PURIFICATION AND CHARACTERIZATION OF THERAPEUTIC PROTEIN FROM *NERIUM ODORUM*

A THESIS

Submitted in partial fulfilment of the requirements for the award of the degree

of

DOCTOR OF PHILOSOPHY

in

BIOTECHNOLOGY

By

SEEMA PARVEEN

CENTRAL LIBRARY
ACC. No. G14067
DATE 21/7/09
IIT ROORKEE
23/7/09

DEPARTMENT OF BIOTECHNOLOGY
INDIAN INSTITUTE OF TECHNOLOGY ROORKEE
ROORKEE-247 667 (INDIA)

MARCH, 2008

©INDIAN INSTITUTE OF TECHNOLOGY ROORKEE, ROORKEE, 2008
ALL RIGHTS RESERVED

INDIAN INSTITUTE OF TECHNOLOGY ROORKEE
ROORKEE

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in this thesis entitled, **PURIFICATION AND CHARACTERIZATION OF THERAPEUTIC PROTEIN FROM *NERIUM ODORUM*** in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Biotechnology of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during a period from January 2003 to March 2008 under the supervision of Dr. Ramasare Prasad, Associate Professor, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee.

The matter in this thesis has not been submitted by me for the award of any other degree of this or any other Institute.

Seema Parveen
10/03/2008
(SEEMA PARVEEN)

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

(Ramasare Prasad)
Supervisor

Dated: 10.3.2008

The PhD Viva-Voce Examination of **Ms. Seema Parveen**, Research Scholar has been held on .....30.06.2008.....

Signature of Supervisor

Signature of External Examiner

ACKNOWLEDGEMENTS

It is a great pleasure to acknowledge those individuals who inspired and encourages me to fulfill this herculian task and without their help and co-operation this work would not have been possible, therefore, I take this opportunity to express my feelings and sense of gratitude to all of them.

I express my deepest and profound gratitude to my supervisor Dr. Ramasare Prasad, Department of Biotechnology, Indian Institute of Technology Roorkee, for his miraculous guidance discreet support and keen interest throughout the course. His unbounded affection, inspirational guidance, unfailing enthusiasm, and tireless interest make me remain indebted to him. His earnest desire for my success will always be my pride and my devotion and respect for him will be forever.

I have deep and sincere sense of gratitude towards Prof. Ben. M. J. Pereira, Head, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee, for his constant encouragement and providing me all in-house research facilities and an excellent working environment in the department. I would like to take the opportunity to thank Prof. G.S. Randhawa, Prof. H.S. Dhaliwal, Prof. R.P. Singh, Dr. V. Pruthi, and Dr. P. Roy, faculty members of this department for their valuable suggestions and encouragement during this period.

I would like to express sincere thanks to Dr. Sujata Sharma, and Dr. Mau Sinha for N-terminal sequencing of the protein in the Department of Biophysics. All India Institute of Medical Sciences (AIIMS), New Delhi. I thank Mrs. Chanda for Transmission Electron Microscope facility in the Department of Microscopy, All India Institute of Medical Sciences (AIIMS), New Delhi. I would like to acknowledge Dr.

Som Dutta, Head of the proteomic facility, and other members of the facility in The Centre of Genomic application (TCGA), New Delhi for helping mass spectrometry analysis and internal sequencing of the protein.

I am thankful to Dr. Hriyadesh Prakash, Mrs. Suwagmani Das, and Mr. Santosh Kumar for helping me to carry out some of the Immunology and Animal tissue culture experiments.

This work would not be possible without the help and support of all the members of the office staff. I appreciate and wish to express my heartfelt thanks to my colleagues Ajeet Singh, Ashish Deep Gupta, Nilesh Kumar Sharma, Garima Sharma, Sreela Dey, Sunity, Alok Jha, Anju Bhalla, P. Osman Basha, and Anindita Mukerjee for their complete support in all possible ways during this period.

Last, but not least I want to thank my father, mother, brother, sister and my whole family and close relatives for their blessing and unconditional support, who provided me strength and confidence to attain this academic level.

Finally, I thankfully acknowledge All India Council for Technical Education (AICTE) for providing me financial support during this period.

Seema Parveen
10/03/2008

(SEEMA PARVEEN)

CONTENTS

LIST OF FIGURES

LIST OF TABLES

INDIAN INSTITUTE OF TECHNOLOGY

ABBREVIATIONS USED

MTCC	Microbial Type Culture Collection
ITCC	Indian Type Culture Collection
CFU	Colony Forming Unit
LPS	Lipopolysaccharide
PHA	Phytohemagglutinin
Con A	Concanavalin A
TNF-α	Tumor necrosis Factor-alpha
IL-2	Interleukin-2
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
MALDI-TOF	Mass assisted Laser Desorption Ionization- Time of flight
LC-MS/MS	Liquid Chromatography-Mass Spectrometry/Mass Spectrometry
NCBI	National Center for Biotechnology Information
MSDB	Mass spectrometry data base
BLAST	Basic Local Alignment and Search Tool
EDTA	Ethylenediamine tetraacetic acid
DEAE	Diethylaminoethyl
CM	Carboxymethyl
RPMI 1640	Roswell Park Memorial Institute Media 1640
PDA	Potato dextrose agar
YPD	Yeast peptone dextrose
TFA	Trifluoroacetic acid
pNPP	*p*-nitrophenyl phosphate
SDS	Sodium dodecyl sulfate
PAGE	Poly-acrylamide Gel Electrophoresis
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochlroide
ELISA	Enzyme linked immunosorbant assay
RBC	Red blood cells

WBC	White blood cells
LDH	Lactate dehydrogenase
S.E	Standard Error
%	Percentage
µg	Microgram
µm	Micrometer
mm	millimetre
cm	centimeter
Fig.	Figure
mg	Milligram
ml	Millilitre
mM	millimole
°C	Degree centigrade
kDa	Kilo dalton
v/v	Volume/Volume
w/v	Weight/Volume
sec	Second
h	Hour
min	Minute
g	Gram
l	Litre
M	Molarity
µ	micron
α	Alpha
β	Beta
Ala, A	Alanine
Arg, R	Arginine
Asn, N	Asparagine
Asp, D	Aspartic acid
Cys, C	Cystine
Gln, Q	Glutamine

Glu, E	Glutamic acid
Gly, G	Glycine
His, H	Histidine
Iso, I	Isoleusine
Leu, L	Leucine
Lys, K	Lysine
Met, M	Methionine
Phe, F	Phenylalanine
Pro, P	Proline
Ser, S	Serine
Thr, T	Threonine
Trp, W	Tryptophan
Tyr, Y	Tyrosine
Val, V	Valine

CHAPTER 1

INTRODUCTION

The world we live in is full of various harmful microorganisms. Our body temperature and wealth of nutrients provide an ideal home for these organisms to thrive. This has lead to the development of various serious infectious diseases which cause great suffering and burden on human health. Besides, some of the harmful microorganisms also cause serious diseases in plants that affect crops productivity. All animals and plants have developed one or other type of defense mechanism to protect themselves from these pathogenic microorganisms (Tenover, 2006; Sanglard and Odds, 2002). However more often these protections failed, pathogen invades the system and we need to use other means to combat them. Chemotherapy has been the common approach to control these diseases and a large number of chemical agents and antibiotics have been developed or synthesized (Lee, 2005). They have been very effective in controlling the infections but widespread and indiscriminate use of these drugs has resulted in emergence of drug resistance (Lee, 2005) and more recently multidrug resistant pathogenic strains for various human and plant pathogens. The control of infection by these resistant and multidrug resistant strains has been a great challenge mainly because of the existing armory of drug/antibiotics becomes failed to control these infections. In addition, these conventional synthetic drugs and microbial origin antibiotics have side effects (Butler and Buss, 2006). Therefore, there is an urgent need to search for a safer and effective alternative to control and treat these infections. The search for new safer and effective drug which do not cause resistant development is one of the thrust area of biomedical and agriculture sciences (Butler and Buss, 2006).

Attempts have been made to isolate therapeutic agents from diverse group of organisms including microbes (Chernin *et al.*, 1997), fungi (Hao *et al.*, 1999; Hao *et al.*,

2000), animals (Iijima *et al.*, 1993) and plants (Lee *et al.*, 2007; Huynh *et al.*, 1992). Because of the highly diversified flora and their diversified phytochemical constituents, plants are thought to be a better choice for search of new therapeutic agents (Butler and Buss, 2006). Among the numerous compounds, bioactive proteins have been found of prime importance because they often have very specific interactions with a macromolecular target in the body. On the basis of their beneficial health effects they may be classified as antimicrobial, antioxidative, antidiabetic, antithrombotic, antihypertensive, anticancer and immunomodulatory (Liu *et al.*, 2007; Ghosh and Maiti, 2007; Hsu *et al.*, 1997; Fisher and Yang, 2002; Sheu *et al.*, 2004). Among the various biologically active proteins identified, the present discussion is mainly inclined towards antimicrobial and immunomodulatory proteins. A large number of antimicrobial proteins/peptides (AMP) have been isolated from various plant sources (Wang and Ng, 2002; Hao *et al.*, 1999; Hao *et al.*, 2000; Lee *et al.*, 2007; Huynh *et al.*, 1992; Iijima *et al.*, 1993) and extensive research has been done on their structure and mode of action (Brogden, 2005; Otvos Jr *et al.*, 2005). The best known plant's antimicrobial proteins comprising thaumatin-like proteins (Chu and Ng, 2003; Graham *et al.*, 1992; Huynh *et al.*, 1992; Pressey, 1997; Vu and Huyhn, 1994; Wurms and Greenwood, 1999; Ye and Ng, 2000), chitinases (Van Damme *et al.*, 1993; Vergauwen, 1993), chitinase-like proteins (Lam *et al.*, 2000; Ye *et al.*, 2000) , chitin binding proteins (Huang *et al.*, 2000; Van Den Bergh *et al.*, 2004), defensins, defensin-like proteins (Ngai and Ng, 2004; Wong and Ng, 2003; Wong and Ng, 2005), miraculin-like proteins (Ye and Ng, 2000), embryo-abundant proteins (Wang and Ng, 2000), ribosome inactivating proteins (Leah *et al.*, 1991), lipid transfer protein-like proteins (Cammue *et al.*, 1995; Wang *et al.*, 2004),

protease inhibitors (Chen et al., 1998; Chilosi *et al.*, 2000; Joshi *et al.*, 1998; Loritoet *et al.*, 1994) and new proteins/peptides (Chu *et al.*, 2005; Ng and Wang, 2000; Xia and Ng, 2005). A 42 kDa chitin-binding proline-rich protein (PRP) from French bean has been characterized through its involvement in plant-pathogen interactions (Bindschedlera *et al.*, 2006). An antifungal protein was purified from *Alocasia esculenta* rhizomes showing activity toward *Botrytis cinerea* and resembles pisavin, the miraculin-like anti-fungal protein from sugar snap in N-terminal sequence (Wang and Ng, 2003). Ginkbilobin, a novel antifungal protein from *Ginkgo biloba* seeds was isolated with sequence similarity to embryo-abundant protein (Wang and Ng, 2000). An antibacterial and antifungal protein was also isolated from *Indigofera oblongifolia* leaves (Dahot, 1999) with activity toward *Escherichia coli*, *Klebsiella aerogenes*, *K. pneumoniae*, *Staphylococcus aureus*, *Bacillus subtilis*, *Aspergillus* fumigatus, *A. niger* and *A. flavus*. A 21 kDa thaumatin-like protein has been isolated from green kiwi fruits, and exhibits HIV-1 reverse transcriptase inhibitory activity and antifungal activity against a number of fungi (Lixin and Ng, 2004). There are several excellent review articles discussing various aspects of AMP (Ghosh and Maiti, 2007; Wasser, 2002; Winder *et al.*, 1998; Wong and Ng, 2005).

The immune system plays an important role in the protection of the body from disease. It protects against not only those diseases which result from an attack by bacteria, virus, and other pathogens, but also cancer, as well as disease states which result from immune imbalance; opportunistic infections, or autoimmune disorders. Modulation of the immune system through pharmaceutically induced stimulation or suppression offers an important approach to the control of diseases. Therefore, search for the novel immunomodulatory agents have been one of the important area of biomedical research. A

number of naturally occurring proteins from plants with *in* vitro and/or *in vivo* immunomodulatory activities have been reported from various plants (Pugh *et al.*, 2001; Wasser, 2002; Hsu *et al.*, 2004; Ou *et al.*, 2005; Liu *et al.,* 2007; Hsu *et al.,* 1997; Vivanco 1997). Some of the known immunomodulatory proteins are 13 kDa LZ-8 protein from *Ganoderma lucidum,* 15 kDa Fip-*vvo* protein from *Volvariella volvacea,* 31.5 kDa and 205 kDa lectins from *Agrocybe cylindracea,* and *Cteropharyngodon idellus*, 13.8 kDa napin-like polypeptide from chinese cabbage seed, arabinogalactan (AG) from *Nerium oleander* and arabinogalactan-proteins (AGPs) from *Echinacea purpure.*

In recent few years, there has been sudden increase in bacterial and fungal infections caused by opportunistic and drug resistant pathogens particularly in immuno-compromised host and patients who has gone for transplant surgery. A therapeutic molecule with inherent properties of both antimicrobial as well as immunomodulatory activity will be more effective in controlling and treating the antimicrobial infections in general and in immunocompromised hosts in particular. As such novel molecules will have dual action, first by killing the pathogen and second by boosting the immune system of the host, but finding such molecules is difficult task. However, the observations that several antimicrobial proteins/peptides besides their antimicrobial action also found to have immunomodulatory effects provide strong basis that such proteins do exist in nature. Keeping this fact in mind, an attempt has been made in the present study to search for such a novel proteins from *Nerium odorum* a well known medicinal plant.

Nerium odorum Syn. *N. indicum (*Mill) common name oleander is a widely distributed evergreen plant belonging to the family of Apocynaceae. It is a large, erect,

stout shrub with milky juice, linear lanceolate, thickly coriaceous, acuminate leaves, with faintly sweet-scented white or red colored flowers. *N. odorum* is well known for its medicinal uses in immune deficient diseases including cancer and AIDS. Whole plant is said to have anticancer properties (Manna et al, 2000). An immunologically active acidic pectic polysaccharide has been isolated and characterized from the leaves (Mueller et al, 1991). Oleandrin, a polyphenolic cardiac glycoside from leaves possess anti-inflammatory and tumor cell growth-inhibitory effects (Stenkvist, 1999). The fresh juice of leaves is dropped into the eyes for inducing lachrymation in ophthalmia (Hussain, and Gorsi, 2004). A decoction of the leaves has been applied externally in the treatment of scabies (Kirtikar et al, 1975). Paste of the root is used as an external application in hemorrhoids, and ulcerations (Chopra et al, 1956). The root of this plant is a good tonic for chronic pain in the abdomen and joints and used as an antidote to snake-venom (Hussain and Gorsi, 2004). Oil extracted from the root bark is used in leprosy and other diseases of a scaly nature. However, there is no report on the study of therapeutic proteins from this plant. The present work was emphasized to search a protein with potential antimicrobial and immunomodulatory activity from *N. odorum*. This study was undertaken with the following objectives:

1. Isolation and purification of antimicrobial protein from the leaves of *N. odorum*.
2. Characterization of purified protein by different biochemical methods (SDS PAGE and HPLC) and biophysical methods (N-terminal sequencing, and mass spectrometric technique).
3. Evaluation of antimicrobial potential of purified protein using standard methods (disk diffusion and broth dilution assays, SEM and TEM).

4. Evaluation of immunomodulatory activity of purified protein in *in vitro* and *in vivo* system.
5. Evaluation of cytotoxicity of purified protein.

