JW and Bentley R. Whats's in a name? – Microbial secondary metabolism. *Adv. Appl. Microbiol.* 34 (1989) 1-28.

Berdy J. Screening, classification and identification of microbial products. Verrall MS (ed). Discovery and isolation of microbial products. Ellis Horwood Ltd, Society of chemical industry, London, (1985) Ch:1, pp 9-31.

Bertolini A, Ottani A and Sandrini M. Dual acting anti-inflammatory drugs: a reappraisal. *Pharmacol. Res*. 44 (2001) 437- 450.

Bertz A, Stierle A, Anton R and Cazenave J. Role of cyclic AMP in the inhibition of human platelet aggregation by Quercetin, a flavonoid that potentiates the effect of prostacyclin. *Biochem. Pharmacol.* 321 (1981) 3597-3600.

Betina V. Bioactive secondary metabolites of microorganisms. *Prog. Ind. Microbiol.* 30 (1994) 16-50.

Beutler E, Duron O and Kelly BM. Improved method for the determination of blood glutathione. *J. Lab. Clin. Med.* 61 (1963) 882-888.

Bezakova L, Misik V, Malekova L, Svajdlenka E and Kostalova D. Lipoxygenase inhibition and antioxidant properties of benzylisoquinoline alkaloids isolated from *Mahonia aquifolium*. *Pharmazie.* 51 (1996) 758-761.

Birkinshaw JH and Chaplen P. Studies in the Biochemistry of Microorganisms. Chemical degradation of sclerotiorin. *Biochem. J*. 69 (1958) 505-509.

Birkinshaw JH. Studies in the biochemistry of microorganisms, metabolic products of *Penicillium multicolor* G-M. and P. with special reference to sclerotiorin. *Biochem. J.* 52 (1952) 283-288.

Birkinshaw JH, Kalyanpur MG and Stickings CE. Studies in the biochemistry of micro-organisms, pencolide, a nitrogen-containing metabolite of *Penicillium multicolor* grigorieva-manilova and poradielova. *Biochem. J*. 86 (1963) 237-243.

Blots MS. Antioxidant determination by the use of stable free radical. *Nature.*181 (1958) 1999-2000.

Boado RJ, Pardridge WM, Vinters HV and Black KL. Differential expression of arachidonate 5-lipoxygenase transcripts in human brain tumors: evidence for the expression of a multitranscript family. *Proc. Natl. Acad. Sci.* 89 (1992) 9044-9048.

Borel C, Welti DH, Fernandez I and Colmenares M. Dicranin, an antimicrobial and 15-lipoxygenase inhibitor from the moss *Dicranum scoparium*. *J. Nat. Prod.* 56 (1993) 1071-1077.

Bortuzzo C, Hanif R, Kashfi K, Staiano-Coico L, Shiff SJ and Rigas B. The effect of leukotrienes B and selected HETEs on the proliferation of colon cancer cells. *Biochim. Biophys. Acta* 1300 (1996) 240-246.

Boyington JC, Geffney BJ and Amzel LM. The three dimensional structure of an arachidonic acid 15-lipoxygenase. *Science.* 260 (1993) 1482-1486.

Boym A. Isolation of lymphocytes, granulocytes and macrophages. *Scand. J. Immunol. Suppl.* 5 (1976) 9-15.

Brammer JP, Maguire MH, Walaszek EJ and Wiley RA. Acetal phosphatidic acids: novel platelet aggregating agents. *Brit. J. Pharmacol.* 79 (1983) 157-166.

Brash, A. R. Lipoxygenase: occurrence, function, catalysis and acquisition of substrate. *J. Biol. Chem.* 274 (1999) 23679-23682.

Brooks CDW, Stewart AO, Basha A, Bhatia P, Ratajczyk JD and Martin JG. R-(+)-N-[3-[5-(4-Fluorophenyl)methyl]-2-thienyl]-1- methyl-2-propynyl]-N-hydroxyurea (ABT-761), a second generation 5- lipoxygenase inhibitor. *J. Med. Chem.* 38 (1995) 4768-4775.

Bruckner B and Blechschmidt A. Nitrogen regulation of gibberellin biosynthesis in *Gibberella fujikuroi*. *Appl. Microbiol. Biotechnol.* 35 (1991) 646-650.

Buege JA and Aust SD. Microsomal lipid peroxidation. *Meth. Enzymol*. 52 (1978) 302-310.

Burg RW, Miller BM, Baker EE, Birnbaum J, Currier SA, Hartman R, Yu-Ling K, Managlan RL, Olson G, Putter I, Tunac JB, Wellicl H, Sampley EO, Oiwa R and Omura S. Avermectins, new family of potent anthelminthic agents, production organism and fermentation. *Antimicrob. Agents. Chemother*. 15 (1979) 361-367.

Butler MS and Buss AD. Natural products-the future scaffolds for novel antibiotics. *Biochem. Pharmacol*. 71 (2006) 919-929.

Butler MS. Natural products to drugs: natural product derived compounds in clinical trials. *Nat. Prod. Rep*. 22(2005) 162-195.

Carroll J, Jonsson EN, Ebel R, Hartman MS, Holman TR and Crews P. Probing sponge-derived terpenoids for human 15-lipoxygenase inhibitors. *J. Org. Chem. 66* (2001) 6847-6851.

Carter GW, Young PR, Albert DH, Bouska J, Dyer R and Bell RL 5-Lipoxygenase inhibitory activity of zileuton. *J. Pharmacol. Exp. Ther.* 256 (1991) 929-937.

Carter P. Spectrophotometric determination of serum Iron at the submicrogram level with a new reagent (Ferrozine). *Anal. Biochem.* 40 (1971) 450-458.

Celotti F and Laufer S. Anti-inflammatory drugs: new multitarget compounds to face an old problem. The dual inhibition concept. *Pharmacol. Res.* 43 (2001) 429-436.

Chen QX and Kubo I. Kinetics of mushroom tyrosinase inhibition by quercetin. *J. Agric. Food. Chem.* 50 (2002) 4108-4112.

Chen X, Li N, Wang S, Wu N, Hong J and Jiao X. Leukotriene A4 hydrolase in rat and human esophageal adenocarcinomas and inhibitory effects of bestatin. *J. Natl. Cancer. Inst.* 95 (2003) 1053-1061.

Chen X, Wang S, Wu N and Yang CS. Leukotriene A4 hydrolase as a target for cancer prevention and therapy. *Curr. Cancer. Drug. Targets.* 4 (2004) 267-283.

Chen YQ, Duniec ZM, Liu B, Hagmann W, Gao X, Shimoji K, Marnett LJ, Johnson C R, and Honn KV. Endogenous 12(*S*)-HETE production by tumor cells and its role in metastasis. *Cancer. Res*. 54 (1994) 1574-1579.

Chodon D, Ramamurty N and Sakthisekaran D. Preliminary studies on induction of apoptosis by genistein on HepG2 cell line. *Toxicology in vitro*. 21 (2007) 887-891.

Choi J, Chon JK, Kim S and Shin W. Conformational flexibility in mammalian 15S-lipoxygenase: Reinterpretation of the crystallographic data. *Proteins.* 70 (2008) 1023-32.

Chong R, King RR and Whalley WB. The chemistry of fungi, the synthesis of (±) sclerotiorin and (±)-4-6-Dimethylocta-trans-2,trans-4-dienoic acid, and of an analog of rotiorin. *J. Chem. Soc* (1971) 3566-3571.

Cillard J, Cillard P, Cormier M and Girre L. α-Tocopherol proxidant effect in aqueous media: increased autoxidation rate of linoleic acid. *J. Am. Oil. Chem. Soc.* 57 (1980) 252-255.

Claesson HE and Dahlen SE Asthma and leukotrienes: antileukotrienes as novel anti-asthmatic drugs. *J. Intern. Med.* 245 (1999) 205-227.

Clardy J and Walsh C. Lessons from natural molecules. *Nature* 432 (2004) 829-837.

Coffa G, Imber AN, Maguire BC, Laxmikanthan G, Schneider C, Gaffney BJ and Brash AR. On the relationships of substrate orientation, hydrogen abstraction and product stereochemistry in single and double dioxygenations by soybean lipoxygenase-1 and its Ala542Gly mutant, *J. Biol. Chem.* 280 (2005) 38756-38766.

Cohen B-S, Grossman S, Pinksky and Klein BP. Chlorophyll inhibition of lipoxygenase in growing pea plants. *J. Agric. Food Chem.* 32 (1984) 516-519.