CHAPTER 2

LITERATURE REVIEW

2.1. Brief overview

The medicinal value of plants has been known since ancient times. People on all continents have used poultices and imbibed infusion from thousands of indigenous plants for various aliments (Lee, 2005; Akinpelu and Onakoya, 2006; Heinrich, 2003). Indian Ayurvedic medicine, Unani and Chinese system of medicines have well documentations of a large number medicinal plants and there possible uses (Fabricant and Farnsworth, 2001; Guleria and Kumar, 2006; Janovská, 2003). Thus, the plants are the oldest source of pharmacologically active compounds, and have provided humankind with many medicinal useful compounds for centuries (Cordell, 1981). However, in spite of rich diversified antimicrobials and other therapeutics constituents, since the advent of antibiotics in the 1950s, the use of plant derivatives as antimicrobials has been virtually ignored, as the emphasis was given to the search and development of microbial origin drugs. Diversified group of bacteria and fungi from various sources were collected from all over the world and screened for new antibiotics which lead to an impressive arsenal of antimicrobial agents such as the cephalosporins, tetracyclines, aminoglycosides, rifamycins, and chloramphenicol by the early 1970s. It was believed that virtually any bacterial infection could be treated by the use of these antibiotics. However, the euphoria over the potential conquest of infectious diseases was proven wrong in short time due to following main factors:

1. Prolong and indiscriminate use of these microbial antibiotics gave rise to various drug resistant and more recently multi drug resistant strains worldwide. Hospitals have become breeding grounds for human associated micro organisms (Mainous and Pomeroy, 2001).

2. The alarming increase in the incidence of bacterial and fungal diseases, due to diseases caused by these drug resistant strains and also by opportunistic pathogens in immunocopromised host, patients with organ transplants and AIDS patients.
3. Nonetheless, the same time-bomb effect is also found to be developing with plant and animal associated pathogens in commercially driven activities, such as aquaculture and confined poultry breeding, where the indiscriminate use of antibiotics is perceived as essential for industries survival.
4. In addition, these side effects are associated with these commonly used antibiotics.

The control and treatment of infectious diseases caused by these resistant strains has presently become one of the major health problems globally. Consequently, there is an urgent need to search for safer alternatives to synthetic antibiotics. Once again the attention has been drawn towards plants, due to its century's old established therapeutic potentials and thought to be relatively safer. A wide variety of active phytochemicals with different biological activity have been reported from plants but the present discussion focuses mainly around the antimicrobial agent.

2.2. Major groups of antimicrobial compounds from plants

Plants have an almost limitless ability to synthesize wide variety of phytochemicals. These phytochemical are divided into different categories according to their mechanism of function like chemotherapeutic, bacteriostatic, bactericidal and antimicrobial (Cowan, 1999). In many cases, these substances serve as plant defense mechanisms against predation by microorganisms, insects and herbivores. Some, such as terpenoids, give plants their odors; others (quinones and tannins) are responsible for plant

pigmentation. Many compounds are responsible for plant flavour (e.g., the terpenoid capsaicin from chili peppers), and some of the herbs and spices used by humans to season food yield useful medicinal compounds. Useful antimicrobial phytochemicals can be divided into several categories, summarized in Table 1. A brief description of various antimicrobial compounds are given below, however, the antimicrobial proteins/peptides have been discussed separately in great details as the focus of the present study is on plant derived antimicrobial proteins.

2.2.1. Phenolics and Polyphenols

2.2.1.1. Simple phenols and phenolic acids

A number of phenol and phenolic acids are thought to be toxic to microorganism. Caffeic acid from terragon and thyme is effective against virus (Wild, 1994), bacteria (Thomson, 1978), and fungi (Duke, 1985). Catechol and pyrogallol both are hydroxylated phenols and are toxic to microorganisms. Eugenol is a phenolic compound from clove oil is having antimicrobial activity (Thomson, 1978; Duke, 1985).

2.2.1.2. Quinones

Quinones are ubiquitous in nature and are highly reactive compounds containing aromatic rings with two ketone substitutions. Anthraquinone isolated from *Cassia italica*, and shows antimicrobial activity against *Pseudomonas pseudomalliae, P. aeruginosa*, and *Corynebacterium pseudodiphthericum,Bacillus anthracis*. (Kazmi *et al.*, 1994).

Table 1. Major classes of antimicrobial compounds from plants (Cowan, 1999)

Class	Subclass	Example(s)	Mechanism	References
Phenolics	Simple phenols	Catechol Epicatechin	Substrate deprivation Membrane disruption	Wild, 1994
	Phenolic acids	Cinnamic acid	-	Duke, 1985
	Quinones	Hypericin	Bind to adhesins, complex with cell wall, inactivate enzymes	Kazmi *et al.*, 1994
	Flavonoids	Chrysin	Bind to adhesions	Hunter, and Hull, 1993
	Flavones	-	Complex with cell wall	Batista *et al.*, 1994
		Abyssinone	Inactivate enzymes Inhibit HIV reverse transcriptase	
	Flavonols	Totarol	-	Fessenden, and Fessenden, 1982
	Tannins	Ellagitannin	Bind to proteins Bind to adhesins Enzyme inhibition Substrate deprivation Complex with cell wall Membrane disruption Metal ion complexation	Scalbert, 1991
	Coumarins	Warfarin	Interaction with eucaryotic DNA (antiviral activity)	O'Kennedy and Thornes, 1997
Terpenoids, essential oils		Capsaicin	Membrane disruption	Cowan, 1999
Alkaloids		Berberine Piperine	Intercalate into cell wall and/or DNA	Omulokoli *et al.*, 1997
Peptides and lectins		Fabatin Mannose-specific agglutinin	Form disulfide bridges Block viral fusion or adsorption	Zhang and Lewis, 1997; Meyer *et al.*, 1997

2.2.1.3. Flavones, flavonoids and flavonols

Flavones are phenolic structures containing one carbonyl group. The addition of a 3-hydroxyl group yields a flavonol (Fessenden and Fessenden, 1982). Phloretin, an isoflavone isolated from apples, has antimicrobial activity against a variety of

microorganisms (Hunter and Hull, 1993). Galangin (3,5,7-trihydroxyflavone) was isolated from *Helichrysum aureonitens*, shows antimicrobial activity against a wide range of gram-positive bacteria as well as fungi and viruses, in particular HSV-1 and coxsackie B virus type 1 (Afolayan, and Meyer, 1997). Catechins is the most reduced form of the C3 unit in flavanoid compounds and showed *in* vitro antimicrobial activity against *Streptococcus mutans* (Batista *et al.,* 1994), *Vibrio cholerae* (Borris, 1996), *Shigella* (Sakanaka *et al.,* 1989; Sakanaka *et al.,* 1992), and other bacteria and microorganisms.

2.2.1.4. Tannins

Tannins are polymeric phenolic substances and are divided into two groups, hydrolyzable and condensed tannins. Alternatively, tannins may be formed by polymerization of quinone units (Geissman, 1963). In 1991, Scalbert (Scalbert, 1991) reviewed 33 studies on antimicrobial properties of tannins, according to these studies, tannins can be toxic to filamentous fungi, yeasts, and bacteria. Condensed tannins bind to the cell walls of ruminal bacteria thereby preventing the growth and protease activity of microorganism (Jones *et al.,* 1994). Tannins are considered to be responsible for the antibiotic activity of methanolic extracts of the bark of *Terminalia alata* (Taylor *et al.,* 1996).

2.2.1.5 Coumarins

Coumarins are phenolic substances made up of fused benzene and α-pyrone rings (O'Kennedy and Thornes, 1997). Coumarin has been used to prevent recurrences of cold sores caused by HSV-1 in humans (Berkada, 1978). Hydroxycinnamic acids are member of coumarins and seem to be inhibitory to gram positive bacteria (Fernandez *et al.,* 1996).

Phytoalexins, which are hydroxylated derivatives of coumarins, isolated from carrots, showed antifungal activity (Hoult and Paya, 1996).

2.2.2. Terpenoids and Essential Oils

The fragrance of plants is due to essential oil fraction. These oils are secondary metabolites that are highly enriched compounds based on an isoprene structure. They are called terpenes, their general chemical structure is $C_{10}H_{16}$, and they occur as diterpenes, triterpenes, and tetraterpenes (C_{20}, C_{30}, and C_{40}), as well as hemiterpenes (C_5) and sesquiterpenes (C_{15}) (Cowan, 1999). When the compounds contain additional elements, usually oxygen, they are termed as terpenoids.

Terpenenes or terpenoids are active against bacteria (Amaral *et al.,* 1998; Barre *et al.*, 1997; Kubo *et al.,* 1992; Mendoza *et al.,* 1997), fungi (Rana *et al.,* 1197; Suresh *et al.,* 1997), viruses (Fujioka and Kashiwada, 1994; Sun *et al.,* 1996; Xu *et al.,* 1996), and protozoa (Ghoshal, 1996). The triterpenoid betulinic acid shows inhibitory activity against HIV. The mechanism of action of terpenes is not fully understood but is speculated to involve membrane disruption by the lipophilic compounds. Food scientists have found the terpenoids present in essential oils of plants to be useful in the control of *Listeria monocytogenes* (Aureli *et al.,* 1992).

2.2.3. Alkaloids

Heterocyclic nitrogen compounds are called alkaloids. The first medically useful alkaloid, morphine was isolated in 1805 from the opium poppy *Papaver somniferum.* Diterpenoid alkaloids, commonly isolated from the plants of the Ranunculaceae, are commonly found to have antimicrobial properties (Omulokoli *et al.,* 1997). Solamargine, a glycoalkaloid from the berries of *Solanum khasianum*, and other alkaloids may be

useful against HIV infection (McDevitt *et al.,* 1996) as well as intestinal infections associated with AIDS. Berberine is an alkaloid, inhibits trypanosomes and plasmodia (Freiburghaus, *et al.,* 1996).

2.3. Antimicrobial peptides and proteins

Plants and their products have been used for centuries to prevent and cure diseases. More than a quarter of all the medicines used in the world today contain ingredients derived from plants (Luzhetskyy *et al.,* 2007). There are two classes of antimicrobial peptides are, non-ribosomally synthesized (Hancock and Chapple, 1999) present in bacteria, lower eukaryotes and plants; and ribosomally synthesized peptides of wider distribution (Broekaert *et al.*, 1997; Zasloff, 2002). The knowledge acquired in the past two decades and the discovery of new groups of antimicrobial peptides makes natural antibiotics, the basic element of a novel generation of drugs for the treatment of bacterial and fungal infections (Butler and Buss, 2006; Chin *et al.*, 2006; Selitrennikoff, 2001). Because of their important role in protection against infections, the search for antimicrobial peptides (AMP) and proteins have been one of the thrust areas of research globally and large number of AMP have been isolated and characterized from diversified sources (Dahanukar *et al.*, 2000; Fernandez *et al.*, 1996; Guerbette *et al.*, 1999; Guo *et al.*, 1999; Koehn F.E. and Carter G.T. 2005; Ghebremichaela *et al.,* 2005; Sharma *et al.*, 2004; Vivanco *et al.*, 1999; Silphaduang *et al.*, 2006). Over the past 20 years, a wide array of antimicrobial proteins has been discovered throughout plant and animal kingdoms, which provide insights into the innate defensive system and has attracted increasing attention recently (Zasloff, 2002). It is well known that plants have no immune

system but can live in harmony with various microbial pathogens. They have evolved highly effective defense mechanisms to prevent the invasion of microorganisms. The wide spread of antimicrobial proteins, produced either in a constitutive or in an inducible manner, are believed to be involved in these mechanisms (Broekaert *et al.,* 1997; Xiang *et al.,* 2004; Fritig *et al.,* 1998; Agrawal *et al.,* 2000; Almeida *et al.,* 2000). The diversity of AMPs is so high that it is difficult to categorize them except broadly on the basis of their secondary structure (Marshall and Arenas, 2003; Reddy *et al.*, 2004) Basically, AMPs can be classified in four major classes: β-sheet, α-helical, loop and extended peptides (Brodgen, 2005). In addition to the natural peptides, thousands of synthetic variants have been produced which also fall into these structural classes. A rich collection of all known AMPs can be found in the Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.php). The Antimicrobial Peptide Database offers an interface to predict the antimicrobial activity of any submitted sequence, based on a simple residue analysis and count method and some useful statistical information on peptide. Extensive studies have been performed revealing their structure and mode of action which has been described in detail in several recent reviews (Marshall and Arenas, 2003; Reddy *et al.*, 2004; Brodgen, 2005). Based on its amino acid composition, structural features and mode of action, antimicrobial proteins are classified in different groups. The best known plant antimicrobial peptides and proteins are pathogenesis related proteins (PR), hydroxyproline rich glycoproteins, cycophilin-like proteins, ribosome-inactivating proteins, lipid transfer proteins, lectins and protease inhibitors. These groups of protein are described below in bit more details.

2.3.1. Pathogenesis related protein (PR protein)

Plants when exposed to microbes such as fungi and viruses produce low molecular weight antimicrobial compounds called phytoalexins, antimicrobial peptides, and small proteins (e.g., heveinlike proteins, and knottin-like peptides (Segura *et al.,* 1993), defensins (Broekaert *et al.,* 1995), thionins (Bloch *et al.,* 1998; Florach and Stiekema, 1998)) and large antimicrobial proteins. These plant proteins, called pathogenesis-related (PR) proteins are classified on the basis of serological and amino acid sequence analysis, PR have been classically divided into five groups, PR-1, 2, 3, 4, and 5 (Van Loon, 1985). Each of the five classical groups of PR proteins has two subclasses: a basic subclass found in the plant cell vacuole and an acidic subclass usually found in the extracellular space (Kitajima and Sato, 1999). Each group has members with antimicrobial activity, and cognates of most groups have been found in a diversity of other organisms.

2.3.1.1. PR-1 proteins

PR-1 proteins are produced after pathogen infection and are antifungal both in plants (transgenic plants overexpressing tobacco PR-1) and *in* vitro (Niderman *et al.,* 1995). PR-1 proteins have been found in rice, wheat, maize, tobacco, *Arabidopsis thaliana* barley, and many other plants. PR-1 proteins have antifungal activity at the micromolar level against a number of plant pathogenic fungi, including *Uromyces fabae, Phytophthora infestans*, and *Erysiphe graminis* (Niderman *et al.,* 1995). PR-1 proteins have molecular masses of 15 to 17 kDa and have homology to the superfamily of cysteine-rich proteins. Precise mechanism of antifungal activity is not understood for plant PR-1 proteins.

2.3.1..2. PR-2 proteins (β -glucanases)

PR-2 proteins have β-endoglucanase *in vitro* activity and on the basis of amino acid sequence PR-2 have been categorized into three classes (Beffa and Meins, 1996; Leah, 1991; Nielsen, 1997). Class I glucanases are basic proteins of 33 kDa and are found in the plant vacuole, while Classes II and III are acidic, extracellular proteins of 36 kDa. PR-2 proteins have been found in a wide variety of plants, including tobacco, *Arabidopsis thaliana*, peas, grains, and fruits (Kim and Hwang, 1997; Rezzonico *et al.*, 1998). The PR-2 proteins are active against number of fungi at micromolar levels (~50 μg/ml), including human and plant pathogens (e.g., *Rhizoctonia solani, Candida. albicans*, and *Aspergillus fumigatus*). PR-2 proteins by hydrolyzing the structural (1-3) β-glucan present in the fungal cell wall, particularly at the hyphal apex of filamentous molds where glucan is most exposed, resulting in cell lysis and cell death.

2.3.1.3. PR-3 proteins (chitinases)

PR-3 proteins have chitinase activity *in vitro*. Most of the PR-3 proteins have molecular mass of between 26 and 43 kDa (Nielsen, 1997; Watanabe *et al.*, 1999). Chitinases (both from plant and other sources) have been divided into five groups. class I chitinases contain an N-terminal cysteine-rich domain of 40 amino acids, a chitin-binding hevein-like domain, a highly conserved central portion, and a hinge region; most class I proteins have molecular mass of 32 kDa. Class II proteins are similar in amino acid sequence to class I proteins, but they lack the N-terminal cysteine-rich domain and have molecular mass of 27 to 28 kDa. Class III proteins do not share amino acid sequence homology to any other class and have molecular mass of 28 to 30 kDa. Class IV proteins resemble class I chitinases but are significantly smaller due to four major deletions. Class

V chitinases show sequence similarities to bacterial exo-chitinases and have molecular mass of 41 to 43 kDa. Chitinases have been isolated from fungi (Kang *et al.,* 1999), grains (Huynh *et al.*, 1992) cucumber, beans (Ye *et al.,* 2000), tobacco (Melchers *et al.,* 1994), peas, and many others (Lee *et al.,* 1999) and bacteria (Chernin *et al.,* 1997) which have potent antifungal activity against a wide variety of human and plant pathogens, including *Trichoderma reesei, Botrytis cinerea, Alternaria solani, A. radicina, Fusarium oxysporum, Rhizopus solani, Guignardia bidwellii,* and *Coprinus comatus*. By analogy with β-glucanases, the mode of action of PR-3 proteins is relatively straightforward, PR-3 proteins are endochitinases that cleave cell wall chitin polymers *in situ*, resulting in a weakened cell wall and lysed fungal cells osmotically.

2.3.1.4. PR-4 (chitin-binding) proteins

PR-4 proteins are chitin-binding proteins, have molecular mass of 13-14.5 kDa, and have been categorized into two groups (Van Loon, 1985). Class I proteins have amino acid sequence similarities to hevein (a chitin binding polypeptide) and belong to the superfamily of chitin binding lectins, but class II proteins lack the chitin binding domain. PR-4 proteins have been isolated from potato, tobacco, barley, tomato and many other plants (Van Damme *et al.,* 1999; Ponstein *et al.,* 1994). Both classes of PR-4 proteins have potent antifungal activity against a wide variety of human and plant pathogens (e.g., *Fusarium culmorum, Trichoderma harzianum, Bacillus cinerea* and *F. graminearum*). The mechanism of action of PR-4 proteins is not understood.

2.3.1.5. PR-5 (TL) proteins

PR-5 proteins share significant amino acid homology to thaumatin protein from the South African ketemfe berry bush and are known as thaumatin like (TL) proteins. TL

proteins have been isolated from tobacco (Koiwa *et al.,* 1997), *A. thaliana* (Hu and Reddy, 1997), corn (Huynh *et al.,* 1992), flax (Borgmeyer *et al.,* 1992), soybeans, rice, wheat, tomato (Singh *et al.,* 1987), pumpkin (Cheong *et al.,* 1997), beans (Ye *et al.,* 1999), barley (Hejgaard *et al.,* 1991) and many other plants. Most of PR-5 proteins have molecular masses of 22 kDa and are stabilized by eight disulfide bonds. This highly stabilized structure allows PR-5 proteins to be very resistant to protease degradation (Roberts and Selitrennikoff, 1990), although the precise mechanism of action of PR-5 proteins is not completely understood.

2.3.2. Defensins

Defensins are a diverse group of low-molecular mass cysteine rich proteins found in plants (Broekaert *et al.*, 1995), fungi (Ng, 2004), mammals, and insects (Lamberty *et al.,* 1999). Plant defensins are cysteine rich proteins ranging from 45 to 54 amino acids, are positively charged, and in most cases, it contain four disulfide bonds (Almeida *et al.,* 2000; Liu *et al.,* 2000). Defensins are categorized into four groups. Group I defensins cause morphological changes in microbes and are known as morphogenic defensins, group II proteins inhibit fungal/bacterial growth but do not cause morphological changes, group III are inactive against test fungi but inhibit α-amylases *in vitro*, and group IV are unique in terms of antimicrobial specificity and structure. Defensins are broadly active, inhibiting a large number of human and plant pathogens, including *F. solani*, *B. cinerea, F. culmorum, F. oxysporum, Alternaria brassicola,* and *C. albicans* at micromolar levels.

Table 2. Major classes of antimicrobial proteins from plants

Antimicrobial proteins	Molecular weight	Mechanism of action	Source	Example	References
Pathogenesis-related protein (PR)	6 - 43 kDa	Permeability changes in fungal cell wall,	*Vitis vinifera, Nicotiana tobacum, Arabidopsis thaliana*	Thaumatin like protein, chitinases, β-lucanases, cysteine-rich proteins	Koiwa *et al.*, 1997; Hu and Reddy, 1997; Chu and Ng, 2003; Bindschedler *et al.*, 2006
Ribosomal inactivating proteins (RIPs)	11 - 60 kDa	Inactivate ribosomes, RNA damage	*Mirabilis expansa, Pisum sativum, Momordica charantia*	-	Vivanco *et al.*, 1999; Lam *et al.*, 1998
Protease inhibitors	4.5 -55 kDa	Membrane channel and pore formation	*Eleusine coracana, Triticum aestivum, Hordeum vulgare*	Serine proteases cysteine proteases	Joshi *et al.*, 2000; Park *et al.*, 2000
Cyclophilin-like protein	5 - 18 kDa	Not known	*Phaseolus mungo*	mungin	Ye and Ng, 2000; Lee *et al.*, 2007
Glycine/ histidine-rich proteins	8 kDa	Not known	*Holotrichia diomphalia, Sarcophaga peregrina, Tenebrio molitor*	Holotrichin,	Lee *et al.*, 1995; Lee *et al.*, 1999
Defensins	3 - 5 kDa	Morphological changes	*Raphanus sativas, Arabidopsis thaliana, Brassica napus*	thionin	Wong and Ng, 2005; Liu *et al.*, 2000; van Dijk *et al.*, 2007
Lipid transfer proteins (LTPs)	8.7 kDa	Penetrate into the cell, membrane, forms a pore and allowing efflux of intracellular ions	*Beta vulgaris, Spinacia oleracea, Lycopersicon esculentum*	-	Guerbette *et al.*, 1999
Killer proteins	10.7 - 156.5 kDa	Disrupt cell wall synthesis, DNA synthesis, β-glucan synthesis, arrest the cell cycle	*Saccharomyces cerevisiae, Ustilago maydis, Hanseniaspora uvarum*	-	Hiraga *et al.*, 1999; Komiyama *et al.*, 1998
Other proteins	6 - 65 kDa	Not known	*Trichoderma viride, Solanum tuberosum, Engelmannia pinnatifida*	Viridin, Snakin-1 Vicilin	Hao *et al.*, 2000; Segura *et al.*, 1999; Ribeiro *et al.*, 2007

2.3.3. Cyclophilin-like protein

Cyclophilins are a highly conserved group of proteins that are the intracellular receptors for cyclosporine. They have been found in a wide variety of organisms, including bacteria, plants, animals, and fungi (Ostoa-Saloma *et al.,* 2000; Lee *et al.,* 2007). A 18 kDa with antimicrobial activity has been isolated from mung bean (*Phaseolus mungo*) and showed activity against *R. solani, F. oxysporum, B. cinerea* and *Coprinus comatus* (Ye and Ng, 2000). This protein called mungin, showed significant homology to cyclophilins and inhibit β-glucosidases *in vitro*. However, the antifungal mechanism of action of mungin is not known.

2.3.4. Glycine/histidine-rich proteins

Insects synthesize a number of glycine/histidine-rich antimicrobial proteins and polypeptides, including *Sarcophaga peregrina* (Iijima *et al.,* 1993), *Holotrichia diomphalia* larvae (Lee *et al.,* 1995) and *Tenebrio molitor* (Lee *et al.*, 1999). These proteins have been reported to inhibit the most common human pathogen like *C. albicans*. The mechanism of action of these proteins is not understood.

2.3.5. Ribosome Inactivating Protein (RIPs).

Ribosomal inactivating proteins are RNA *N*-glycosidases that function to depurinate rRNA results in the arrest of protein synthesis due to ribosome damage (Barbieri *et al.,* 1993; Hwu *et al.,* 2000). Plant RIPs inhibit mammalian, bacterial, fungal, and plant protein synthesis *in vitro* and *in vivo* (Iglesias *et al.,* 1993). RIPs have been categorized into three classes. Type 1 RIPs are single-chain *N*-glycosidases with molecular mass of 11 to 30 kDa. Type 2 RIPs contain two chains, a cell-binding lectin (B chain) and an *N*-glycosidase (A chain), with molecular masses of 60 kDa (Zhang *et al.,*

1999). Type 2 RIPs include toxic members such as ricin and nontoxic members such as ebulin 1 (Girbes *et al.,* 1993) and nigrin b. Type 3 RIPs consist of four chains organized as two dimers of type 2 RIPs. RIPs have been isolated from a number of plants like (*Momordica charantia* (Leung *et al.,* 1997), *Mirabilis expansa* (Vivanco *et al.,* 1999), *Pisum sativum* (Lam *et al.,* 1998), *Ricinus communis* (Arias *et al.,* 1994), *Viscum album*, and many others) as well as from fungi, e.g., *Aspergillus giganteus* (α-sarcin (Krietensen *et al.,* 1999))

2.3.6. Lipid transfer proteins (LTPs)

Lipid transfer proteins have the ability to transfer phospholipids between membranes. LTPs are small proteins (8.7 to 10 kDa) stabilized by four disulfide bonds with a central tunnel-like hydrophobic cavity. They have been isolated from a number of sources, including mammals, plants, fungi and bacteria (Nielsen *et al.,* 1996; Tassin *et al.,* 1998). This may also play several *in vivo* roles, such as lipid exchange between cytoplasmic organelles and importantly defense against pathogens (Guerbette *et al.,* 1999). A 10 kDa LTP has been isolated from onion seed and showed antimicrobial activity against different bacterial and fungal strains. LTPs are active in *in vitro* against a number of bacteria and fungi, however, the mechanism of action is still unknown. It may be that these proteins insert themselves into the cell membrane and the central hydrophobic cavity forms a pore which allows efflux of intracellular ions and finally leading to cell death. How this is related to their lipid transfer function is unclear.

2.3.7. Killer proteins (killer toxins)

A number of yeasts secrete proteins that are lethal to bacterial and fungal cells. These proteins, called killer proteins or killer toxins, are encoded either by double-

stranded RNA, linear double-stranded plasmid DNA, or nuclear genes (Ahmed *et al.,* 1999; Clausen *et al.,* 1999; Izgu *et al.,* 1999). More than 20 killer toxins have been identified, varying in molecular mass from 10.7 to 156.5 kDa. The killer toxins have broad and potent antimicrobial activity against a number of human and plant pathogens (Hiraga *et al.,* 1999; Komiyama *et al.,* 1998), their MICs vary from 20 μg/ml to far less. Although they have varied mechanisms of action, the first step of killer protein activity involves binding of the protein to specific cell surface receptors. Once bound, killer proteins are internalized and can disrupt cell wall synthesis, DNA synthesis, and K1 channel activity, inhibit (1-3) β-glucan synthesis, or arrest the cell cycle (Ahmed *et al.,* 1999). Any one of these effects leads to inhibition of growth and to cell death.

2.3.8. Protease inhibitors

Serine (e.g., trypsin and chymotrypsin) and cysteine proteases and have emerged as a class of antimicrobial proteins that have potent activity against number of human and plant pathogens. Cysteine protease inhibitors have been isolated from a number of plants and form a group of cystatins called phytocystatins (Joshi *et al.,* 2000; Kouzuma *et al.,* 2000; Park *et al.,* 2000). These proteins are bifunctional as they inhibit enzymes as well as inhibiting cell growth. Blanco-Labra (1980) identified a bifunctional α-amlyase/trypsin inhibitor from corn (Blanco-Labra *et al.,* 1980). The mechanism of antimicrobial activity of these proteins is not fully understood.

2.3.9. Other proteins

There are number of new proteins that have antimicrobial activity but do not neatly fall into any of the above classes are being discovered at a rapid pace. Only a few can be mentioned here. Vicilins, 7S globulin protein with antimicrobial activity has been

isolated from different legume seeds. The mechanism of its action is based on the ability to bind to the cell wall and the plasma membrane of fungi (Ribeiro *et al.*, 2007). Viridin, a novel protein isolated from the culture medium of *Trichoderma viride*, has a molecular mass of 65 kDa and is active at 6 mM against number of fungi (Hao *et al.*, 1999; Hao *et al.*, 2000). Snakin-1 has been isolated from potato, has a molecular mass of 6.9 kDa and is active at 10 mM (Segura *et al.*, 1999). A 30-kDa protein with very potent antimicrobial activity (50 ng/disk in an agar diffusion assay) was isolated from Engelmann's daisy (*Engelmannia pinnatifida*). The mechanism of action of none of these proteins is known.

2.4 Mode of action

Although the exact mechanism of action of AMPs remains a matter of controversy, there is consensus that these peptides selectively disrupt the cell membrane. The molecular mechanism of membrane permeation and disruption of AMPs depends on a number of parameters such as the amino acid sequence, membrane lipids and peptide concentration. These studies have indicated that all AMPs interact with membranes and tend to divide peptides into two mechanistic classes: 1) membrane disruptive and 2) non-membrane disruptive (intracellular targets) (Brodgen, 2005). Various models of mode of killing by antimicrobial proteins and peptides are shown in Table 3 and figure 1.

Table 3. Membrane and intracellular models of antimicrobial peptide killing and lysis (Brodgen, 2005)

Mode of Antimicrobial activity	Synonym	Examples of peptides
Transmembrane pore forming mechanisms		
Toroidal pore	Wormhole disk	Magainin 2, proteogrin-1, Melititin, LL-37 and MSI-78
Carpet		Dermasceptin S, cercopin, melititin, Caerin 1.1 and ovisprin
Barel stave	Helical-bundle model	Alamethicin
Mode of Intracellular killing		
Rocculation of intracellular contents		Anionic peptide
Alter cytoplasmic membrane septum formation		PR-39, PR-26, Indolicidin and microcin 25
Inhibit cell-wall synthesis		Mersacidin
Binds nuclec acids		Buforin II and tachyplesin
Inhibit nucleic acid synthesis		Pleurocidin, dermasceptin, PR-39, HNP-1,-2 and indolicidin
Inhibit enzymatic activity		Histatins, pyrrhocoricin, drosocin, and apidaecin

Antimicrobial peptides must first be attracted to bacterial/fungal cell surfaces, and one obvious mechanism of action is electrostatic bonding between anionic or cationic peptides and structures on the bacterial surface were formed. Once close to the microbial surface, peptides must traverse capsular polysaccharides before they can interact with the outer membrane, which contains LPS in Gram-negative bacteria and, teichoic acids and lipoteichoic acids in Gram-positive bacteria. The initial association between the cationic peptide and anionic lipopolysaccharide, a major Gram-negative bacterial component is stabilized by interactions between divalent cations such as Mg^{2+} and Ca^{2+} (Brodgen, 2005). Removal of these ions or their displacement by cationic AMPs facilitates the formation of destabilized areas through which the peptide translocates the outer membrane in a process termed self-promoted uptake. Once peptides have gained access to the cytoplasmic membrane they can interact with lipid bilayers. *In vitro* studies of antimicrobial peptides incubated with single or mixed lipids in membranes or vesicles

show that peptides bind in two physically distinct states (Otvos *et al.*, 2005). At low peptide/ lipid ratios, α-helical peptides, β-sheet peptides and θ-defensins adsorb and embed into the lipid head group region in a functionally inactive state (surface or S state) that stretches the membrane (Reddy *et al.*, 2004). The extent of membrane thinning is specific to the peptide and directly proportional to the peptide concentration. At high peptide/lipid ratios, peptide molecules are orientated perpendicularly and insert into the bilayer, forming transmembrane pores (I state). The peptide/lipid ratio varies with both the peptide and target lipid composition (Brodgen, 2005).