Corey EJ and Nagata RYU. Evidence in favor of an organoiron-mediated pathway for lipoxygenation of fatty acids by soybean lipoxygenase *J. Am. Chem. Soc.* 109 (1987) 8107-8108.

Crawley GC, Dowell RI, Edwards PN, Foster SJ, McMillan RM and Walker ER. Methoxytetrahydropyrans. A new series of selective and orally potent 5-lipoxygenase inhibitors. *J. Med. Chem.* 35 (1992) 2600-2609.

Curtin TP and Reilly J. Sclerotiorin, $C_{20}H_{20}O_5Cl$, a chlorine-containing metabolic product of *Penicillium sclerotiorum* van Beyma. *Biochem. J.* 34 (1940) 1419-1421.

Daniel NG, Goulet J, Bergeron M, Paquin R and Landry PE. Antiplatelet drugs: Is there a surgical risk? *J. Can. Dent. Assoc.* 68 (2002) 683-687.

Dean FM, Eade RA, Moubasher RA, and Robertson A. Fulvic acid, its structure and relationship to citromycetin and fusarubin. *Nature*. 179 (1957) 366.

Dean FM, Staunton J and Whalley WB. The chemistry of fungi, a revised structure for sclerotiorin. *J. Chem. Soc*. (1959) 3004- 3009.

Degreef H, Dockx P, De-Doncker P, De-Beule K and Cauwenbergh G. A double-blind vehicle-controlled study of R 68 151 in psoriasis: a topical 5-lipoxygenase inhibitor. *J. Am. Acad. Dermatol.* 22 (1990) 751-755.

Demain AL. (1992) Microbial secondary metabolism: a new theoretical frontier for academia, a new opportunity for industry. In Secondary Metabolites: Their Function and Evolution (Chadwick, D.J. and Whelan, J., eds), pp. 3–23, John Wiley and Sons.

Demain AL. Achievements in microbial technology. *Biotechnol. Adv*. 8 (1990) 291-301.

Demain AL. Contributions of genetics to the production and discovery of microbial pharmaceuticals. *Pure. Appl. Chem.* 60 (1988) 833-836.

Demain AL. In Chadwick DJ and Whelan J (eds). Secondary metabolites: their function and evolution. John Wiley and Sons, Chichester. (1992) pp 3.

Demain AL. Microbial natural products: alive and well in 1998. *Nat. Biotechnol*. 16 (1998) 3-4.

Denduluri N. Phase II trial of ixabepilone, an epothilone B analog, given daily for three days every three weeks, in metastatic breast cancer. *Invest. New. Drugs.* 25 (2007) 63~67.

Ding XZ, Iversen P, Cluck MW, Knezetic JA and Adrian TE. Lipoxygenase inhibitors abolish proliferation of human pancreatic cancer cells. *Biochem. Biophys. Res. Commun.* 261 (1999) 218-223.

Ding XZ, Talamonti MS, Bell RH, Jr and Adrian TE. A novel anti-pancreatic cancer agent, LY293111. *Anticancer. Drugs.* 16 (2005) 467-473.

Djuric SW, Collins PW, Jones PH, Shone RL, Tsai BS, Fretland DJ, Butchko Gm, Villani-Price D, Keith RH, Zemaitis JM, Metcalf L and Bauer RF. 7-[3-(4-Acetyl-3-methoxy-2-propylphenoxy)propoxy]-3,4-dihydro-8-propyl-2*H*-1-benzopyran-2carboxylic acid: an orally active selective leukotriene B_4 receptor antagonist. *J. Med. Chem.* 32 (1989) 1145-1147.

Ducharme Y, Brideau C, Dube D, Chan CC, Falgueyret JP and Gillard JW. Naphthalenic lignan lactones as selective, nonredox 5-lipoxygenase inhibitors. Synthesis and biological activity of (methoxyalkyl) thiazole and methoxytetrahydropyran hybrids. *J. Med. Chem.* 37 (1994) 512-518.

Duran N, Teixeira MFS, Conti RS and Esposito E. Ecological- friendly pigments from fungi. *Crit. Rev. Food. Sci. Nutr*. 42 (2002) 53-66.

Eade RA, Page H, Robertson A, Turner K, and Whalley WB. The chemistry of fungi. XXVIII. Sclerotiorin and its hydrogenation products. *J. Chem. Soc*. (1957) 4913-4924.

Ellestad GA and Whalley WB. The chemistry of fungi LII (-) sclerotiorin. *J. Chem. Soc*. (1965) 7260-7261.

Ellman GL, Courtney KD, Andres V Jr. and Featherstone RM. New and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* 7 (1961) 88-95.

Fan XM, Tu SP, Lam SK, Wang WP, Wu J and Wong WM. 5-Lipoxygenase-activating protein inhibitor MK-886 induces apoptosis in gastric cancer through upregulation of p27kip1 and bax. *J. Gastroenterol. Hepatol*. 19 (2004) 31-37.

Ferry DR, Deakin M, Baddeley J, Daryanani S, Bramhall S and Anderson DA. A phase II study of the 5-lipoxygenase inhibitor, CV6504, in advanced pancreatic cancer:correlation of clinical data with pharmacokinetic and pharmacodynamic endpoints. *Ann. Oncol.* 11 (2000) 1165-1170.

Feussner I and Wasternack C. The lipoxygenase pathway. *Annu. Rev. Plant. Biol*. 53 (2002) 275-95.

Fiorucci S, Meli R, Bucci M and Cirino G. Dual inhibitors of cyclo-oxygenase and 5-lipoxygenase. A new avenue in anti-inflammatory therapy. *Biochem. Pharmacol.* 62 (2001) 1433-1488.

Fischer L, Szellas D, Radmark O, Steinhilber D and Werz O. Phosphorylation-and stimulus-dependent inhibition of cellular 5-lipoxygenase activity by nonredox-type inhibitors. *FASEB. J.* 17 (2003) 949-951.

Ford-Hutchinson AW, Gresser M and Young RN. 5-Lipoxygenase. *Annu. Rev. Biochem*. 63 (1994) 384-417.

Forman BM, Chen J and Evans RM. Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors a and d. *Proc. Natl. Acad. Sci.* 94 (1997) 4312-4317.

Frankel EN, Huang SW, Kanner J and German JB. Interfacial phenomena in the valuation of antioxidants: bulk oils versus emulsions. *J. Agric. Food Chem*. 42 (1994) 1054-1059.

Frasier PJ. Lipoxygenase action and lipid binding in bread making. *Bakers. Digest.* 53 (1979) 8-29.

Fujimoto H, Matsudo T, Yamaguchi A and M. Yamazaki: Two new fungal azaphilones from *Talaromyces luteus*, with monoamineoxidase inhibitory. *Heterocycles.* 30 (1990) 607-616.

Fukuyama Y, Kiriyama Y, Kodama M, Iwaki H, Hosozawa S, Aki S and Matsui K. Total synthesis of ardisiaquinone A, a potent 5-lipoxygenase inhibitor, isolated from *Ardisia sieboldii*, and degree of 5-lipoxygenase inhibitory activity of its derivatives. *Chem. Pharm. Bull.* 42 (1994) 2211-2213.

Funk CD. The molecular biology of mammalian lipoxygenases and the quest for eicosanoid functions using lipoxygenase-deficient mice. *Biochim. Biophys. Acta.* 1304 (1996) 65-84.

Funk CD. Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science* 294 (2001) 1871-1875.

Funk Jr. MO, Andre JC and Otsuki T. Lipoxygenation of trans polyunsaturated fatty acids by lipoxygenase reveals steric features of the catalytic mechanism. *Biochemistry.* 26 (1987) 6880-6884.

Gabbay KH. The sorbitol pathway and the complication of diabetics. *N. Engl. J. Med.* 288 (1973) 831-836.

Gardner HW. Recent investigations into the lipoxygenase pathway of plants. *Biochim. Biophys. Acta.* 1084 (1991) 221-239.

Gerwick WH. Structure and biosynthesis of marine algal oxylipins. *Biochim. Biophys. Acta.* 1211 (1994) 243-55.

Ghosh J and Myers CE. Arachidonic acid stimulates prostate cancer cell growth: critical role of 5-lipoxygenase. *Biochem. Biophys. Res. Commun*. 235 (1997) 418–423.

Gillard J, Ford-Hutchinson AW, Chan C, Charleson S and Denis D. L-663,536 (MK-886)(3-[1-(4-chlorobenzyl)-3-t-butyl-thio-5-isopropylindol-2-yl]-2,2- dimethylpropanoic

acid), a novel, orally active leukotriene biosynthesis inhibitor. *Can. J. Physiol. Pharmacol.* 67 (1989) 456-464.

Gillmor SA, Villasenor A and Fletterick R. The structure of mammalian 15-lipoxygenase reveals similarity to the lipases and the determinants of substrate specificity. *Nat. Struct. Biol.* 4 (1997) 1003-1009.

Grechkin A. Recent developments in biochemistry of plant lipoxygenase pathway. *Prog. Lipid. Res*. 37 (1998) 317-352.

Gregory EM and Turner WB. 7-epi-sclerotiorin. *Chem. and Ind*. (1963) 1625.

Griffiths RJ, Smith MA, Roach ML, Stock JL, Stam EJ and Milici AJ, Collagen-induced arthritis is reduced in 5-lipoxygenaseactivating protein-deficient mice. *J Exp Med* 185 (1997) 1123-1129.

Grosch W and Schwarz J. Linoleic and linolenic acid as precursors of the cucumber flavour. *Lipids.* 6 (1971) 351-352.

Grosch W, Laskawy G and Weber F. Formation of volatile carbonyl compounds and cooxidation of β-carotene by lipoxygenase from wheat, potato, flax and beans. *J. Agric. Food. Chem.* 24 (1976) 456-459.

Grossman S and Waksman EG. New aspect of the inhibition of soybean lipoxygenase by α-tochopherol: evidence for existence of a specific complex. *Int. J. Biochem.* 6 (1984) 281-289.

Guindon Y, Girard Y, Maycock A, Ford-Hutchinson AW and Atkinson JG. L-651,392: A novel, potent and selective 5-lipoxygenase inhibitor. *Adv. Prostaglandin. Thromboxane. Leukot. Res.* 17 (1987) 554-557.

Gupta S, Srivastava M, Ahmad N, Sakamoto K, Bostwick DG and Mukhtar H. 5-lipoxygenase is overexpressed in prostate adenocarcinoma. *Cancer.* 91 (2001) 737-743.

Gvozdkova TS, Chernook TV and Kapich AN. Lipid antioxidant activity of some fungi of different taxonomic groups. *Vestisi Nats. Akad. Navuk Belarusi Ser. Biyal. Navuk.* 4 (2000) 50-53. CA: 134 204855.

Haeggstrom JZ. Leukotriene A_4 hydrolase/aminopeptidase, the gatekeeper of chemotactic leukotriene B_4 biosynthesis. *J. Biol. Chem.* 279 (2004) 50639-50642.

Hakonarson H, Thorvaldsson S and Helgadottir A. Effects of a 5-lipoxygenase activating protein inhibitor on biomarkers associated with risk of myocardial infarction. A randomized trial. *J. Am. Med. Assoc.* 293 (2005) 2245-2256.

Halladay SC, Ryerson BA, Smith CR, Brown JP and Parkinson TM. Comparison of effects of dietary administration of butylated hydroxytoluene or a polymeric antioxidant on the hepatic and intestinal cytochrome P-450 mixed function oxygenase system of rats. *Food. Cosmet. toxicol.* 18 (1980) 569- 74.

Hamberg M and Samuelsson B. Prostaglandin endoperoxides. Novel transformations of arachidonic acid in human platelets. *Proc. Natl. Acad. Sci.* 71 (1974) 3400-3404.

Hawkey CJ, Dube LM, Rountree LV, Linnen PJ, and Lancaster JF. A trial of zileuton versus mesalazine or placebo in the maintenance of remission of ulcerative colitis. The European Zileuton Study Group For Ulcerative Colitis. *Gastroenterology.* 112 (1997) 718-724.

Hayman S and Kinoshita JH. Isolation and properties of lens aldose reductase. *J. Biol. Chem.* 240 (1965) 877- 882.

Heep M, Beck D, Bayerdoerffer E and Lehn N. Rifamin and rifabutin resistance machanism in *Helicobacter pylori*. *Antimicrob. Agents. Chemother*. 43 (1999) 1497-1499.

Hennig R, Ding XZ, Tong WG, Schneider MB, Standop J and Friess H. 5-Lipoxygenase and leukotriene B(4) receptor are expressed in human pancreatic cancers but not in pancreatic ducts in normal tissue. *Am. J. Pathol.* 161 (2002) 421-428.

Hennig R, Grippo P, Ding XZ, Rao SM, Buchler MW and Friess H. 5-Lipoxygenase, a marker for early pancreatic intraepithelial neoplastic lesions. *Cancer. Res.* 65 (2005) 6011-6016.

Holgate ST, Peter Bradding P, Anthony P and Sampson AP. Leukotriene antagonists and synthesis inhibitors: new directions in asthma therapy. *Journal of Allergy and Clinical Immunology*. 98 (1996) 1-13.

Holker JSE, Ross WJ, Staunton J and Whalley WB. The chemistry of fungi, part XL. Further evidence for the structure of sclerotiorin. *J. Chem. Soc*. (1962) 4150-4154.

Hong SH, Avis I, Vos MD, Martínez A, Treston AM and Mulshine JL. Relationship of arachidonic acid metabolizing enzyme expression in epithelial cancer cell lines to the growth effect of selective biochemical inhibitors. *Cancer Res*. 59 (1999) 2223-2238.

Honn KV, Tang DG, Gao X, Butovich IA, Liu B, Timar J and Hagmann W 12 Lipoxygenases and 12 (*S*)-HETE: role in cancer metastasis. *Cancer. Metastasis. Rev*. 13 (1994) 365-396.

Hoque A, Lippman SM, Wu TT, Xu Y, Liang ZD and Swisher S, Increased 5-lipoxygenase expression and induction of apoptosis by its inhibitors in esophageal cancer: a potential target for prevention. *Carcinogenesis.* 26 (2005) 785-791.

Horton AA and Fairhurst S. Lipid peroxidation and mechanism of toxicity. *Crit. Rev. Toxicol*. (1987) 27-29.

Huang SW, Hopia A, Schwarz K, Frankel EN and German JB. Antioxidant activity of r-tocopherol and trolox in different lipid substrates: bulk oils vs oil-in-water emulsions. *J. Agric. Food. Chem*. 44 (1996) 444-452.

Irvin CG, Tu YP, Sheller JR and Funk CD. 5-Lipoxygenase products are necessary for ovalbumin-induced airway responsiveness in mice. *Am. J. Physiol.* 16 (1997) 1053-1058.

Ishikawa Y, Ito T and Lee KH. Inhibition of sardine flesh lipoxygenase by a new antioxidant from *Aspergillus terreus*. *Nihon. Yukagakkaishi.* 45 (1996) 1321-1325.

Ito C, Abe N and Hirota A. Isolation and structure of a novel indophenol-reducing and 1,1-diphenyl-2-picrylhydrazyl radical-scavenging compound from a fungus. *Biosci. Biotechnol. Biochem.* 63 (1999) 1993-1996.

Jackman GB, Robertson A, Travers RB, and Whalley WB. The chemistry of fungi. XXXIV. Rotiorin, a metabolite of *Penicillium sclerotiorum* van Beyma. *J. Chem. Soc.* (1958) 1825-1832.

Janssen-Timmen U, Vickers PJ, Wittig U, Lehmann WD, Stark HJ and Fusenig NE, Expression of 5-lipoxygenase in differentiating human skin keratinocytes. *Proc. Natl. Acad. Sci.* 92 (1995) 6966-6970.

Jiang J, Shan LVH , Lin JH, Jiang DF and Chen ZK. LTB_4 can directly stimulate human osteoclast formation from PBMC independent of RANKL. *Artificial Cells, Blood Substitutes, and Biotechnology.* 33 (2005) 391-403.

Jong SC and Donovick R. Antitumor and antiviral substances from fungi. *Adv. Appl. Microbiol.* 34 (1989) 183-262.

Jongrungruangchok S, Kittakoop P, Yongsmith B, Bavovada R, Tanasupawat S, Lartpornmatulee N and Thebtaranonth Y. Azaphilones pigments from a yellow mutant of the fungus *Monascus kaoliang*. *Phytochemistry.* 65 (2004) 2569-2575.

Kador PE, Robinson WG and Kinoshita JH The pharmacology of aldose reductase inhibitors. *Ann. Rev. Pharmacol. Toxicol*. 25 (1985) 691-714.

Kador PE. The role of aldose reductase in the development of diabetic complications. *Med. Res. Rev*. 8 (1988) 325-352.