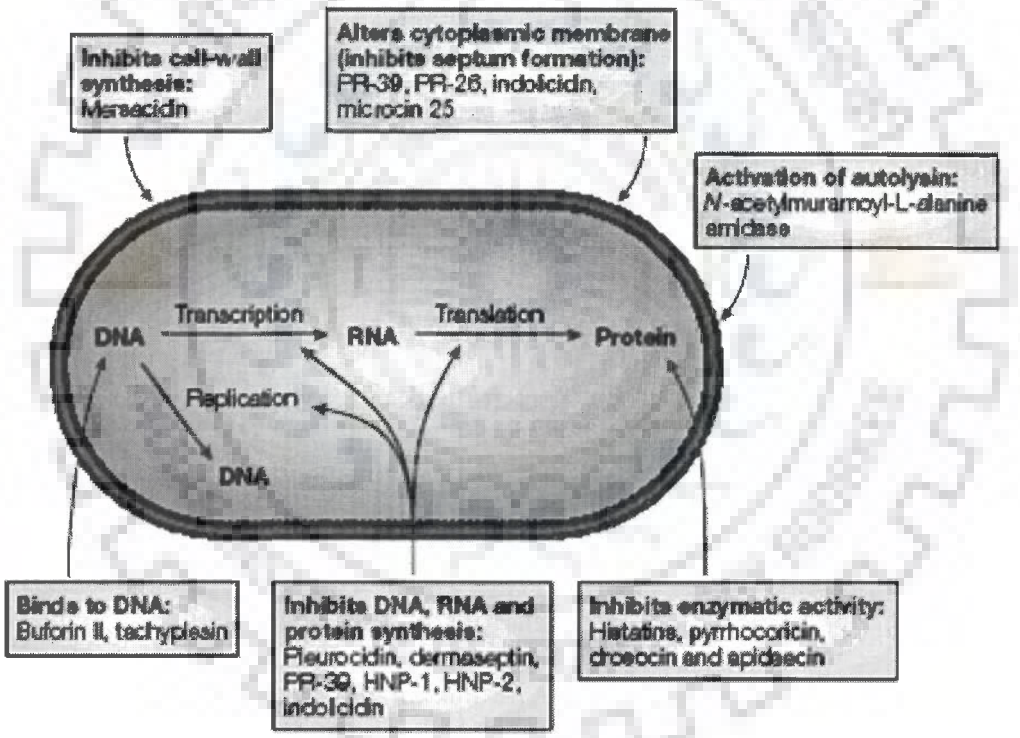

Figure 1. Mode of action for intracellular antimicrobial peptide activity. In this figure *Escherichia coli* is shown as the target microorganism. (Brodgen, 2005)

2.4.1 Membrane disruptive mechanisms

A number of models for antimicrobial peptide membrane permeabilization and subsequent disruption have been proposed (barrel stave, toroidal, carpet and micellar aggregate models) however, there is no universal consensus among investigators in this regard.

2.4.1.1. Barrel stave model

The barrel stave mechanism is proposed for a selected group of peptides. This model involves the perpendicular insertion and aggregation of a relatively small number of individual peptides (or peptide complexes) also referred as staves in a barrel-like ring inside the membrane leading to a transmembrane pore or channel with a cylindrical structure. The transmembrane pore formation involves the following steps; binding of peptide monomers to the membrane in a helical fashion followed by insertion of the helices into the hydrophobic core of the membrane (He *et al.*, 1995). Progressive recruitment of additional monomers increases the pore size leading to leakage of cell contents and thereby death of the cell. Pore formation accompanies the reorientation of the helix from the parallel state to the perpendicular membrane spanning state. Alamethicin, a member of the peptabiol family, behaves in a similar manner (Yang *et al.*, 2001). This peptide forms an open pore consisting of 3–11 helical rods arranged around a water filled pore that forms transmembrane helical bundles or barrel staves.

2.4.1.2. Carpet Model

This model was proposed for the first time to describe the mode of action of dermaseptin (Dagan *et al.*, 2002) and has also reported in cecropin P1 (Gazit *et al.*, 1995) and ovispirin (Yamaguchi, 2001). In the 'carpet model', peptides accumulate on the

bilayer surface (Wildman *et al.*, 2003). This model explains the activity of antimicrobial peptides such as ovispirin that orientate parallel ('in-plane') to the membrane surface. Peptides are electrostatically attracted to the anionic phospholipid head groups at numerous sites covering the surface of the membrane in a carpet-like manner. At high peptide concentrations, surface-oriented peptides are thought to disrupt the bilayer in a detergent-like manner, eventually leading to the formation of micelles (Ladokhin and White, 2001). At a critical threshold concentration, the peptides form toroidal transient holes in the membrane, allowing additional peptides to access the membrane. Finally, the membrane disintegrates and forms micelles after disruption of the bilayer curvature (Bechinger, 1999).

2.4.1.3. Toroidal pore model

More recent studies often support the toroidal pore model (Brodgen, 2005). This model is an extension of the transmembrane helical bundle in which the pores are lined with peptides and lipids. In this model, antimicrobial peptide helices insert into the membrane and induce the lipid monolayers to bend continuously through the pore so that the water core is lined by both the inserted peptides and the lipid head groups (Matsuzaki *et al.*, 1996). This type of transmembrane pore is induced by magainins, protegrins and melittin (Matsuzaki *et al.*, 1996). In forming a toroidal pore, the polar faces of the peptides are associated with the polar head groups of the lipids. The lipids in these openings tilt from the lamellar and connect the two leaflets of the membrane, forming a continuous bend from the top to the bottom in the fashion of a toroidal hole. The pore is lined by both the peptides and the lipid head groups, which are likely to screen and mask cationic peptide charges (Yang *et al.*, 2001). The toroidal model differs from the barrel-

stave model as the peptides are always associated with the lipid head groups even when they are perpendicularly inserted in the lipid bilayer (Yang *et al.*, 2001). The formation of the toroidal pore and translocation of the peptide depend on a critical peptide-lipid ratio.

It has been suggested that ion channels, transmembrane pores and extensive membrane rupture do not represent three completely different modes of action, but instead are a continuous graduation between them (Dathe and Wieprecht, 1999). This concept correlates with the observation that the formation of peptide induced ultrastructural lesions behind the loss of cell viability (Lehrer *et al.*, 1989).

2.4.2 Alternative mechanism of action (intracellular target)

Although the formation of ion channels, transmembrane pores and extensive membrane rupture eventually leads to the lysis of microbial cells, there is increasing speculation that these effects are not the only mechanisms of microbial killing. Peptides must cross the cytoplasmic membrane and they have developed unique mechanisms to translocate to the cytoplasm. Buforin II, which is a linear, α-helical peptide with a proline hinge, does not permeabilize the cytoplasmic membrane but penetrates it and accumulates in the cytoplasm. Arginine-rich peptide groups, such as arginine-rich TAT-related peptides, NLS peptides, RNA-binding peptides, DNA-binding peptides and polyarginine and arginine-rich antimicrobial peptides, all readily and efficiently translocate across both cellular and nuclear membranes (Futaki *et al.*, 2001). In eukaryotic cells, the TAT peptide are internalized by endocytosis (Richard *et al.*, 2003), and TAT-fusion proteins are internalized by lipid-raft-dependent macro pinocytosis (Wadia *et al.*, 2004). Apidaecin, a short, proline-rich antibacterial peptide, is translocated by a permease/transporter-mediated mechanism (Casteels *et al.*, 1993). Once in the

cytoplasm, translocated peptides can alter the cytoplasmic membrane septum formation, inhibit cell-wall synthesis, nucleic-acid synthesis, protein synthesis or enzymatic activity. PR-39, which is a proline-arginine-rich neutrophil peptide, induces filamentation of *Salmonella typhimurium*, and indolicidin was proposed to inhibit DNA synthesis leading to filamentation in *E. coli* (Shi *et al.*, 1996; Subbalakshmi and Sitaram, 1998). Cells exposed to these peptides have an extremely elongated morphology, which indicates that the peptide-treated cells are unable to undergo cell division. Some AMPs were found to interfere with the metabolic processes of microbes; an example is the glycine-rich attacins that have been shown to block the transcription of the *omp* gene in *E. coli* (Carlson *et al.*, 1991), whereas magainins and cecropins induce selective transcription of its stress-related genes *micF* and *osmY* at non-bactericidal concentrations (Oh *et al.*, 2000). Buforin II, a histone H2A-derived antimicrobial peptide originally isolated from the Asian toad, inhibits the cellular functions of *E. coli* by binding to DNA and RNA after penetrating the cell membranes (Park *et al.*, 1999). Two bovine bacteriocins, Bac5 and Bac7, inhibit protein and RNA synthesis of *E. coli* and *Klebsiella pneumoniae* and inhibit their respiration inducing a drop in ATP content (Skerlavag *et al.*, 1990). Histatins bind to a receptor on the fungal cell membrane, enter the cytoplasm and induce the nonlytic loss of ATP from actively respiring cells. Their action can also disrupt the cell cycle and lead to the generation of reactive oxygen species. The class of short, proline-rich antimicrobial peptides secreted by insects, such as pyrrhocoricin, apidaecin, and drosocin, are thought to kill bacteria by entering cells and inhibiting the molecular chaperone DnaK (Otvos *et al.*, 1999). DnaK is a 70-kDa molecular chaperone that functions co-translationally and post-translationally to promote protein folding and to

inhibit the formation of toxic protein aggregates. The killing of bacteria and DnaK binding are related events, as an inactive pyrrhocoricin analogue made of D-amino acids failed to bind to DnaK. These inhibitors could represent an important new class of antimicrobial agents, since they demonstrate to selectively bind to the prokaryotic but not to the eukaryotic chaperone (Chesnokova *et al.*, 2004). Alternative, mechanisms of action may act independently or synergistically with membrane permeabilization. Nevertheless, the interaction with the bacterial cell membrane appears to be the killing mechanism of the vast majority of AMPs (Toke, 2005).

2.5 The basis of peptide activity and specificity

Although many relationships between peptide/protein structure and antibacterial activity have been described (Boman, 1995; Tossi *et al.*, 2000) but little is known about the molecular basis of the marked differences in peptide/protein activity and specificity. The susceptibility of AMPs to the bacterial and fungal membrane compared to mammalian membrane is due to the membrane composition. Bacterial membranes are negatively charged with lipids bearing phospholipid headgroups such as phosphatidylglycerol, cardiolipin, or phosphatidylserine. On the contrary, mammalian membranes are enriched in zwitterionic phospholipids (neutral in net charge) as phosphatidylethanolamine, phosphatidylcholine, or sphingomyelin. Moreover, the presence of cholesterol, a major constituent of mammalian cellular membranes, can reduce the activity of AMPs stabilizing the lipid bilayer or by directly interacting and neutralizing them. Composition likely provides an important determinant by which AMPs selectively target microbial versus host membranes.

The differences in the susceptibility of a single microorganism to a panel of antimicrobial peptides indicates that the size, sequence, degree of structuring (for example, helical content), charge, overall hydrophobicity, amphipathicity and respective widths of the hydrophobic and hydrophilic faces of the helix are all important (Boman, 1995). Additional traits like γ-loop sequence found in defensins, brazzein and scorpion neurotoxin, charybdotoxin, are also important and found to correlate with antimicrobial activity (Matsuzaki *et al.*, 1995). Altering any of the above parameters, including the hydrophobic moment and the angle subtended by charged residues can modify antimicrobial and haemolytic activity of peptides (Dathe *et al.*, 2002).

On the other hand, the differences in the susceptibility of a panel of microorganisms to a single peptide indicate that the composition of the microbial surface and cytoplasmic membrane is equally important (Powers and Hancock, 2003). A certain amount of innate antimicrobial resistance is related to the structure and composition of the LPS molecule in the outer membrane of Gram-negative bacteria and phospholipids in the cytoplasmic membrane of both Gram-negative and Gram-positive bacteria. Membrane lipid composition is important (Matsuzaki *et al.*, 1995) and bacteria with cytoplasmic membranes that are enriched in acidic phospholipids are more susceptible to antimicrobial peptides (Costerton *et al.*, 1999). Finally, effective definitions of antimicrobial peptide activity and specificity should take into consideration the physiological conditions *in vivo*. This includes the concentrations of antimicrobial peptides at the sites of infection, the role of synergistic substances that might be present in tissues and fluids (for example, the presence of lysozyme, other antimicrobial peptides and proteins and the absence of divalent cations), the role of inhibiting substances that

might be present (for example, physiological concentrations of salts and serous proteins) and the unusual characteristics of bacteria replicating *in vivo*, particularly those in biofilms (Matsuzaki *et al.*, 1997)

2.6. Other biological activities of antimicrobial proteins/peptides

It has been known for quite some time that antibiotics may also exert other biological effects other than direct antimicrobial ones. Beyond their antimicrobial function, these peptides are known to be multi-functional. Modulation of the immune system through pharmaceutically induced stimulation or suppression offers an important approach to the control of diseases. Compounds which nonspecifically stimulate the immune system are of potentially significant medicinal importance and have been the object of lengthy research efforts. In fact, it has been demonstrated that their multiple role as mediators of inflammation with effects on epithelial and inflammatory cells, and their roles over such diverse processes as proliferation, immune induction, wound healing, cytokine release, chemotaxis, protease-antiprotease balance, and redox homeostasis (Ganz, 2002; Cole *et al.*, 2003; Com *et al.*, 2003; Liu *et al.*, 2003). There are few review articles highlighting various biological activities of antimicrobial peptides (Marshall and and Arenas, 2003; Brodgen, 2005; Butler, 2006; Reddy *et al.*, 2004). Some of the other activities of antimicrobial proteins /peptides are describe below.

2.6.1. Immunomodulatory Activity

In order to survive in a world laden with microorganisms, most multi-cellular organisms ought to depend on a network of host defense mechanisms which in most cases, involves several levels of interacting systems. In higher vertebrates such as

mammals, there are two types of immunity: innate (or natural) and adaptive (or acquired). Innate immune system is primitive type and wide spread in all organisms including plants. In complex system such as humans, an invading microorganism can simply be eliminated by this primary reaction, without requiring an activation of the adaptive immunity, the next step in this complex cascade (Bals, 2000). If the invading microbe outgrows the innate host defense, endogenous effector mechanisms of the innate system are up-regulated and have direct antimicrobial activity and mediator functions to attract inflammatory cells and cells of the adaptive immune system. In lower eukaryotes, mostly invertebrates, the adaptive system is non existent, thus innate system control the invasiveness of pathogens (Otvos, 2000).