Kador PF, Akagi Y, Takahashi Y, Ikebe H, Wyman M and Kinoshita JH. Prevention of retinal vessel change associated with diabetic retinopathy in galactose fed dogs by aldose reductase inhibitors. *Arch. Opthalmol.* 108 (1990) 1301-1309.

Kawasumi T, Sato M, Tsuriya Y and Ueno S. Antioxidant activity of a medicine based on *Aspergillus oryzae* NK *koji* measured by a modified t-butyl peroxy radical scavenging assay. *Biosci. Biotechnol. Biochem.* 63 (1999) 581-584.

Kemp JP. Recent advances in the management of asthma using leukotriene modifiers. *Am. J. Respir. Med.* 2 (2003) 139-156.

Kennedy TJ, Chan CY, Ding XZ and Adrian TE. Lipoxygenase inhibitors for the treatment of pancreatic cancer. *Expert. Rev. Anticancer. Ther.* 3 (2003) 525-536.

Kim HY and Oh JH. Screening of Korean forest plants for rat lens aldose reductase inhibition. *Biosci. Biotechnol. Biochem.* 63 (1999) 184-188.

King AD, Hocking AD and Pitt JI. Dichloran-rose bengal medium for enumeration and isolation of molds from foods. *Appl. Environ. Microbiol.* 37 (1979) 959-964.

Kirst HA, Thompson DG and Nicas TI. Historical yearly usage of vancomycin. *Antimicrob Agents Chemother*. 42 (1998) 1303-4.

Kishimura Y, Kawashima A, Kagamizono T, Yamagishi M, Matsumoto K, Kawashima Y and Hanada. K PI-200 and PI-201, new platelet aggregation inhibitors produced by *Streptomyces* sp. A7498. *J. Antibiot.* 45 (1992) 892-898.

Kitamura S, Hashizume K, Iida T, Miyashita E, Shirahata K and Kase H. Studies on lipoxygenase inhibitors. II. KF8940 (2-n-heptyl-4-hydroxyquinoline-N-oxide), a potent and selective inhibitor of 5-lipoxygenase, produced by *Pseudomonas methanica*. *J. Antibiot.* 39 (1986)a 1160-1166.

Kitamura S, Iida T, Shirahata K and Kase H. Studies on lipoxygenase inhibitors. I. MY3-469 (3-methoxytropolone), a potent and selective inhibitor of 12-lipoxygenase, produced by *Streptoverticillium hadanonense* KY11449. *J. Antibiot.* 39 (1986)b 589-593.

Knapp HR. Reduced allergen-induced nasal congestion and leukotriene synthesis with an orally active 5-lipoxygenase inhibitor. *New. Engl. J. Med.* 323 (1990) 1745-1748.

Kobayashi A, Koguchi Y, Takahashi S, Kanzaki H and Kawazu K. St-1, a novel radical scavenger from fungus F-124. *Biosci. Biotechnol. Biochem.* 57 (1993) 1034-1036.

Kohyama N, Nagata T, Fujimoto S and Sekiya K. Inhibition of arachidonate lipoxygenase activities by 2-(3,4-dihydroxyphenyl) ethanol, a phenolic compound from olives. *Biosci. Biotechnol. Biochem.* 61 (1997) 347-350.

Komoda T, Morimitsu Y, Hirota H and Hirota A. USF-19A, a new lipoxygenase inhibitor from *Streptomyces* sp. *Biosci. Biotechnol. Biochem.* 59 (1995) 924-926.

Komoda T, Yoshida K, Abe N, Sugiyama Y, Imachi M, Hirota H, Koshino H and Hirota A, Novel Lipoxygenase Inhibitors, Tetrapetalone B, C, and D from *Streptomyces* sp. *Biosci. Biotechnol. Biochem.* 68 (2004) 903-908.

Kono K, Tanaka M, Ono Y, Hosoya T, Ogita T and Kahama T. S-15183 a & b, new sphingosine kinase inhibitors, produced from fungi. *J. Antibiot.* 54 (2001) 415-420.

Krell RD, Aharony D, Buckner CK, Keith RA, Kusner EJ, Snyder DW, Bernstein PR, Matassa VG, Yee YK, Brown FJ, Hesp B and Giles RE. The preclinical pharmacology of ICI 204,219: a peptide leukotriene antagonist. *Am. Rev. Respir. Dis.* 141 (1990) 978-987.

Kuhn H and Thiele BJ. The diversity of the lipoxygenase family. Many sequence data but little information on biological significance. *FEBS Lett.* 449 (1999) 7-11.

Kuhn H, Heydeck D, Wiesner R and Schewe T. The positional specificity of wheat lipoxygenase; the carboxylic group as signal for the recognition of the site of the hydrogen removal. *Biochim. Biophys. Acta.* 830 (1985) 25-29.

Kuhn H, Jan J, Eibach S, Holzhütter HG, Ivanov I and Walther M. Structural biology of mammalian lipoxygenases: Enzymatic consequences of targeted alterations of the protein structure. *Biochem. Biophys. Res. Commun.* 338 (2005) 93-101.

Kuhn H, Schewe T, and Rapoport SM. The stereochemistry of the reactions of lipoxygenases and their metabolites. Proposed nomenclature of lipoxygenases and related enzymes. *Adv. Enzymol. Relat. Areas. Mol. Biol.* 58 (1986) 273-311.

Kuhn H, Sprecher H and Brash AR. On singular or dual positional specificity of lipoxygenases. The number of chiral products varies with alignment of methylene groups at the active site of the enzyme. *J. Biol. Chem.* 265 (1990) 16300-16305.

Kusner EJ, Buckner CK, Dea DM, DeHaas CJ, Marks RL and Krell RD. The 5-lipoxygenase inhibitors ZD2138 and ZM230487 are potent and selective inhibitors of several antigen-induced guinea-pig pulmonary responses. *Eur. J. Pharmacol.* 257 (1994) 285-292.

Lalithakumari K, Krishnaraju AV, Sengupta K and Subbaraju GV. Safety and toxicological evaluation of a novel, standardized 3-o-acetyl-11-keto-beta-boswellic acid (AKBA)-enriched boswellia serrata extract (5-Loxin). *Toxicology Mechanisms and Methods.* 16 (2006) 199-226.

Lam KS. New aspects of natural products in drug discovery. *Trends in Microbiology.* 15 (2007) 279-289.

Lau CK, Belanger PC, Scheigetz J, Defresne C and Williams HWR. Synthesis and structure activity relationships of a novel class of 5-lipoxygenase inhibitors. 2-(Phenylmethyl)-4-hydroxy-3,5-dialkylbenzofurans: the development of L-656,224. *J. Med. Chem.* 32 (1989) 1190-1197.

Laufer SA, Augustin J, Dannhardt G and Kiefer W. (6,7-Diaryldihydropyrrolizin- 5-yl) acetic acids, a novel class of potent dual inhibitors of both cyclooxygenase and 5-lipoxygenase. *J. Med. Chem.* 37 (1994) 1894-7.

Lehnigk B, Rabe KF, Dent G, Herst RS, Carpentier PJ and Magnussen H. Effects of a 5-lipoxygenase inhibitor, ABT-761, on exerciseinduced bronchoconstriction and urinary LTE4 in asthmatic patients. *Eur. Respir. J.* 11 (1998) 617−623.

Li N, Sood S, Wang S, Fang M, Wang P and Sun Z. Overexpression of 5-lipoxygenase and cyclooxygenase 2 in hamster and human oral cancer and chemopreventive effects of zileuton and celecoxib. *Clin. Cancer. Res*. 11 (2005) 2089-2096.

Liu B, Maher RJ, Hannun YA, Porter AT and Honn KV. 12(*S*)-HETE enhancement of prostate tumor cell invasion: selective role of PKCa. *J. Natl. Cancer. Ins.* 86 (1994) 1145-1151.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193 (1951) 265-75.

Lucas EMF, Monteiro-de-CastroMC and Takahashi JA. Antimicrobial properties of sclerotiorin, isochromophilone VI and pencolide, metabolites from a brazilian cerrado isolate of *Penicillium sclerotiorum* van beyma *Brazilian Journal of Microbiology*. 38 (2007) 785-789.

Luchese RH and Harrigan WF. Biosynthesis of aflatoxin-the role of nutritional factors. *J. Appl. Bacteriol.* 74 (1993) 5-14.

Mac Leod G and Ames J. Soy flavour and its improvement. *CRC Crit. Rev. Food. Sci. Nutr.* 27 (1988) 219-400.

Maccarone M, Salucci ML, van Zadelhoff G, Malatesta F, Veldink G, Vliegenthart JFG, and A Finazzi-Agro A. Tryptic digestion of soybean lipoxygenase-1 generates a 60 kda fragment with improved activity and membrane binding ability. *Biochemistry.* 40 (2001) 6819-6827.

Malterud KE. and Rydland KM. Inhibitors of 15-Lipoxygenase from Orange Peel. *J. Agric. Food. Chem.* 48 (2000) 5576-5580.

Mancini JA, Prasit P, Cappolino MG, Charlson P and Leger S. 5-Lipoxygenase-activating protein is the target of a novel hybrid of two classes of leukotriene biosynthesis inhibitors. *Mol. Pharmacol.* 41 (1992) 267-272.

Maron DM and Ames BN. Revised methods for salmonella mutagenicity test. *Mutation Research.* 113 (1983) 173-215.

Marques RCP, De-Medeiros SRB, Da-Silva Dias C, Barbosa – Filho JM and Agnez – Lima LF. Evaluation of the Mutagenic potential of yangambin and of the hydroalcoholic extracts of *Ocotea duckei* by the Ames test. *Mutation Research.* 536 (2003) 117-120.

Martin J P. Use of acid rose bengal, and streptomycin in the plate method for estimating soil fungi. *Soil. Sci.* 69 (1950) 215-32.

Matoba T, Hidaka H, Narita H, Kitamura K, Kaizuma N and Kito M. Lipoxygenase-2 isozyme is responsible for generation of hexanal in soybean homogenate. *J. Agric. Food. Chem.* 33 (1985) 852-855.

Matsuo M. *In vivo* antioxidant activity of *Okara koji*, a fermented Okara, by *Aspergillus oryzae*. *Biosci. Biotechnol. Biochem.* 61 (1997) 1968-1972.

Matsuyama M, Yoshimura R, Mitsuhashi M, Tsuchida K, Takemoto Y and Kawahito Y. 5-Lipoxygenase inhibitors attenuate growth of human renal cell carcinoma and induce apoptosis through arachidonic acid pathway. *Oncol. Rep.* 14 (2005) 73-79.

Matsuzaki K, Ikeda H, Masuma R, Tanaka H and Omura S. Isochromophilones I and II, novel inhibitors against gp120-CD_4 binding produced by *Penicillium multicolor* FO-2338. Screening, taxonomy, fermentation, isolation and biological activity. *J. Antibiot.* 48 (1995)a 703-707.

Matsuzaki K, Tanaka H and Omura S. Isochromophilones I and II, novel inhibitors against gpl20-CD_4 binding produced by *Penicillium multicolor* FO-2338. II Structure elucidation. *J. Antibiot.* 48 (1995)b 708-713.

Mc Cord JM. Human disease, free radicals and the oxidant/antioxidant balance. *Clin. Biochem.* 26 (1993) 351-357.

McGill KA and Busse WW. Zileuton. *Lancet.* 348 (1996) 519-524.

McMillan RM and Walker ERH. Designing therapeutically effective 5-lipoxygenase inhibitors. *Trends. Pharmacol. Sci.* 13(1992) 323-330.

Mehrabian M, Allayee H, Wong J, Shi W, Wang XP and Shaposhnik Z. Identification of 5-lipoxygenase as a major gene contributing to atherosclerosis susceptibility in mice. *Circulation. Res.* 91 (2002) 120−126.

Michael A.P, Grace E, Kotiw M and Barrow RA. Isochromophilone IX, a novel GABA-containing metabolite isolated from a cultured fungus, *Penicillium* sp. *Aust. J. Chem*. 56 (2003) 13-15.

Minor W, Steczko J, Stec B, Otwinowski Z, Bolin JT, Walter R, and Axelrod B. Crystal structure of soybean lipoxygenase L-1 at 1.4 Å resolution. *Biochemistry*. 35 (1996) 10687-10701.

Moody TR, Leyton J, Martinez A, Hong S, Malkinson A and Mulshine JL. Lipoxygenase inhibitors prevent lung carcinogenesis and inhibit non-small cell lung cancer growth. *Exp. Lung Res.* 24 (1998) 617-628.

Mortelmans K and Zeiger E. The Ames *Salmonella*/microsome mutagenicity test. *Mutation Research.* 455 (2000) 29-60.

Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods.* 16 (1983) 55-63.

Muller-Peddinghaus R, Kohlsdorfer C, TheisePopp P, Fruchtmann R, Perzborn E, Beckermann B, Buhner K, Ahr H-J and Mohrs K-H. BAY-X1005, a new inhibitor of leukotriene synthesis: *in vivo* inflammation, pharmacology and pharmacokinetics. *J. Pharmacol. Exp. Ther.* 267 (1993) 51-57

Myers CE and Ghosh J. Lipoxygenase inhibition in prostate cancer. *Eur. Urol.* 35 (1999) 395−398.

Nakamura M, Kino T, Niko K, Kyotto S and Okuhara M.. Phospholipase A2 inhibitors contaning sclerotiorin from *Penicillium sclerotiorum* for treatment of inflammatory pancreatitis, and allergy. *Japan Kokai Tokyo Koho*. JP 02255615A2 (1990).

Nam JY, Kim HK, Kwon JY, Han MY, Son KH, Lee UC, Choi JD and Kwon BM. 8-O-Methylsclerotiorinamine Antagonists of Grb2-SH2 domain isolated from *Penicillium multicolor. J. Nat. Prod*. 63 (2000)*a* 1303-1305.

Nam JY, Son KH, Kim HK, Han MY, Kim SU, Choi JD and Kwon BM. Sclerotiorin and isochromophilone IV: Inhibitors of Grb2-Shc interaction, isolated from *Penicillium multicolor* F1753. *J. Microbiol. Biotechnol*. 10 (2000)b 544-546.

Natsume N, Takahashi Y and Maruma S. Chlamydospore –like cell inducing substance of fungi: clso correlation between chemical reactivity with methylamine and biological activity. *Agri. Biol. Chem*. 52 (1988) 307-312.

Negishi Y, Matsuo N, Miyadera K. and Tanishima M. Lipase inhibitors containing sclerotiorin. *Japan Kokai Tokyo Koho*. JP 200091527A2. (2000).

Nelson MJ, Cowling RA and Seitz SP. Structural characterisation of alkyl and peroxyl radicals in solutions of purple lipoxygenase. *Biochemistry.* 33 (1994) 4966-4973.

Nelson MJ, Seitz SP and Cowling RA. Enzyme-bound pentadienyl and peroxyl radicals in purple lipoxygenase *Biochemistry.* 29 (1990) 6897-903.

Newman DJ. Cragg GM and Snader KM. Natural products as sources of new drugs over the period 1981–2002. *J. Nat. Prod*. 66 (2003) 1022-1037.

Nicholos MA. A spectrophotometer assay for iodide oxidation by thyroid peroxidase. *Anal. Biochem*. 4 (1962) 311- 345.

Nihei Y, Yamamoto H, Hasegawa M, Hanada M, Fukogawa Y and Oki T. Epocarbazolins A and B, novel 5-lipoxygenase inhibitors taxonomy, fermentation, isolation, structures and biological activities. *J. Antibiot.* 46 (1993) 25-33.

Nishio M, Hasegawa M, Suzuki K, Sawada Y, Hook J and Oki T. Nitrosoxacins A, B and C, new 5-lipoxygenase inhibitors. *J. Antibiot.* 46 (1993) 193-195.

Nutter CLN and Medvedeff ED The effect of leukotriene synthesis inhibitors in models of acute and chronic inflammation. *Arthritis. Rheum*. 39 (1996) 515-521.

Ohd JF, Nielsen CK., Campbell J, Landberg G, Lofberg H and Sjolander A. Expression of the leukotriene D_4 receptor CysLT1, COX-2, and other cell survival factors in colorectal adenocarcinomas. *Gastroenterology* 124 (2003) 57−70.

Ohkawa H, Ohishi, N and Yagi K. Assay for lipid peroxides in animal tissue by thiobarbituric acid reaction. *Anal. Biochem.* 95 (1979) 351-358.

Ohkuma H, Hoshino Y, Sawada Y and Hook D. Antibiotic 5-lipoxygenase inhibitors and their isolation from *Streptomyces*. US Patent 5208364 (1993).

Omaye ST, Turnbull JD and Sauberlich HE. Selected methods for the determination of ascorbic acid in animal cells, Tissues and Fluids. *Meth. Enzymol*. 62 (1973) 3-11.