Antimicrobial peptides, due to their membrane permeabilizing properties towards a wide spectrum of microbes, are classified as effector molecules of innate immunity. Besides direct antimicrobial properties, these peptides are able to modulate other components of innate immunity. Efficacy of an antimicrobial drug depends on both its antimicrobial potential as well as the host immune system. It has been suggested long back that at the bottom there is only one genuine scientific treatment for all diseases, and that is to stimulate the phagocytes. On other hand, the response of the host to infection may be deleterious as pro-inflammatory cytokines (e.g. TNF-α, IL-1, IL-8) and secretory products of WBC (reactive oxygen metabolites, chloramines) are able to produce serious tissue damage. Lysis of micro-organisms by host factors (complement, lysosomal enzymes, perforins etc) as well as by antibiotics may increase these deleterious effects. Therefore to cope up with these effects, the drug that had immunomodulatory activity

have been searched for. In general, immunomodulatory effect of drugs fall into one of the following categories or combinations thereof;

1. Stimulation of the inflammatory response either by increasing the pro-inflammatory cytokines (TNF-α, il-1, IL-8) or by augmenting the phagocytes or T-cell function.
2. Inhibition of the inflammatory response either by decreasing the pro-inflammatory cytokines or by inhibiting phagocytes or T-cell function or by inducing apoptosis of inflammatory cells.
3. Stimulation of the counter regulatory ant-inflammatory responses by increasing anti-inflammatory cytokines such as IL-4, IL-10 & TGF-β.
4. Inhibition of the counter anti-inflammatory response by inhibiting an anti-inflammatory cytokines (eg. IL-10, IL-4, TGF-β).

A number of antimicrobial peptides/ proteins with immunomodulatory activity have been reported from various sources including plant preparations (Hsu *et al.*, 1997; Tan and Vanitha, 2004; Liu *et al.*, 2007). HBD1 and LL37 are produced constitutively by keratinocytes and other epithelial cells and contribute to the barrier functions in the first line of antimicrobial defense (Nizet *et al.*, 2001; Elsbach, 2003). Defensins and PR-39 act as chemoattractants for monocytes and neutrophils, respectively (Territo *et al.*, 1989; Huang *et al.*, 1997). Since neutrophils and monocytes are phagocytes, such action enhances the innate immunity. Moreover, defensins influence the production of several cytokines. In monocytes, TNF and IL-1 expression is stimulated by defensins (Chaly *et al.*, 2000), while in lung epithelial cells defensins enhance the expression of IL-8 (van Wetering *et al.*, 1989). This indicates that defensins could exert chemotaxic activity not

only towards nonspecific phagocytes but also towards cells engaged in adaptive immune response. Human hBD2 and LL37 induce the activation and degranulation of mast cells resulting in the release of histamine and PDG2 (Galkowska.and Olszewski, 2003). Because mast cell granule products increase neutrophil influx, antimicrobial protein/peptides can indirectly promote accumulation of these cells at inflammatory sites. Degranulation of neutrophils releases more defensins and upregulates innate host inflammatory defense against microbial invasions. HNP1 and HNP2 are chemotactic for human monocytes, dendritic cells and T cells (Elsbach, 2003). Murine β-defensin 2 acts directly on immature dendritic cells as an endogenous ligand for Toll-like receptor 4 (TLR-4), inducing up-regulation of co-stimulatory molecules and, subsequently, dendritic cell maturation, acting as the natural adjuvant (Elsbach, 2003). In addition to their role in host defense, defensins may also contribute to some pathophysiologic mechanisms. Neutrophil defensins can inhibit fibrinolysis and modulate tissue-type plasminogen activator and plasminogen binding to fibrin and endothelial cells (Galkowska.and Olszewski, 2003.). Cathelicidin LL37 can also play a part in wound closure and its reduction in chronic wounds can impair re-epithelialization (Elsbach, 2003). Defensins also act as adjuvants (Tani *et al.,* 2000). Mice, when immunized and treated with defensins, increase the production of antigen-specific IgG antibodies. At a molecular level, production of IFN-γ and IL-4 was increased. Thus, defensins enhance the antigen-specific immune response by inducing the production of lymphokines, which promote the adaptive immune response.

Among the many classes of compounds which non-specifically stimulate the immune system is a number of naturally occurring proteins form plants. In the literature,

several proteins were reported to have immunomodulatory activities *in vitro* and/or *in vivo* (Pugh *et al.*, 2001; Wasser, 2002; Brown and Gordon, 2003; Hsu *et al.*, 2004; Ou *et al.*, 2005; Liu *et al.*, 2007; Hsu *et al.*, 1997; Vivanco 1997). LZ-8, a protein from *Ganoderma lucidum* with a molecular mass of 13 kDa, exhibited mitogenic activity toward spleen cells (Kino *et al.*, 1989). Fip-*vvo*, from *Volvariella volvacea* with a molecular mass of 15 kDa, exhibited proliferation activity in human peripheral blood lymphocytes and enhanced the IL-2, IL-4, IFN-γ, TNF-a gene expressions by RT-PCR in mouse spleen cells (Hsu *et al.*, 1997). The lectin from *Agrocybe cylindracea* with molecular mass of 31.5 kDa (Wang et al., 2002) or from *Cteropharyngodon idellus* with a molecular mass of 205 kDa (Ng *et al.*, 2003) exhibited mitogenic activity toward mouse splenocytes. A napin-like polypeptide (13.8 kDa), from Chinese cabbage seeds, stimulated nitric oxide production from murine peritoneal macrophages (Ng and Ngai, 2004). FIP-*fvo*, with its 114 amino acids from *Flammulina velutipes*, was able to inhibit the development of allergic reactions in mice by oral administration (Hsieh *et al.*, 2003) and stimulated IFN-γ and IL-4 secretions in peripheral blood mononuclear cells (Wang *et al.*, 2004). The lactotransferrin (or lactoferrin) (Zeimeck *et al.*, 1991), a component of milk with a molecular mass of 80 kDa, and its peptic hydrolysates (nor casein hydrolysates or whey protein hydrolysates) have been reported to have immunomodulatory activities (Miyauchi *et al.*, 1997). The oral administration of recombinant human lactoferrin could stimulate IL-18 secretions, systemic NK cell activation, circulating CD8+ T-cell expansion and inhibit the growth of established tumors in mice. The immunity-related gene was expressed in the small intestines of BALB/c mice after oral administration of lactoferrin (Varadhachary *et al.*, 2004). The

influences of arabinogalactan (AG) and arabinogalactan-proteins (AGPs) from *Nerium oleander* and *Echinacea purpurae* on proliferation and IgM-production of mouse lymphocytes as well as nitrite and IL-6 production of mouse macrophages were investigated *in vitro* and *in vivo* (Linda *et al.*, 2002; Classen *et al.*, 2006). AGPs from cell culture of *E. purpurea* stimulate nitrite and IL-6 productions in alveolar mouse macrophage culture.

2.6.2 Antitumor Activity

Antimicrobial peptides/proteins exert their effect by interacting with negatively charged molecules in a target membrane and its specificity depends on the membrane composition. Tumor cells can differ in membrane composition from non-transformed cells and such differences can result in higher susceptibility of tumor cells to membrane permeabilizing peptides. The difference in phosphatidylserine content has been reported in the membrane of melanoma and carcinoma cells in comparison to that in normal human keratinocytes (Utsugi *et al.* 1991). Magainins a cationic peptide isolated from the skin of amphibians has been reported to lyse many types of tumour cells at a much lower concentration than that for normal cell lysis (Jacob & Zasloff, 1994). Magainin 2 and magainins' synthetic analogues have been studied both *in vitro* and *in vivo,* and showed antitumour activity towards murine ascites tumours, leukaemia, and spontaneous ovarian tumor cells (Baker *et al.* (1993). Defensin found to induce tumour cell lysis in the concentration dependent manner. *In vitro* incubation of murine teratocarcinoma cells with defensins reduces their oncogenecity *in vivo* (Lichtenstein *et al.*, 1986). *In vitro* experiments proved that Cecropins (insect derived peptides 0 are able to lyse multidrug-resistant tumor cell lines (Moore *et al.*, 1994). Expression of antimicrobial peptides has

been reported in several tumor cell lines where depending on their concentrations, they exert mitogenic or necrotic activity (Muller *et al.*, 2002).

2.6.3 Role as signaling molecules

Another aspect of antimicrobial peptides activity is their possible role in modulation of signal transduction. The molecule, which was first discovered and described as the EGF receptor kinase inhibitor has been reported to have antimicrobial properties towards different species of Gram-positive and Gram-negative bacteria (Pogrebnoy *et al.*, 1998). Several others antimicrobial proteins/peptides have been reported to influence the activity of kinases. Human defensins are potent inhibitors of protein kinase C (PKC), whereas they have little or no effect on the activity of myosin light chain kinase and protein kinase A (Charp *et al.*, 1988). PR-39 is a proline and arginine-rich peptide which can bind to the adapter protein p130 (Cas) (Tanaka *et al.*, 2001), involved in various cellular processes including cell adhesion, migration and transformation (Kirsch *et al.*, 2002). It has been reported bind to a subunit of phosphoinositide 3-kinase (PI3-kinase) and thereby inhibiting kinase activity (Tanaka *et al.*, 2001). The *PR-39* gene transfected cells showed a reorganisation of actin structure and suppression of cell proliferation. These cells have also been characterized by decreased activity of c-Jun N-terminal kinase (JNK), mitogen-activated protein kinase (MAP) and decreased expression of cyclin D. The epidermoid carcinoma derived antimicrobial peptide (ECAP) found to be responsible for inhibition of EGFR auto phosphorylation and decreased activity of non-receptor kinases like Lyn and Syk (Hobta *et al.*, 2001). Such results may suggest that protein kinases are the physiological targets for antimicrobial peptides.

2.7 Potential as therapeutics

2.7.1. Clinical application of antimicrobial protein

In order to be a good candidate for therapeutic use, a drug needs to show good activity, appropriate function, low toxicity and have stability *in vivo* and be reasonably inexpensive to manufacture. So far only a few protein and peptides have made their way to clinical trials.

As the problem of emergence of bacterial and fungal resistance to current antibiotic drugs continues to grow, there has been considerable interest in the development of antimicrobial proteins as a novel therapeutic approach to treat infections. To date, several antimicrobial proteins have been developed and entered into clinical trials. Antimicrobial protein based therapies are attractive candidates as alternative antibiotic treatments, since they offer several potential advantages over currently used classes of drugs. First, they represent a naturally occurring means of combating pathogenic challenge by rapid microbicidal activity. In addition, the emergence of resistance is thought to be considerably reduced compared with that for many current antibiotics, which have more specific molecular targets (Tenover, 2006). This prediction has been substantiated in several studies in which, despite serial passage in sub-inhibitory concentrations of peptide, resistant bacteria did not arise (Chopra *et al.,* 1997). Interestingly, a number of evidence has shown efficacy of some antimicrobial proteins and peptides against systemic infections, including α-helical-peptide (SMAP29) efficacy against *P. aeruginosa* peritoneal infections, β-sheet-protegrin efficacy against methicillin-resistant *S. aureus* (MASA), vancomycin-resistant *Enterococcus faecalis* (VRE) *and P.*

aeruginosa infections, and indolicidin in liposomal formulation against *Aspergillus* fungal infections (Thies *et al.*, 2005).

The new generation of native protein/peptide molecules, also known as antimicrobial proteins and peptides isolated from a full range of organisms and species from bacteria to man, seems to fit this description. As a consequence, they have been termed "natural antibiotics", because they are active against a large spectrum of microorganisms, including bacteria and filamentous fungi in addition to protozoan and metazoan parasites (Butler and Buss, 2006). All of these molecules are key elements directly implicated in the innate immune response of their hosts, which includes the expression of fluid phase proteins that recognize pathogen associated molecular patterns, instead of specific features of a given agent to promote their destruction. As a result, the response is very fast, highly efficient and applicable to a wide range of infective organisms (Lewis and Ausubel, 2006).

2.7.2. Development of disease resistance plants

A number of antibacterial and antifungal proteins have been identified from plants and other sources, and have been used for developing disease resistant transgenic plants. In plants, tobacco has been the target for successful engineered-production of mammalian AMPs (Morassutti *et al.*, 2002) as well as amphibian anti microbial peptides, where vertical transmission of resistance occurs. In addition, AMPs from other origins have been added to confer disease resistance in transgenic tobacco, banana and potato, thus opening unsuspected alternatives to provide agronomically relevant levels of disease control worldwide. Besides, number of antimicrobial protein of plant origin have also been used for developing disease resistant plants (Devdas and Raina, 2002; Jagdeeswaran

et al., 2007). Poplar tree has been transformed in the transgenic tobacco and was resistant to bacterial pathogens but not to the fungal pathogen (Mentag *et al.*, 2003). Cotton plants have been transformed similarly with the gene coding for D4E1 showed resistance to fungi including *Fusarium*, *Verticillium* and *Aspergillus*, hence the synthetic peptide was proposed to be effective against mycotoxin-causing fungal pathogens (Rajaskaran *et al.*, 2005). Meanwhile, researchers at the National Agricultural Research Center, Niigata, in Japan have created transgenic rice with genes of the antimicrobial peptide, defensin, from *Brassica*. The transgenic rice plants were resistant to rice blast disease caused by the fungus *Magnaporthe grisea*. The researchers went a step further and systematically altered the genetic code for defensin to produce synthetic peptides that were far more toxic to the fungus than the natural peptides (Kawata *et al.*, 2003). Rice with the synthetic genes and peptides are being proposed for field-testing prior to commercial release in Japan, and little effort appears to have been devoted to evaluate the safety for human health and the environment.

2.7.2. Introduction of therapeutic protein antibiotics on the market

The widespread increase of microbial resistance towards many conventional antibiotics has resulted in an intensive search for alternative antimicrobial agents. In this respect, antimicrobial proteins are on the brink of a breakthrough. Due to the increasing interests, many companies are making efforts to introduce the antimicrobial protein products on the market. Natural antimicrobial peptides have potential application in food preservation as they specifically kill microbial cells by destroying their unique membranes. Interest in LAB bacteriocins has been sparked by growing consumer demands for natural and minimally-processed foods. LAB bacteriocins have well

documented lethal activity against food borne pathogens and spoilage microorganisms, and can play a vital role in the design and application of food preservation technology (Cotter *et al.,* 2005). Currently, nisin has been approved as a food preservative in more than 40 countries (Montville and Chen, 1998) and the use of pediocin PA-1 is covered by several European and US patents (Montville and Chen, 1998). Both nisin and pediocin PA-1 have applications in dairy and canned products. Studies of model food systems demonstrate that pediocin-like bacteriocins are better at killing pathogens in meat products, where nisin is ineffective (Vandenbergh, 1993).

The potential to develop antimicrobial protein/peptide with broad spectrum characteristics, especially in multiple pathogens could potentially be targeted with one treatment possessing antibacterial, antiviral, or antifungal activity. Novel antimicrobial treatments could also potentially be used in conjunction with existing drugs as part of a "combination therapy" to create an additive or synergistic effect. The multidrug target approach has been successful in improving the efficacy of and reducing emergence of resistance to HIV therapies (De Clercq, 2004). Thus, in combination, peptides have the potential to ultimately reduce the rate of emergence of resistant microbes, since selective pressure is deviated away from one specific molecular target. The potential for additional antiseptic activity (Gough *et al.,* 1996) also proved to be defined asset; for example, researchers at Migenix (Vancouver, British Columbia, Canada) have developed a peptide "indolicidin" with anti-inflammatory activity which able to cure acne. Antimicrobial pharma have developed human lactoferrin, hLF1-11 that has proved efficacious in animal models of osteomyelitis (Faber *et al.,* 2005) and other bacterial infections (Nibbering *et al.,* 2001). Iseganan, an antimicrobial protein was originally developed by Intrabiotics

Pharmaceuticals Inc. (Mountain View, CA) as a mouth rinse to prevent polymicrobial infection associated with oral mucositis in patients receiving chemotherapy (Trotti *et al.*, 2004). Another antimicrobial peptide in mouth wash, used for the treatment of oral candidiasis, was developed using a variant of histatins, which are naturally occurring cationic peptides in saliva. This has been recently licensed to the Vancouver, Canada, company Pacgen.