Omura S, Tanaka H, Matsuzaki K, Ikeda H and Masuma R Isochromophilones I and II, novel inhibitors against gp120-CD_4 binding from *Penicillium* sp. *J. Antibiot.* 46 (1993) 1908-1911.

Otitow JCG and Glathe H. Rose bengal-malt extract-agar, a simple medium for the simultaneous isolation and enumeration of fungi and actinomycetes from soil. *Appl. Microbiol.* 16 (1968) 170-171.

Owais M, Sharad KS, Shehbaz A and Saleemuddin M Antibacterial efficacy of *Withania somnifera* (ashwagandha) an indigenous medicinal plant against experimental murine Salmonellosis. *Phytomedicine.* 12 (2005) 229-235.

Oxford AE, Raistrick H, and Simonart P. Studies in the biochemistry of microorganisms. LX. Griseofulvin, C17H1706C1, a metabolic product of *Penicillium griseofulvum* Dierckx. *Biochem. J*. 33 (1939) 240-248.

Ozasa T, Yoneda T, Hirasawa M, Suzuki K, Tanaka K, Kadota S and Iwanami M. New aldose reductase inhibitors from *Actinosynema* sp. *J. Antibiot.* 44 (1991) 768-773.

Pairet L, Wrigley SK, Chetland I, Reynolds EE, Hayes MA, Holloway J, Ainsworth AM, Katzer W, Cheng X-M, Hupe DJ, Charlton P and Doherty AM. Azaphilones with endothelin receptor binding activity produced by *Penicillium sclerotiorum*: taxonomy, fermentation, isolation, structural elucidation and biological activity. *J. Antibiot.* 48 (1995) 913-923.

Parker CW. Lipid mediators produced through the lipoxygenase pathway. *Annu. Rev. Immunol.* 5 (1987) 65-84.

Parks DR, Bryan VM, Oi VM, Oi VT and Herzenberg LA. Antigen-specific identification and cloning of hybridomas with a Fluorescence Activated Cell Sorter (FACS). *PNAS*, 76 (1979) 1962-66.

Pearce C. Biologically active fungal metabolites. *Adv. Appl. Microbiol.* 44 (1997) 1-80.

Petty MA An introduction to the origin and biochemistry of microbial halometabolites. *Microbiol. Mol. Biol. Rev*. 25 (1961) 111-130.

Peypoux F, Bonmatin JM and Wallach J. Recent trends in the biochemistry of surfactin. *Appl. Microbiol. Biotechnol.* 51 (1999) 553-563.

Pinto MC, Garcia-Barrado JA and Macias P. Resveratrol is a potent inhibitor of the dioxygenase activity of lipoxygenase. *J. Agric. Food Chem.* 47 (1999) 4842-4846.

Pistorius EK and Axelord B. An essential component of lipoxygenase. *J. Biol. Chem.* 249 (1974) 3183-3186.

Porta H and Rocha-Sosa M. Lipoxygenase in bacteria: a horizontal transfer event? *Microbiology.* 147 (2001) 3199-200.

Porter WL, Black ED and Drolet AM. Use of polyamide oxidative fluorescence test on lipid emulsions: contrast in relative effectiveness of antioxidants in bulk versus dispersed systems. *J. Agric. Food. Chem*. 37 (1989) 615-623.

Pourplanche C, Lambert C, Berjot M, Marx J, Chopard C, Alix A JP and Garde VL. Conformational changes of lipoxygenase (LOX) in modified environments. *J. Biol.Chem.* 369 (1994) 31585-31591.

Prasit P, Belley M, Blouin M, Brideau C, Chan C, Charleson S, Evans JF, Frenette R, Gauthier JY and Guay J. A new class of leukotriene biosynthesis inhibitor: the development of MK-0591. *J. Lipid Mediat.* 6 (1993) 239-244.

Prieto P, Pineda M and Aquilar M. Spectrophotometric quantification of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to determination of Vitamin E. *Anal. Biochem.* 269 (1999) 337-341.

Prigge ST, Boyington JC, Faig M, Doctor KS, Gaffney BJ and Amzel LM. Structure and mechanism of lipoxygenases. *Biochimie*. 79 (1997) 629-636.

Pryor WA, Strickland T and Church DF. Comparison of the efficiencies of several natural and synthetic antioxidants in aqueous sodium dodecyl sulfate micelle solutions. *J. Am. Chem. Soc*. 10 (1988) 2224-2229.

Przylipiak A, Hafner J, Przylipiak J, Kohn FM, Runnebaum B, and Rabe T. Influence of 5-lipoxygenase on *in vitro* growth of human mammary carcinoma cell line MCF-7. *Gynecol. Obstet. Invest.* 46 (1998) 61-64.

Qiu H, Gabrielsen A and Haeggstorm JZ. Expression of 5-lipoxygenase and leukotriene A_4 hydrolase in human atherosclerotic lesions correlates with symptoms of plaque instability. *Proc. Natl. Acad. Sci*. 103 (2006) 8161-8166.

Rader DJ and Daugherty A. Translating molecular discoveries into new therapies for atherosclerosis. *Nature* 451 (2008) 904-913.

Ramanarayan K, Bhat A, Shripathi V, Swamy GS and Rao KS. Triacontanol inhibits both enzymatic and nonenzymatic lipid peroxidation. *Phytochemistry* 55 (2000) 59-66.

Rao KCS, Divakar S, Rao AGA. Karanth NG, Suneetha WJ, Krishnakantha TP and Sattur AP Asperenone: an inhibitor of 15-lipoxygenase and human platelet aggregation from *Aspergillus niger*. *Biotechnol. Lett* 24 (2002)a 1967-1970.

Rao KCS, Divakar S, Babu KN, Karanth NG and Sattur AP Nigerloxin, a novel inhibitor of aldose reductase and lipoxygenase with free radical scavenging activity from *Aspergillus niger* CFR-W-105. *J. Antibiot* 55 (2002)b 789-793.

Rao KCS, Divakar S, Srinivas M, Babu KN, Karanth NG and Sattur AP. Asperaldin, a new aldose reductase inhibitor from *Aspergillus niger* CFR-1046. *J. Antibiot.* 56 (2003) 173-176.

Raper, KB and Thom C. A Manual of the Penicillia. London:Bailliere, Tindall and Cox (1949).

Ratty AK, Sunamoto J and Das NP. Interaction of flavonoids with 1,1-diphenyl-2-picrylhyrazyl free radical. Liposomal membranes and soybean lipoxygenase-1. *Biochem. Pharmacol.* 37 (1988) 989-995.

Reddy GR, Ueda N, Hada T, Sackeyfio AC, Yamamoto S, Hano Y, Aida M and Nomura T. A prenylflavone, Artonin E, as arachidonate 5-lipoxygenase inhibitor. *Biochem. Pharmacol*. 41 (1991) 115-118.

Reilly D and Curtin TP The Influence of Halide Concentration on the Metabolism of *Penicillium sclerotiorum* van Beyma. *Biochem. J.* 37 (1943) 36-39.

Reilly D, Long T and Curtin TP. The influence of sodium nitrate concentration on the metabolism of *Penicillium sclerotiorum* van beyma. *Biochem. J.* 38 (1944) 37-39.

Rioux N and Castonguay A. Inhibitors of lipoxygenase: a new class of cancer chemopreventive agents. *Carcinogenesis.* 19 (1998) 1393-1400.

Robak J, Shridi F, Wolbis M and Krolikowska M. Screening of the influence of flavonoids on lipoxygenase and cyclooxygenase activity, as well as on nonenzymatic lipid oxidation. *Pol. J. Pharmacol. Pharm.* 40 (1988) 451-458.

Roberts WG, Simon TJ, Berlin RG, Haggitt RC, Snyder ES and Stenson WF. Leukotrienes in ulcerative colitis: results of a multicenter trial of a leukotriene biosynthesis inhibitor, MK-591. *Gastroenterology.* 112 (1997) 725-732.

Robinson DS, Wu Z, Domoney C and Casey R. Lipoxygenase and the quality of foods. *Food Chem.* 54 (1995) 33-43.

Romano M, Catalano A, Nutini M, D'Urbano E, Crescenzi C and Claria J. 5 Lipoxygenase regulates malignant mesothelial cell survival: involvement of vascular endothelial growth factor. *FASEB J.* 15 (2001) 2326-2336.

Safayhi H, Boden SE, Schweizer S and Ammon HP. Concentration-dependent potentiating and inhibitory effects of Boswellia extracts on 5-lipoxygenase product formation in stimulated PMNL. *Planta. Med.* 66 (2000) 110-113.

Sailer ER, Subramanian LR, Rall B, Hoernlein RF, Ammon HP and Safayhi H. Acetyl-11-keto-beta-boswellic acid (AKBA): structure requirements for binding and 5-lipoxygenase inhibitory activity. *Brit. J. Pharmacol.* 117 (1996) 615-618.

Sameer AS, Mapari AS,.Meyer AS and Thrane U. Colorimetric characterization for comparative analysis of fungal pigments and natural food colorants. *J. Agric.Food Chem.* 54 (2006) 7027-7035.

Samson RA, Pitt JI, Advances in *Penicillium* and *Aspergillus*. Systematic north atlantic treaty, Plenum press, (1986) 483 pp.

Samuelsson B, Dahlen SE, Lindgren JA, Rouzer CA and Serhan CN. Leukotrienes and lipoxins: structures, biosynthesis, and biological effects. *Science.* 237 (1987) 1171-1176.

Sato Y, Taketomo T, Yoshiyama Y and Yokota I. Catechol derivative, its manufacture with *Streptomyces*, and its uses as anti-inflammatory and antiallergy agents. *Jpn. Kokai Tokkyo Koho* JP0436085 A2 (1992) Heisei, pp 6. CA: 118 167620.

Satosh Y and Yamazaki M. Studies on the monoamine oxidase (MOA) inhibitory potency of TL-1, isolated from a fungus *Talaromyces luteus*. *Chem. Pharm. Bull.* 31 (1989) 206-207.

Schewe T, Halangk W, Hiebsch Ch and Rapoport SM. A lipoxygenase in rabbit reticulocytes which attacks phospholipids and intact mitochondria. *FEBS Lett*. 60 (1975) 149-152.

Schewe T, Rapoport SM and Kuhn H. Enzymology of reticulocyte lipoxygenase: comparision with other lipoxygenases. *Adv. Enzymol. Relat. Areas. Mol. Biol.* 58 (1986) 191-272.

Schroder O, Sjostrom M, Qiu H, Stein J, Jakobsson PJ, and Haeggstrom JZ. Molecular and catalytic properties of three rat leukotriene C(4) synthase homologs. *Biochem. Biophys. Res. Commun.* 312 (2003) 271-276.

Sedlock J and Lindsay RH. Estimation of total, protein bound and nonprotein sulfhydryl groups in tissues with Ellman's reagent. *Anal. Biochem*. 25 (1968) 192-205.

Sekiguchi J and Gaucher GM. Conidiogenesis and secondary metabolism in *Penicillium urticae*. *Appl. Env. Microbiol.* 33 (1977) 147-158.

Sekiya K and Okuda H. Selective inhibition of platelet lipoxygenase by baicalein. *Biochem. Biophys. Res. Commun.* 5 (1982) 1090-1095.

Sekiya K, Okuda H and Arichi S. Selective inhibition of platelet lipoxygenase by esculetin. *Biochim. Biophys. Acta.* 713 (1982) 68-72.

Shannon VR, Chanez P, Bousquet J and Holtzman MJ. Histochemical evidence for induction of arachidonate 15-lipoxygenase in airway disease. *Am. Rev. Respir. Dis*. 147 (993) 1024-1028.

Shibata D and Axelrod B. Plant lipoxygenases. *J. Lipid. Mediat. Cell. Signal.* 12 (1995) 213-28.

Siedow JN. Plant lipoxygenase: structure and function. *Annu. Rev. Plant. Physiol. Plant. Mol. Biol.* 42 (1991) 145-188.

Simon A, Najid A, Chulia AJ, Delage C and Rigaud M. Inhibition of lipoxygenase activity and HL60 leukemic cell proliferation by ursolic acid isolated from heather flowers (*Calluna vulgaris*). *Biochim. Biophys. Acta.* 1125 (1992) 68-72.

Singh H and Soni SK. Production of starch-gel digesting amyloglucosidase by *Aspergillus oryzae* HS-3 in solid state fermentation. *Process. Biochem.* 37 (2001) 453-459.

Singh SA, Ramakrishna M and Rao AGA. Optimization of downstream processing parameters for the recovery of pectinase from the fermented bran of *Aspergillus carbonarius*. *Process. Biochem.* 35 (1999) 411-417.

Skrzypczak- Jankun E, Mc-Cabe NP, Selman SH, Jankun H. Curcumin inhibits lipoxygenase by binding to its central cavity: theoretical and X-ray evidence. *Int. J. Mol. Med*. 6 (2000) 521-526.

Snedecor GW and Cochran WG. *Statistical Methods*. 6th Edn., Ames, Iowa State Univ. Press, 1976, p.298.

Steele VE, Holmes CA, Hawk ET, Kopelovich L, Lubet RA, Crowell JA, Sigman CC and Kelloff GJ. Lipoxygenase inhibitors as potential cancer chemopreventives. *Cancer. Epidemiol. Biomarkers. Prevention* . 8 (1999) 467-483.

Stein GE and Craig WA. Tigecycline: a critical analysis. *Clin. Infect. Dis*. 43 (2006) 518-524.

Steinberg D At last, direct evidence that lipoxygenase a role in atherogenesis. *J. Clin. Invest*. 103 (1999) 1487-1488.

Strohl, WR. (1997) Biotechnology of Antibiotics, 2nd edn, Marcel Dekker.

Sudharshan E and Rao AGA. Rapid method to separate the domains of soybean lipoxygenase-1: identification of the interdomain interactions. *FEBS Lett*. 406 (1997) 184-188.

Summerfield FW and Tappel AL. Determination of fluorescence quenching of DNA cross-links made by malondialdehyde. *Biochim. Biophys. Acta*. 740 (1983) 185-189.

Tabata Y, Ikegami S, Yaguchi T, Sasaki T, Hashiko S, Sakma S, Shia-Ya K and Seto H. Diazaphilonic acid, a new azaphilone with telomerase inhibitory activity. *J. Antibiot*. 52 (1999**)** 412-414.

Takahashi N, Tamagawa K, Kawai K-I and Fukui T. Antioxidant properties of a new type of polyene, falconensone A and its derivatives. *Biol. Pharm. Bull.* 23 (2000) 989-994.

Takahashi N, Wahori A, Kawai K, and Tetsuya Fukui. Induction of differentiation in human promyelocytic cell line HL60 by a new type of polyenes, Falconensone A and Its derivatives Leukemia. *Arch. Biochem. Biophys*. 360 (1998) 113-120.

Takenaka Y, Tanahashi T, Nagakura N and Hamada N. Production of xanthones with free radical scavenging property, emodin and sclerotiorin by cultured lichen mycobionts of *Pyrenula japonica*. *Z. Naturforsch.* 55 (2000) 910-14.

Tang W and Eisenbrand G. (1992) Chinese drugs of plant origin: chemistry, pharmacology, and use in traditional and modern medicine. New York: Springer-Verlag

Tateson JE, Randall RW, Reynolds CH, Jackson WP, Bhattacherjee P and Salmon JA. Selective inhibition of arachidonate 5- lipoxygenase by novel acetohydroxamic acids: biochemical assessment *in vitro* and *ex vivo*. *Br. J. Pharmacol.* 94 (1988) 528-539.

Taussky HH and Shorr E. A micro colorimetric method for the determination of inorganic phosphorous. *J. Biol. Chem*. 202 (1953) 675-685.

Tomoda H, Matsushima C, Tabata N, Namatame I, Tanaka H, Bamberger MJ, Arai H, Fukazawa M, Inoue K and Omura S Structure-specific inhibition of cholesteryl ester transfer protein by azaphilones. *J. Antibiot* 52 (1999)160-170.

Tudhope SR, Cuthbert NJ, Abram TS, Jennings MA Maxey RJ and Thompson AM. BAYu9773, a novel antagonist of cysteinylleukotrienes with activity against two receptor subtypes. *Eur. J. Pharmacol.* 264 (1994) 317-323.

Turner G. In Chadwick DJ and Whelan J (eds), Secondary metabolites: their function and evolution. John Wiley and Sons, Chichester. (1992) pp 113.

Udagawa S. (-)-Sclerotiorin, a major metabolite of *Penicillium hirayamae*. *Chem. Pharm. Bull.* 11 (1963) 336-367.

Ulusu NN, Ercil D, Sakar MK and Tezcan EF. Abietic acid inhibits lipoxygenase activity. *Phytother. Res.* 16 (2002) 88-90.

Umezawa H. Low molecular weight enzyme inhibitors of microbial origin. *Annu. Rev. Microbiol.* 36 (1982) 75-99.