2.8. Limitations as therapeutics

The future of antimicrobial protein as antibiotics appears to be great and as mentioned above, a number of antimicrobial proteins with therapeutic potential have been developed in the past two decades. Antimicrobial proteins and peptides are generally considered to be highly selective antimicrobial agents. However, they do not discriminate absolutely between eukaryotes and prokaryotes, although the former are less sensitive. Several studies showed that simple eukaryotes, such as yeasts, fungi, parasites (Jaynes *et al.,* 1988; Ahmad *et al.,* 1995), and large ones as planaria (Zasloff, 2002) are effectively killed. In addition, their unique pharmacological properties have limited their application to topical use. Recent publications describe also changes in bacterial cell wall components induced by environmental conditions, which may be involved in bacterial resistance towards antimicrobial protein (Groisman *et al.*, 1997; Wösten and Groisman, 1999). Technical difficulties and high production costs have made the pharmaceutical industry reluctant to invest much effort in the development of antibiotic protein/peptide therapeutics so far. The antimicrobial proteins are too expensive or with a too limited spectrum to be used on a large scale commercially. The biggest challenge of the near

future will be to overcome the pharmacological limitations of these interesting molecules and to develop them into therapeutics.

2.9. Future Prospects

Several applications of natural occurring antimicrobial proteins have been discussed during the last two decades. They seem to be an attractive alternative for chemical food additives and may also be a new source of clinically useful therapeutics.

Antimicrobial proteins and polypeptides have been isolated from diverse groups of organisms including plants, fungi, bacteria, insects and animals (both vertebrates and invertebrates). The mechanisms of action of these proteins are as varied as their sources and include cell wall polymer degradation, membrane channel and pore formation, damage to cellular ribosomes, inhibition of DNA synthesis and inhibition of the cell cycle. The mode of action of many proteins remains unknown and is the subject of active research. The range of inhibition by antimicrobial proteins is extremely broad, with plant pathogens and human's pathogens being sensitive at micromolar levels; in some cases, even more potent inhibition was found. Food preservation by use of antimicrobial proteins is not a totally new concept. The use of the antimicrobial protein nisin, which is produced by several *Lactococcus lactis* strains, has been approved by eight European countries. The protein inhibits the growth of a wide range of gram positive bacteria (Hurst, 1981), and its mode of action is comparable to magainin (Breukink and De Kruijff, 1999). It has been shown that an additional plasma membrane-based factor, named Lipid II, is needed for pore formation (Brotz *et al.*, 1998), which might also be a reason for the lack of antifungal activity of nisin.

The genes encoding many antimicrobial proteins are being currently used by agribusiness to create genetically modified plants that have increased fungal resistance in the field. Whether these transgenic plants and the crops derived from them gain acceptance in the market place remains to be seen. Antimicrobial proteins and peptides are being tested for use as pharmaceutical agents for the treatment of human and animal fungal diseases. This is particularly exciting since the modes of action of these proteins are vastly different from the currently used therapeutics, resistance to which is becoming a clinical problem. There are a number of antimicrobial proteins in various stages of preclinical development and the results of these experiments and of the subsequent human clinical trials are eagerly anticipated.

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

The certified plants of *Nerium odorum* used in the present study were procured from Dr. B. K. Pandey, Research Institute of Medicinal Plants, Shantikunj, Haridwar. The bacteria *Staphylococcus aureus* (MTCC 2940), *Bacillus substilis* (MTCC 2423), *Pseudomonas putida* (MTCC 2453), *Escherichia coli* (MTCC 739)) and fungi *Aspergillus fumigatus* (ITCC 4880), *Aspergillus flavus* (ITCC 5290), *Aspergillus niger* (ITCC 5454), *Fusarium oxysporum* (ITCC 4998) and *Candida albicans* (MTCC 227) were purchased from Institute of Microbial Technology, Chandigarh, India and Indian Agriculture Research Institute, Pusa New Delhi, India. All chemicals used were of analytical grades and purchased from Sigma (USA), Merck (Germany) and Hi Media (India). Cytokines estimation ELISA kits were purchased from Endogen, Pierce, USA. SGPT, SGOT, Bilirubin and urea estimation kits were purchased from Span diagnostics, Surat, India.

3.2 Methods

(A) Isolation, purification and characterization of antimicrobial protein

3.2.1 Protein extraction

One hundred gram of fresh leaves of *Nerium odorum* was grounded to powder in liquid nitrogen and mixed with 100 ml of extraction buffer (50 mM Tris-HCl pH 8.0; 10 mM EDTA; 0.1 % β- mercaptoethanol; and 1 % (w/v) Triton X-100) containing 1 mM phenylmethylsulfonyl fluoride and incubated at 4°C for 3 h with occasional stirring. The homogenate was centrifuged at 14,000 x g for 20 min and the supernatant which designated as the crude extract was collected. The amount of protein and antimicrobial

activity of the crude protein extract were determined using standard methods described in later sections.

3.2.2. Ammonium sulfate fractionation

The proteins present in the crude extract were fractionated using 10% to 80% ammonium sulfate saturation series. The pellet of each fraction was collected by centrifugation at 14,000 x g for 20 min, and resuspended in 50 mM Tris-HCl, pH 8.0, dialyzed over night against distilled water in a cold room. Each fraction was tested for antimicrobial activity and the fractions (20-50%) having antimicrobial activity were pooled and concentrated by ultrafilteration using Amicon filter (100 kDa cut off) (Millipore, USA). The fraction was stored at -80°C in aliquots for further use.

3.2.3. Gel filtration and ion-exchange chromatography

The ammonium sulfate precipitated active fraction (20-50% saturation) was further purified by gel filtration chromatography. The concentrated protein fraction was applied directly to gel filtration Sephadex G-100 column (1.5 × 100 cm), and pre-equilibrated with 50 mM Tris-HCl buffer (pH 8.0). The proteins were eluted using same buffer and 2 ml fractions were collected and the elution was monitored at 280 nm. Each elution peak was tested for its protein content and antimicrobial activity. The elution peak with antimicrobial activity was further purified by CM-cellulose ion-exchange column (1.0 x 30 cm), which had been pre-equilibrated, with 50 mM Tris-HCl buffer (pH 8.0). The proteins were eluted using NaCl step gradient (0 to 1 M) in the same buffer. 2 ml fractions were collected at a flow rate of 22.5 ml/h, and the elution profile was monitored at 280 nm. The protein peaks were tested for antimicrobial activity and the peak with

antimicrobial activity was dialyzed and concentrated using Amicon filter (100 kDa cut off) (Millipore, USA). The purity of protein was tested and used for further studies.

3.2.4. Protein estimation

The concentration of protein in the sample was estimated by Bradford (1976) assay. BSA was used as protein standard.

3.2.5. Polyacrylamide gel electrophoresis (SDS-PAGE & Native-PAGE)

The purity test and further analysis of purified protein were performed using SDS and Native PAGE Laemmli (1970) with little modifications. The various gel electrophoresis constituents and its compositions of the gel are listed below.

3.2.5.1. Preparation of reagents

The stock solutions of various gel components were prepared as mentioned below.

1. **Acrylamide solution (30%):** 30% w/v acrylamide solution containing 0.8% w/v N, N-methylene-bis-acrylamide: 29.2 g of acrylamide and 0.8 g of bisacrylamide was dissolved in 70 ml of deionised water. When acrylamide was completely dissolved, deionised water was added to make a final volume of 100 ml. Solution was filtered with a whatman No.1 paper and was stored at 4°C in a dark bottle.

2. **Resolving buffer (1.5 M Tris-HCl, pH 8.8):** 18.2 g of Tris base was dissolved in 80 ml of deionised water and the pH was adjusted to 8.8 with HCl and deionised water was added to make a final volume of 100 ml and was stored at 4°C.

3. **Stacking buffer (0.5 M Tris-HCl, pH 6.8):** 6.1 g of Tris base was dissolved in 80 ml of deionised water and pH was adjusted to 6.8 with HCl and deionised water was added to make a final volume of 100 ml and was stored at 4°C.

4. **Sodium dodecyl sulphate (SDS, 10% w/v):** 10 g SDS was dissolved in 60 ml of deionised water and kept at room temperature overnight without shaking. Then the volume was leveled at 100 ml by deionised water and was stored at room temperature.

5. **Catalyst: 10% ammonium per sulphate (APS):** 10 mg APS was dissolved in 100 µl of deionised water (Freshly prepared solution was used).

6. **TEMED (N, N, N, N-tetra methylethylenediamine):** It was used undiluted from the bottle stored at cool, dry and dark place.

7. **Electrode buffer:** (0.025 M Tris, 0.192 M glycine, 0.1% (w/v) SDS, pH 8.3) 0.3 g Tris base, 1.4 g glycine, 1 ml 10% SDS/100 ml electrode buffer. Electrode buffer was also prepared as stock solution 5X concentration, consisting of 15 g Tris base, 72 g glycine and 5 g SDS/litre. It was stored at room temperature and was diluted to 5 times by adding 4 parts of deionised water before use.

8. **Stock sample buffer (2X)** (0.125 M Tris pH 6.8, 2.5% SDS, 20% glycerol, 0.002% Bromophenol blue, 10% β-mercaptoethanol).

   The sample buffer (2X) was prepared by mixing the stock solutions as per the given composition.

3.2.5.2. Preparation of sample buffer

Ingredient	Volume
Water	3.0 ml
0.5M Tris-HCl, pH 6.8	2.5 ml
10% SDS	2.5 ml
Glycerol	2.0 ml
0.5% Bromophenol blue (w/v)	0.5 mg

Stored at room temperature, SDS-reducing sample buffer was prepared by adding 100 µl of β-mercaptoethanol to each 0.9 ml of stock sample buffer, before use.

3.2.5.3. Casting of gel

Twelve percent denaturing discontinuous gel was prepared by mixing gel stock solutions as per given composition. The monomer solution was prepared for resolving gel by mixing all of the reagents given below except the ammonium per sulfate and TEMED.

Recipe for resolving gel (12%): (10 ml)

Ingredient	Volume
Acrylamide solution 30%	3.3 ml
1.5 M Tris-HCl pH 8.8	2.5 ml
10% SDS	0.1 ml
10% APS	0.1 ml
Water	4.0 ml
TEMED	4.0 µl

CENTRAL LIBRARY
ACC.No. G14067
Date.....
I.I.T. ROORKEE

Recipe for stacking gel (5%): (5 ml)

Ingredient	Volume
Acrylamide solution 30%	0.83 ml
0.5 M Tris-HCl pH 6.8	0.63 ml
10% SDS	0.05 ml
10% APS	0.05 ml
Water	3.40 ml
TEMED	5.00 µl

The solution was degassed for at least 3-5 min. The APS and TEMED were gently mixed into the degassed monomer solution. The solution was well mixed uniformly and poured gently in between the plates. The resolving gel was cast up to 2/3 height on pre marked plates followed by layering of 200 µl butanol overlaying solution. After 15 min, the demarcation occurred between the acrylamide layer and butanol layer indicated the complete polymerization of gel. Butanol was decanted and the space was washed with distilled water. Similarly 5% stacking gel was also layered on top of the

resolving gel. The wells were cast in stacking gel by placing the teflon comb in between and at the top of the two plates. After 15-20 min the comb was removed carefully.

3.2.5.4. Sample preparation

Protein concentration 150 μg/50 μl were mixed with 1 volume of sample treatment buffer (0.125 M Tris pH 6.8, 2.5% SDS, 20% glycerol, 0.002% bromophenol blue, 10% β-mercaptoethanol) and heated to boiling point for 5 min.

3.2.5.5. Electrophoresis

Electrophoretic separation was done by using BIO-RAD Mini-PROTEAN® 3 Cell electrophoresis unit. The prepared samples were loaded into the wells with different protein concentration of each sample and electrophoresed at 80 V through stacking gel. Once the sample was concentrated at the interface of the stacking and separating gel as sharp blue line, the voltage was increased to 120 V and the electrophoresis was continued until the tracking dye reached at the bottom of the gel.

3.2.5.6. Staining and destaining of gel

After the run, gel was removed from plates and kept in staining solution (0.1% coomassie brilliant blue R-250 w/v in 40% methanol, 10% acetic acid w/w) for 4-6 h with mild shaking at room temperature. Then, the gel was destained with several changes of destaining solution I (40% methanol, 10% glacial acetic acid) and finally kept in destain II (10% glacial acetic acid).

Gels after electrophoresis were photographed and analyzed using Gel documentation system (BioRad, USA). Native-PAGE was performed similarly except the SDS and other denaturing agents in gel and samples buffers were not added.

3.2.6. Silver staining

Since the silver staining is more sensitive compared to coomassie blue to ensure the purity of the protein, gel was also analyzed after silver staining. Silver staining was performed according to Wray's method (1981). Sample was separated on 12% SDS-PAGE and fixed for 20 min in a solution containing 50% (v/v) methanol, 10% (v/v) acetic acid and deionised water. The gels were washed for 30 min in 10% (v/v) methanol, 5% (v/v) acetic acid and deionized water. The gels were then soaked in a 3.4 mM potassium dichromate solution containing 3.2 mM nitric acid for 5 min. The gels were rinsed with deionized water and placed in 12 mM silver nitrate for 20 min. The Image development was achieved by rinsing the gel in 0.28 M sodium carbonate containing 0.5 ml formaldehyde per liter of deionized water. Image development was stopped when slightly yellowish background appears by placing the gel in 3% acetic acid for 5 min. Gels were stored in 7% glycerol (v/v) and 10% (v/v) acetic acid. Gels were photographed and analyzed using under Gel documentation system (BioRad, USA).

3.2.7. Reverse phase high performance liquid chromatography (RP-HPLC)

Purified protein was diluted in a solution containing 0.1% (v/v) TFA and injected onto C18 column (25 x 0.4 cm) equilibrated with (0.1% (v/v) TFA) on reverse phase high performance liquid chromatography (Agilent series 1100 Model, USA). The column was developed at 1 ml/min with a linear gradient of (0.1% (v/v) TFA to 99.9% (v/v) acetonitrile in 0.1% (v/v) TFA) over 60 min. The elution was monitored by absorbance at 280 nm. Protein concentration of the elution peak was determined by the method of Bradford (Bradford, 1976) using bovine serum albumin (BSA) as a standard. A part of

the eluates was used for protein determination and electrophoresis, and the other was filtered through a 0.45 μm syringe filter and used for antimicrobial activity assays.

3.2.8. Molecular mass determination

MALDI-TOF studies were carried out for molecular mass determination at The Centre for Genomics Application (TCGA), New Delhi, India. For MALDI-TOF analysis, 1 μl of sample was mixed with 1 μl of saturated solution of matrix (sinnapinic acid in 0.1% TFA:Acetonitrile, 2:1) and spotted on MALDI target plate. The instrument (Bruker Daltonik Ultraflex TOF/TOF, USA) was calibrated by using protein calibration standard II Bruker Daltonics. The spectrum was acquired using Flexcontrol 2.2 software with ion source voltage 25 KV and accelerating voltage 23.2 KV in linear mode. The spectrum was processed using flexianalysis^{TM} 2.2 software.

(B) Evaluation of Antimicrobial activity of Purified protein

3.2.9 Growth and maintenance of bacteria and fungi

All bacteria (*P. putida, S. aureus, B. substilis* and *E. coli)* used in the present study were maintained on nutrient agar (Hi Media) media for routine maintenance. For experimental work, the bacteria were grown in nutrient broths at 37°C on incubator shaker at 100 rpm, unless otherwise mentioned. All the filamentous fungi (*A. fumigatus, A. flavus, A. niger and F. oxysporum)* and *C. albicans* used in the present study were maintained on PDA and YPD agar plates, respectively. For the experiments the filamentous fungi were grown in PD broths while *C. albicans* in YPD medium, unless otherwise mentioned. All the bacteria and fungi were preserved in lyophilized condition and also in glycerol containing medium at -80°C.