Van de Kerkhof PC, van Pelt H, Lucker GP, Steijlen PM, and Heremans A. Topical R-85355, a potent and selective 5-lipoxygenase inhibitor, fails to improve psoriasis. *Skin. Pharmacol*. 9 (1996) 307-311.

Vandamme EJ. The search for novel microbial fine chemicals, agrochemicals and biopharmaceuticals. *J. Biotechnol.* 37 (1994) 89-108.

Van-der-Zee J, Eling TE and Mason RP. Formation of free radical metabolites in the reaction between soybean lipoxygenase and its inhibitors. An ESR study. *Biochem*. 28 (1989) 8363-8367.

Veldink GA, Vliegenthart JFG and Bolding J. Plant lipoxygenases. *Prog. Chem. Fat. Other. Lipids.* 15 (1977) 131-166.

Vick BA and Zimmerman DC. Pathways of fatty acid hydroperoxide metabolism in spinach chloroplasts. *Plant. Physiol.* 85 (1987) 1073-1078.

Vila L. Cyclooxygenase and 5-lipoxygenase pathways in the vessel wall: role in atherosclerosis. *Med. Res. Rev.* 24 (2004) 399-424.

Wachtershauser A, Steinhilber D, Loitsch SM and Stein J. Expression of 5-lipoxygenase by human colorectal carcinoma Caco-2 cells during butyrate-induced cell differentiation. *Biochem. Biophys. Res. Commun*. 268 (2000) 778-783.

Wedi B and Kapp A. Pathophysiological role of leukotrienes in dermatological diseases: potential therapeutic implications. *Bio. Drugs.* 15 (2001) 729-743.

Weibel EK, Hadvary P, Hochuli E, Kupfer E and Lengsfeld H. Lipstatin, an inhibitor of pancreatic lipase, produced by *Streptomyces toxitricini*. I. Producing organism, fermentation, isolation and biological activity. *J. Antibiot.* 40 (1987) 1081-1085.

Weinblatt ME, Kremer JM, Coblyn JS, Helfgott S, Maier AL and Petrillo G. Zileuton, a 5-lipoxygenase inhibitor in rheumatoid arthritis. *J. Rheumatol.* 19 (1992) 1537−1541.

Weng SH, Su NW, Choong YM and Lee MH. HPLC/colorimeter combination method for quantitative analysis of sclerotiorin produced by *Penicillium sclerotiorum*. *Journal of Food and Drug Analysis*. 12 (2004) 199-204.

Werz O and Steinhilber D. Therapeutic options for 5-lipoxygenase inhibitors. *Pharmacology and Therapeutics* 112 (2006) 701-718.

Werz O, Szellas D, Henseler M and Steinhilber D. Nonredox 5- lipoxygenase inhibitors require glutathione peroxidase for efficient inhibition of 5-lipoxygenase activity. *Mol. Pharmacol.* 54 (1998) 445-451.

Whalley WB, Ferguson G, Marsh R and Restivo RJ. The chemistry of fungi. Part LXVIII. The absolute configuration of (+)- sclerotiorin and of the azaphilones. *J. Chem. Soc., Perkin I.* 13 (1976) 1366-1389.

Whitehead IM, Muller BL and Dean C. Industrial use of soybean lipoxygenase for the production of natural green note flavour compounds. *Cereal Foods World.* 40 (1995) 193-197.

Wilson B, Abraham G, Manju VS, Mathew M, Vimala B, Sundaresan S and Nambisan B. Antimicrobial activity of *Curcuma zedoaria* and *Curcuma malabarica* tubers. *J. Ethnopharmacol.* 99 (2005) 47-151.

Woodmansee DP and Simon RA. A pilot study examining the role of zileuton in atopic dermatitis. *Ann. Allergy. Asthma. Immunol.* 83 (1999) 548-552.

Yagi K. Assay of lipid peroxides in blood plasma or serum. *Meth. Enzymol.* 105 (1984) 328-331.

Yamamoto S, Suzuki H and Ueda N. Arachidonate 12-lipoxygenases. *Prog. Lipid. Res.* 36 (1997) 23-41.

Yamamoto Y and Nishikawa N. Studies on the metabolic product of *Penicillium implicatum* Biourge. II. The structure of sclerotiorin. *J. Pharm. Soc. Japan* 79 (1959) 297-302.

Yang DJ, Tomoda H, Tabata N, Masuma R and Omura S. Isochromophilones VII and VIII Produced by Penicillium sp. FO-4164. *J. Antibiot.* 49 (1995) 223-229.

Yasukwa K, Takahashi M, Natori S, Kawai K, Yamazaki M, Takeuchi M and Takido M. Azaphilones inhibit tumor promotion by 12-O- tetradecanoylphorbol-13-acetate in two stage carcinogenesis in mice. *Oncology.* 51 (1994) 108-112.

Yasumoto BK, Yamamoto A and Mitsuda H. Effect of phenolic antioxidants on lipoxygenase reaction. *Agric. Biol. Chem.* 34 (1970) 1162-1168.

Yen GC, Chang YC, Sheu F and Chiang HC. Isolation and characterisation of antioxidant compounds from *Aspergillus candidus* broth filterate. *J. Agric.Food Chem.* 49 (2001) 1426-1431.

Yokomizo T, Izumi T, Chang K, Takuwa Y, and Shimizu T. A G protein coupled receptor for leukotriene B_4 that mediates chemotaxis. *Nature.* 387 (1997) 620-624.

Yoshimoto T, Yokoyama C, Ochi K, Yamamoto S, Maki Y, Ashida Y, Terao S, and Shiraishi M. 2,3,5-Trimethyl-6-(12-hydroxy-5,10-dodecadiynyl)-1,4-benzoquinone (AA861), a selective inhibitor of the 5-lipoxygenase reaction and the biosynthesis of slow-reacting substance of anaphylaxis. *Biochim. Biophys. Acta.* 713 (1982) 470-473.

Yu BY. Cellular defenses against damage from reactive oxygen species. *Physiol. Rev.* 74 (1994) 139-162.

Zhi H, Zhang J, Hu G, Lu J, Wang X and Zhou C. The deregulation of arachidonic acid metabolism-related genes in human esophageal squamous cell carcinoma. *Int. J. Cancer.* 106 (2003) 327-333.

List of Publications

1. **C. Chidananda,** L. Jagan Mohan Rao and A.P. Sattur. Sclerotiorin, from *Penicillium frequentans,* a potent inhibitor of aldose reductase. *Biotchnol. Lett.* 2006*,* 28,1633-36.

2. **C. Chidananda** and A.P Sattur. Sclerotiorin, a novel inhibitor of lipoxygenase from *Penicillium frequentans. J. Agric. Food. Chem.* 2007**,** 55, 2879-2883.

3. **C. Chidananda,** C. Mohan Kumar and A.P. Sattur. Strain Improvement of *Aspergillus niger* for the Enhanced Production of Asperenone. *Indian Journal of Microbiology.* (**In press**)

Papers to be communicated:

4. **C. Chidananda**, H. Manjunatha, K. Srinivasan and A.P.Sattur. Antioxidant potential of sclerotiorin from *Penicillium frequentans. J. Nat. Prod.* (**communicated**)

5. **C. Chidananda**, Saleem Javeed and A.P. Sattur A fungal azaphilone, Sclerotiorin, an inhibitor of human PMNL 5 –Lipoxygenase. *Appl. Biochem. Biotechnol.* (**communicated**)

6. **C. Chidananda** and A.P. Sattur. Anticancer property of sclerotiorin from *Penicillium frequentans* **(Under preparation)**

7. **C. Chidananda** and A.P. Sattur Process optimization for sclerotiorin production from *Penicillium frequentans*. *Process biochemistry*. (**Under preparation**)

Papers presented at Conferences / Symposia:

1. C. Chidananda, L. Jagan Mohan Rao and A.P. Sattur
Sclerotiorin, from *Penicillium frequentans,* is a potent inhibitor of rat eye lens aldose reductase.
Presented at International conference on New Bioactive Molecules In Pharmaceutical Research, Contribution Of Natural Products. (INDO-US-CCNP-2006) 13th-14th Nov-2006, IICT, Hydra bad, INDIA

2. Chidananda C and A.P Sattur.
Sclerotiorin, a potent novel inhibitor of lipoxygenase and a free radical former
Presented at AMI 46th Annual conference (Micro-Biotech 2005) 8th–10th Dec 2005,Osmania University, Hydra bad, INDIA