3.2.10. Antimicrobial activity assay using disk method

Antibacterial activity of crude protein fraction and purified protein (NOP-51) towards *P. putida, S. aureus, B. substilis* and *E. coli* was carried out by disk diffusion method as described (Espinel-Ingroff, 2003). Mid log phase grown cultures of respective bacteria were used for testing antibacterial activity. Briefly, nutrient agar plates were prepared under aseptic condition and 100 µl from a freshly grown mid log phase culture stocks (1 x 10^6 cell /ml) of respective bacteria were spread on plates. Several sterile Whatman no.1 filter paper disks (6 mm dia) were placed on each plate and loaded with different amount of crude (10, 20, 30, 40, and 50 µg /disk) and purified protein (NOP-51) (10, 15, 20, 25, and 30 µg /disk). Tris-HCl (50 mM, pH 8.0) and Gentamicin (30 µg/disk) were used as negative and positive control, respectively. The plates were incubated at 37°C for 24 h. Antibacterial activity was evaluated by measuring the zone of inhibition around the discs.

The antifungal activity of crude protein fraction and NOP51 against *A. fumigatus, A. flavus, A. niger, F. oxysporum* and *C. albicans* was tested using disk diffusion method as described (Espinel-Ingroff, 2003). The PDA plates for *A. fumigatus, A. flavus, A. niger, F. oxysporum* and YPD agar plates for *C. albicans,* respectively, were prepared and used for the study. After solidification of the plates 100 µl volume of freshly prepared fungal spore suspension stocks (1.0 x 10^5 spores/ml) of *A. fumigatus, A. flavus, A. niger and F. oxysporum* and *Candida albicans* (1 x 10^6 cell/ml) were spread on respective plates. Several sterile Whatman no.1 filter paper disks (6 mm dia) were placed on each plate and loaded with different amounts of crude (10, 20, 30, 40 and 50 µg/disk) and NOP-51 (10, 15, 20, 25 and 30 µg/disk). Negative and positive controls were 50 mM

Tris-HCl (pH 8.0) buffer and Fluconazole (20 µg/disk), respectively. The plates were incubated at 28 °C for 72 h and 48 h for *C. albicans*. Plates were examined for the presence of zones of inhibition to evaluate the antifungal activity.

3.2.11. Minimum inhibitory concentration (MIC) determination

Minimum inhibitory concentrations (MICs) of crude protein fraction and NOP-51 against various bacteria used in the study were determined by microdilution broth method as per method described (Espinel-Ingroff, 2003)). From the master stocks of the crude protein and NOP-51, a series of dilutions (ranging from 1 to 200 µg/ml) were prepared using sterile nutrient broth medium. In 96 well round bottom microtiter plate, 0.1 ml of various serial dilutions of crude protein and NOP-51 were taken in triplicate in their respective wells as per predetermined plan. Pre-inoculum of various bacteria used were prepared in nutrient broth. From the pre-inoculum, the final inoculum of each bacteria were prepared using sterile broth to give 10^5 cells/ml, as was determined by counting using hematocytometer. To the microtiter plate wells containing 0.1 ml of serially diluted protein, 0.1 ml of each inoculum was added. For control, 0.1 ml of medium alone and 0.1 ml of inoculum were taken. Gentamicin was used as reference drug. The experiment was carried out in triplicates. The microtiter plates were incubated at 37°C for 24 h. The growth in each well was determined visually. The MIC was defined as lowest crude and NOP-51 concentration which resulted in complete inhibition of visible growth.

The susceptibility testing of crude protein fraction and NOP-51 against *A. fumigatus, A. flavus, A. niger, F. oxysporum* and *C. albicans* was studied. MICs were determined by microdilution broth method as per standard norms. Like the bacteria MIC determination, a series of dilutions (1 to 200 µg/ml) were prepared from the master stock

of the crude protein and NOP-51 using sterile nutrient broth medium. In 96 well round bottom microtiter plate 0.1 ml of various serial dilution of crude protein and NOP-51 were taken in triplicate. From the pre-inoculum a final inoculum (1 x 10^5 spores/ml) using potato dextrose broth for filamentous fungi (*A. fumigatus, A. flavus, A. niger, F. oxysporum*) and YPD broth for *Candida albicans* (1 x 10^6 cells/ml) were determined by using hematocytometer. To the microtiter plate wells containing 0.1 ml of serially diluted proteins, 0.1 ml of each inoculum was added. For control 0.1 ml of medium alone and 0.1 ml of inoculum were taken. Fluconazole was used as standard drug for reference. The experiment was carried in triplicate. The microtiter plates were incubated at 28°C for 72 h and 48 h for Candida. The growth in each well was determined visually. The MIC was defined as lowest protein concentration in complete inhibition of visible growth.

3.2.12. Determination of MBC and MFC

The minimum bactericidal concentration (MBC) was determined by measuring the number of CFU on nutrient agar plates. Just after determination of the MICs, 20 µl of respective bacteria sample from wells that showed no growth were spread on plate and incubated at 37°C for 24 h. The MBC was established at lowest concentration of protein at which CFU was found to be negative.

The minimum fungicidal concentration (MFC) was determined by measuring the number of CFU on PDA and YPD plates. Just after determination of the MIC, 20 µl of respective fungi samples from wells that showed no growth were spread on PDA or YPD plate and incubated at 28°C for 72 h. The MFC was established at lowest concentration of protein at which CFU found to be negative.

3.2.13. IC_{50} Determination

To determine the IC_{50} of NOP-51 against various bacteria, nutrient agar plates containing different concentrations of NOP-51 (10, 20, 30, 40 and 50 μg /ml) were prepared. The experiments were performed in triplicate for each concentration. Briefly, to the autoclaved nutrient agar medium which was cooled down to 45°C different amount of protein were added to give above mentioned final concentrations, mixed rapidly and poured into Petri dishes. After solidification of the medium, 0.1 ml (1 x 10^5 cells) of the respective bacteria was spread on plates. Buffer alone without protein served as a control. After incubation at 37°C for 24 h, the numbers of colonies were counted and the percentage inhibition of bacterial growth was calculated as per below mentioned formula.

$$\text{Percent inhibition of bacterial growth} = \frac{\text{No. of colonies in absence} - \text{presence of purified protein}}{\text{No. of colonies in absence of purified protein}} \times 100$$

Similarly the IC_{50} of protein against fungi were also determined. Briefly, the PDA plates for filamentous fungi and YPD agar plates for *C. albicans* were used. To the autoclaved PDA and YPD agar media which were cooled down to 45°C, different amount of NOP-51 were added to give final concentrations (10, 20, 30, 40 and 50 μg/ml), mixed rapidly and poured into Petri dishes. After solidification of the medium, the same amount of mycelia and 0.1 ml (1 x 10^5 cells) in case of yeast was inoculated on plates. Buffer alone without protein served as a control. After incubation at 28°C for 72 h, the area of the mycelia was measured and the percentage inhibition of fungal growth was determined by given formula. In case of Candida the YPD plates were incubated at 28°C for 48 h, the no. of colonies was measured and the percentage inhibition of *C. albicans* growth was determined similarly as in case of bacteria.

$$\text{Percent inhibition of fungal growth} = \frac{\text{Area of mycelial colony in absence} - \text{presence of purified protein}}{\text{Area of mycelial colony in absence of purified protein}} \times 100$$

A graph between percentage inhibition of bacterial growth and the concentration of NOP-51 was plotted and used to determine the IC_{50} of NOP-51. IC_{50} value was defined as the concentration of protein at which 50% inhibition was observed.

3.2.14. Concentration dependent time-killing curve

Time killing curve studies of purified protein (NOP-51) were performed according to Mangoni *et al.*, (2004). *S. aureus, B. substilis, P. aeroginosa* and *E. coli* were grown at 37°C in nutrient broth medium until an absorbance at λ_{590} reached to one. The cells were centrifuged, washed and resuspended in 10 mM sodium phosphate buffer (pH 7.4). Approximately, 1 x 10^6 cells were incubated with different concentrations (10, 20, 30, 40, 50 µg/ml) of purified protein at 37°C. Aliquots of 10 µl were withdrawn at different time intervals and spread on nutrient agar plates. After overnight incubation at 37°C, the surviving bacteria expressed as colony forming units (CFU) were counted. The media containing protein solvent alone (50 mM Tris-HCl, pH 8.0) and inoculum was used as controls.

The time killing curve of purified protein (NOP-51) against *A. fumigatus, A. flavus, A. niger,* and *F. oxysporum and C. albicans* was also determined as per method described (Chryssanthou and Sjölin, 2004). Approximately, 1 x 10^5 spores/ml were incubated with different concentrations of purified protein (10, 20, 30, 40, 50 µg/ml) at 28°C. Aliquots of 10 µl were withdrawn at different time intervals and spread on potato dextrose agar plates. After incubation at 28°C for 72 h, the surviving fungi expressed as

CFU (colony forming units), were counted. *C. albicans* were grown at 28°C in yeast peptone dextrose broth medium similarly. Aliquots of 10 µl were withdrawn at different intervals and spread on YPD agar plates. After overnight incubation at 28°C, the surviving yeast expressed as CFU (colony-forming unit) were counted. The media containing protein solvent alone (50 mM Tris-HCl, pH 8.0) and inoculum was used as control.

3.2.15. Electron microscopy study

To evaluate the affect of protein on the cell surface morphology and anatomy of bacteria and fungi, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) studies were performed.

3.2.15.1. Scanning electron microscopy

Bacteria from the exponential phase were incubated with 10, 30 and 50 µg/ml of purified protein for 30 min. After 30 min incubation, the cells were harvested by centrifugation at 2000 x g for 5 min, washed three times with phosphate buffer saline and resuspended in the same buffer. Controls were run in the presence of 50 mM Tris-HCl buffer, pH 8.0. Each sample was spread on a poly (L-lysine)-coated glass slide (18 mm × 18 mm) to immobilize bacterial cells. Glass slides were incubated at 30°C for 90 min. Slide-immobilized cells were fixed with 2.5% (w/v) glutaraldehyde in 0.1 M sodium phosphate buffer, extensively washed with the same buffer and dehydrated with a graded ethanol series (70%, 80%, 90% for 5 min each and 100% 3 x 5 min each). They were finally dried under vacuum. Samples were then mounted, sputter coated with gold and examined under scanning electron microscope, (LEO, model 435 VF, England.

Fresh fungal culture of *A. fumigatus, A. flavus, A. niger* and *F. oxysporum* on potato dextrose agar were used for scanning electron microscopy studies. A 5 x 5 mm segment were cut from culture growing on potato dextrose agar plates and treated with 10, 30, 50 µg/ml of purified protein for 30 min and was promptly placed in vials containing 2.5 % (w/v) glutaraldehyde in 0.1 M sodium phosphate buffer pH 6.8 at 4 °C, samples were kept in this solution for 24 h for fixation and then washed with distilled water three times for 20 min each. Following, they were dehydrated in ethanol series (70%, 80%, 90%) for 20 min each and finally with absolute alcohol for 45 min. They were finally dried under vacuum. Samples were then mounted, sputter coated with gold and examined under scanning electron microscope, (LEO, model 435 VF, England).

Cells of *C. albicans* from the exponential phase were incubated with 10, 30, 50 µg/ml of purified protein for 30 min and were harvested by centrifugation at 2000 x g for 5 min, washed three times with phosphate buffer saline and resuspended in the same buffer. Controls were run in the presence of 50 mM Tris-HCl buffer pH 8.0. Each sample was spread on a poly (L-lysine)-coated glass slide (18 mm × 18 mm) to immobilize yeast cells. Glass slides were incubated at 30°C for 90 min. Slide-immobilized cells were fixed with 2.5% (w/v) glutaraldehyde in 0.1 M sodium phosphate buffer, extensively washed with the same buffer and dehydrated with a graded ethanol series (70%, 80%, 90% for 5 min each and 100% 3 x 5 min each). They were finally dried under vacuum. Samples were then mounted, sputter coated with gold and examined under scanning electron microscope, (LEO, model 435 VF, England).

3.2.15.2 Transmission electron microscopy

TEM analysis was performed at the Department of Microscopy, All India Institute of Medical Sciences (AIIMS), New Delhi, India. Samples containing *S. aureus, B. substilis* and *E. coli* (1 x 10^6 cells/ml) were incubated at 37°C for up to 30 min with 50 µg/ml of purified protein (NOP-51) and were centrifuged at 2000 x g. Controls were run in the presence of 50 mM Tris-HCl buffer pH 8.0. The bacterial cells were fixed by incubation with 2% glutaraldehyde, 2% paraformaldehyde in PBS for 20 min. After washing with 0.1 M sodium phosphate buffer pH 6.8, four times 10 min each, samples were post fixed with osmium tetroxide in the same buffer (1% osmium tetroxide, 0.8% potassium ferrocyanide) for 3 h at room temperature. They were washed three times with buffer and dehydrated in acetone series and embedded in epon resin. Ultrathin sections were mounted on copper grid and stained with 2% (w/v) phosphotungstic acid. The grids were examined by using a transmission electron microscope (Phillips, model CM 10, Holland).

Fresh fungal culture of *A. fumigatus, A. flavus, A. niger* and *F. oxysporum* was treated with 50 µg/ml of purified protein, incubated at 28°C for 30 min. After treatment, culture were fixed in 2% glutaraldehyde, 2% paraformaldehyde in PBS for 20 min and then processed for TEM by using an agar bubble to enclose the sample. After washing with 0.1 M sodium phosphate buffer pH 6.8, four times 10 min each, samples were post fixed with osmium tetroxide in the same buffer (1% osmium tetroxide, 0.8% potassium ferrocyanide) for 3 h at room temperature. They were washed three times with buffer and dehydrated in acetone series, and embedded in epon resin. Ultrathin sections were mounted on regular copper grids and stained with uranyl acetate and lead acetate. The

grids were examined by using a transmission electron microscope (Phillips, model CM 10, Holland).

Samples containing *C. albicans cells* (1 x 10^6 cells/ml) were incubated at 28°C for up to 30 min with 50 µg/ml of NOP-51 and were centrifuged at 2000 x g. Controls were run in the presence of 50 mM Tris-HCl buffer pH 8.0. The cells were fixed by 2% glutaraldehyde, 2% paraformaldehyde in PBS for 20 min. They were post fixed with osmium tetroxide in the same buffer (1% osmium tetroxide, 0.8% potassium ferrocyanide) for 3 h at room temperature. They were washed three times with buffer and dehydrated in acetone series, and embedded in epon resin Ultrathin sections were mounted on copper grid and stained with uranyl acetate and lead acetate. The grids were examined using a transmission electron microscope (Phillips, model CM 10, Holland).

3.2.16. Effect of temperature and pH on antimicrobial activity

Effect of temperature on the antimicrobial activity of purified protein was studied by heating the protein at different temperature for 20 min. Aliquots of purified protein were heated at 20, 30, 40, 50, 60, 70, 80, 90 and 100°C for 20 min. After incubation, each aliquot was tested for antimicrobial activity. NOP-51 was also stored at room temperature and antimicrobial activity was monitored on alternate days for one month.

The effect of pH on the purified protein preparation were investigated within a pH range between pH 2.2 to pH 12.0 using 0.2 M citrate buffer (pH 2.2, 3.0, 4.0, 5.0 and 6.0), 0.2 M Tris-HCl buffer (pH 7.0, 8.0, and 9.0), and 0.2 M glycine-NaOH buffer (pH 10.0, 11.0, and 12.0). Each sample was incubated at room temperature for 1 h. After the treatment, antimicrobial activity of the purified protein was determined.

(C) Immunomodulatory activity of purified protein (NOP-51):

3.2.17 Animals

The study was conducted on male Swiss albino mice (32 ± 3 g). The animals were bred and maintained under standard laboratory conditions: temperature (25 ± 2°C) and photoperiod of 12 h. Commercial pellet diet and water were given ad libitum. All the animal handling and related experimental work got the approval of the animal ethical committee of the Indian Institute of Technology Roorkee, Uttarakhand, India.

3.2.18. Study of *in vitro* immunomodulatory activity of NOP-51

3.2.18.1. Macrophage cell cultures

A standard and established method for peritoneal macrophage cell culture was adopted (Edelson and Cohn, 1976). Briefly, the mouse peritoneal macrophages were elicited by injecting 4% thioglycollate medium into swiss male albino mice. Peritoneal cells were exudated 72 h after injection by flushing peritoneal cavity with ice-cold RPMI 1640 incomplete medium using a 22 G1 needle. Peritoneal lavage were pooled and collected in 50 ml conical centrifuge tube and centrifuged at 1500 rpm, (10 min, 4°C). The pellet was resuspended in complete RPMI 1640 medium supplemented with 10% heat inactivated FCS containing 15 mM HEPES buffer, 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin. Cells were counted and their viability was checked by Trypan blue dye exclusion method. The peritoneal macrophages were examined microscopically for their morphological identification on phase contrast microscope. Cells (1 x 10^5 /well in a final volume of 200 µl) were seeded in flat bottom 96-well polystyrene microtiter plate (Coster, Corning, NY) and incubated at 37°C, 5% CO_2 and 85% humidity chamber for 3 h to achieve adherence. The non-adherent cells were

removed by repeated washing with ice-cold serum RPMI 1640 medium while adherent cells were cultured for further studies.

3.2.18.2. Measurement of total nitrite

The thioglycollate-elicited peritoneal macrophages (1 x 10^5 /well) were cultured with different concentrations of NOP-51 in presence or absence of LPS (5μg/ml) at 37°C, 5% CO_2 and 85% humidity chamber for 24 h. Cells without protein either in presence or absence of LPS were used as control. NO synthesis was determined by assaying the culture supernatants for nitrite, the stable reaction product of NO with molecular oxygen, using Griess reagent as described by Green *et al.*, (1982) was performed. An equal volume of the culture supernatant (100 μl) from different treatment groups was mixed with 100 μl of Griess reagent (1 % sulphanilamide and 0.1 % N-[naphthyl] ethylenediamine dihydrochloride; 1:1) and the absorbance was measured at 550 nm by ELISA reader (BIOTEK instrument INC, Canada). LPS (5 μg/ml) was used as a positive control for the stimulation of macrophages to produce nitrite. The amount of nitrite was calculated from a $NaNO_2$ standard curve.

3.2.18.3 Quantification of TNF-α

The peritoneal macrophage culture supernatants used for TNF-α assay. The assay was carried out using mouse TNF-α ELISA kit from Endogen, Pierce, USA. The kit was a solid phase sandwich ELISA. A monoclonal antibody specific for mouse TNF-α was coated onto the wells of the microtitre strip plate provided. The antigen and a biotinylated monoclonal antibody specific for mouse TNF-α were simultaneously incubated. Revelation step included Steptavidin-HRP and 3,3,5,5-tetramehylbenzidine (TMB) as chromogen. Results were read immediately at 450 nm using a microtitre plate reader

BIO-TEK instrument Inc., Canada). LPS (10 ng/ml) was used as positive control for the stimulation of TNF-α. A standard curve was obtained by plotting known concentration of TNF-α vs absorbance at 450 nm.

3.2.18.4 Preparation of mouse splenocytes

The spleens were excised from mice aseptically and kept in incomplete RPMI 1640 medium. The single-cell suspension was prepared as per procedure described by Ly & Mishell (1974). The spleen was minced using a pair of scissors and passed through a fine steel mesh to obtain a homogeneous cell suspension and the suspension was layered on incomplete serum free RPMI 1640 medium in a 60 mm Petri dish for 10 min to allow cellular debris to settle down. The suspension was carefully transferred to a 15 ml conical centrifuge tube and centrifuged at 300 x g, (10 min, 4°C). Red blood cells were removed by centrifuging the pellet in RBC lysing buffer (0.1 M NH_4Cl). The cell suspension was again centrifuged at 300 x g, (10 min, 4°C) and the pellet was resuspended in complete RPMI 1640 medium. 1 x 10^6 cells/well in a final volume of 200 µl were seeded in flat bottom 96-well polystyrene microtitre plate and cultured for further studies.

3.2.18.5. Effect on splenocytes proliferation

The proliferation assay of splenocytes was tested according to 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mossmann, 1983). Briefly, 1 × 10^5 cells/well was incubated with various concentration of purified protein (25-150 µg/ml) for 24 h at 37°C in humidified 5% CO_2. Proliferation of cells in the absence and presence of mitogens were investigated by using predetermined optimum doses of lipopolysaccharide (LPS) and concanavalin A (Con A) (5 µg/ml) for stimulating B and T lymphocytes respectively. After 72 h, MTT (5 µg/ml) in PBS was added and the

plate was further incubated for 2-5 h at 37°C. Culture medium was discarded by aspiration after incubation and added with 100 µl of 0.04 M HCl in isopropanol to lyse cells. Then, 100 µl of distilled water was added to dilute the solution and the absorbance was measured at 570 nm.

3.2.18.6. *In vitro* cytotoxicity assay

Viable counts of macrophages and splenocytes were measured by the Trypan blue dye exclusion method described by Ignacio *et al.,* (2001). Thioglycollate-elicited adherent peritoneal macrophages (1 x 10^5 cells/well) and splenocytes (1 x 10^6 cells/well) were placed in microtiter plates with medium supplemented with 10% FCS, containing different concentrations of protein (25-150 µg/ml) and incubated up to 24 h at 37°C. The cell suspensions were mixed with equal volume of dye (0.1% in PBS) and incubated at room temperature for 5 min. The cells were washed to remove the free dye. Viable cell counts were taken by using a haemocytometer under 40 x magnification of the Phase contrast microscope. Cells with dye were excluded and only transparent cells were taken and counted as viable cells. Triplicate wells were set for each concentration.

3.2.19. Study of *in vivo* immunomodulatory activity of NOP-51

3.2.19.1. Treatment of animals

Animals were divided into five groups of six animals each; (Group I) control, received 50 mM Tris-HCl pH 8.0; (Group II) received 25 µg/ml; (Group III) received 50 µg/ml; (Group IV) received 100 µg/ml; and (Group V) received 150 µg/ml, respectively of NOP-51. The dose volume of 200 µl in each group was given intraperitoneally (i.p.) for 15 consecutive days. The animals were sacrificed 24 h after the last dose. Body

weight and organ weight of kidney, liver, spleen and thymus were determined for each animal.

3.2.19.2. Peritoneal macrophage primary cell culture

Mouse peritoneal macrophages from the various animal treatment groups were isolated and cultured similarly as discussed in *in vitro* study (section-3.2.18.1).

3.2.19.3. Macrophage function assay

3.2.19.3.1 Total nitrite estimation

The effect of *in vivo* treatment of NOP-51 on peritoneal macrophages NO production was studied similarly using Griess reagent as described in section (3.2.18.2)

3.2.19.3.2. Quantification of TNF-α

The effect of *in vivo* treatment of NOP-51 on peritoneal macrophages TNF-α was studied similar using mouse TNF-α ELISA kit from Endogen, Pierce, USA as described in section (3.2.18.3)

3.2.19.3.3. Cellular lysosomal enzyme activity assay

The cellular lysosomal enzyme activity (acid phosphatase) was used to determine the phagocytosis activity of peritoneal macrophages as per method described (Suzuki *et al.*, 1988) with little modification. Briefly, 30 µl of macrophage suspension (1×10^5 cells/well), 50 µl of RPMI medium and the culture was incubated at 37°C in 5% CO_2 humidified atmosphere for 24 h. The medium was removed by aspiration and 20 µl of 0.1% Triton X-100, 100 µl of 10 mM *p*-nitrophenyl phosphate (*p*-NPP) (Sigma) solution and 50 µl of 0.1 M citrate buffer (pH 5.0) were added in each well. The plate was further incubated for 30 min, 150 µl of 0.2 M borate buffer (pH 9.8) was then added and the absorbance was measured at 405 nm (BIO-TEK instrument Inc., Canada).

3.2.19.3.4. Assay for candidacidal activity

Candidacidal activity of peritoneal macrophages of *in vivo* NOP-51 treated animals was assayed by the method of Nozawa and Yokata (1982) with slight modifications. Briefly, 200 cells of *C. albicans* in 100 µl of RPMI 1640 were added to the macrophage monolayer (1 x 10^5 cells/well) in 96-well tissue culture plate and incubate at 37°C for 3 h. Then, the cell suspension was spreaded on Sabouraud agar. The number of colonies of *C. albicans* was counted after incubation for 24 h at 28°C. The candidacidal activity was calculated by the following formula:

Candidacidal activity = 200 – Average no colonies/200

3.2.19.4. Splenocyte primary cell culture

The single-cell suspension of splenocytes was prepared as described in section 3.2.17.4.

3.2.19.5. Splenocyte function assay

3.2.19.5.1 Splenocyte proliferation assay

The effect of *in vivo* treatment of NOP-51 on splenocytes proliferation was studied similarly as described in section (3.2.17.5). Briefly, 1 × 10^5 cells/well of different treated animals groups was incubated with various concentrations of purified protein (25-150 µg/ml) for 24 h at 37°C in humidified 5% CO_2. Proliferation of cells in the absence and presence of mitogens were investigated by using predetermined optimum doses of lipopolysaccharide (LPS) and concanavalin A (Con A) (5 µg/ml) for stimulating B and T lymphocytes respectively. After 72 h, MTT (5 µg/ml) in PBS was added and the plate was further incubated for 2-5 h at 37°C. The proliferation was measured similarly as described above.

3.2.19.5.2. Quantification of IL-2

The level of IL-2 was determined in the splenocytes cell culture supernatant of different *in vivo* protein treated animal groups by an enzyme linked immunosorbant assay (ELISA) according to manufacturer's instruction (Endogen, Pierce, USA), A monoclonal antibody specific for mouse IL-2 was coated onto the wells of the microtitre strip plate provided. Revelation step included anti-IL-2-HRP Conjugate and 3,3,5,5-tetramehylbenzidine (TMB) as chromogen. Results were read immediately at 450 nm using a microtitre plate reader. PHA (5 µg/ml) was used as positive control for the stimulation of IL-2. A standard curve was obtained by plotting known concentration of IL-2 vs absorbance at 450 nm.

3.2.19.6 Viability assay

Viable counts of macrophages and splenocytes were measured by the Trypan blue dye exclusion method described by Ignacio *et al.,* (2001). Cells from the different experimental groups were taken as described in the above sections on cell isolation and culture. The cell suspensions were mixed with equal volume of dye and incubated at room temperature for 5 min. The cells were washed to remove the free dye. Viable cell counts were taken by using a haemocytometer under 40 x magnification of the phase contrast microscope. Cells with dye were excluded and only transparent cells were taken and counted as viable cells.

(D) Toxicological investigation

3.2.20. Lactate dehydrogenase assay

The presence of exclusive cytosolic enzyme, LDH, in cell culture medium is the indication of cell membrane damage. The assay of LDH was performed as per methods described (Fry and Hammond, 1993; Freshney, 2000). Splenocytes and peritoneal macrophages cell culture supernatants were collected from each tested group and LDH activity was measured by using Cayman kit, USA as per the manufacturer instructions.

3.2.21. Hematological parameter

Blood sample of each animal was collected in the presence of a drop of anticoagulant (4% sodium citrate). Total WBC count (Turke's method), RBC count (Hayem's method), hemoglobin content (cyanmethemoglobin method) were studied in treated as well control animals.

3.2.22. Liver function tests

Transaminase activity was used as a diagnostic tool for liver tissue destruction. The level of serum glutamate oxalate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), bilirubin and urea measured using standard methods. Liver tissues are rich in GPT and GOT and necrosis to liver tissue results in more rapid changes in serum GPT and GOT level. All tests were performed by using the kits (Span Diagnostics, Surat, India) according to the manufacturer's instructions. For this purpose, five groups of animals (one control + four treatment groups) as described above were used and treated for 15 days with respective doses of purified protein.

(E) Identification of NOP-51 protein from *N. odorum* leaves

3.2.23. N-terminal sequence analysis

To analyze the N-terminal amino acid sequence, purified protein (NOP-51) was subjected to SDS-PAGE and then electroblotted onto polyvinylidene difluoride membrane (Immobilone-P^{SQ}, Millipore, USA) in 100 mM CAPS buffer, pH 11.0. After electroblotting, membrane was stained with 0.1% Coomassie brilliant blue R-250, the protein bands on the membrane were cut out and rinsed with 50% and 100% methanol (three times each) to eliminate the staining reagent. Now protein band was analyzed to determine N-terminal amino acid sequence by using Edman degradation method using an automated protein sequencer (Shimadzu Automated Protein Sequencer (PPSQ-20), USA) at the National facility of Protein Analysis, Department of Biophysics, All India Institute of Medical Sciences (AIIMS), New Delhi, India.

3.2.24. Identification by LC-MS/MS

For LC-MS/MS studies, the purified protein was run on 12% SDS-PAGE and the band containing the 51 kDa protein of *N. odorum* leaves was excised by using a clean scalpel or razor blade from the gel. Band containing gel pieces were successively washed in H_2O/methanol/acetic acid (47.5:47.5:5) and in acetonitrile, dried under a vacuum, rehydrated in 50 mM NH_4HCO_3 containing 10 ng/µl trypsin (Sigma Aldrich), and incubated for 5 h at 37°C. This first supernatant was collected and stored at -20°C. The peptides left in the gel pieces were extracted once with 50 mM NH_4HCO_3 and twice with a water/acetonitrile/formic acid (47.5:47.5:5) solution. These three supernatants were pooled to the first one, concentrated in a vacuum centrifuge to a final volume of 30 µl, acidified by adding 1.8 µl of acetic acid, and stored at - 20°C. The peptide mixtures were

analyzed for high-performance liquid chromatography nanospray ion trap tandem MS analyses (LC-MS/MS).

The peptide mixture resulting from trypsin digestion was analyzed by online capillary high-performance liquid chromatography coupled to a nanospray LCQ ion trap mass spectrometer. Peptides were separated on a 75 μm (inner diameter) by 15 cm C18 PepMap column. The flow rate was 200 nl/min, and peptides were eluted using a 5 to 50% linear gradient of solvent B in solvent A; solvent A was 0.1% (v/v) formic acid in 5% acetonitrile, and solvent B was 0.1% formic acid in 80% acetonitrile. The mass spectrometer was operated at a 1.8 kV needle voltage and a 38 V capillary voltage. Data acquisition was performed in a data dependent mode consisting of a full-scan MS over the range *m/z* 300 to 100,000. Data were analyzed by using the MSDB and NCBI software.

3.2.25 Statistical analysis

All antimicrobial and immunomodulatory activity studies were carried out in triplicate and six replicates respectively, experimental results represents the mean of three/six identical studies. Standard deviation (SD) and standard error (SE) were calculated using following formula:

$$SD = \frac{\sqrt{\sum X^2}}{N}$$

$$SE = \frac{SD}{\sqrt{N}}$$

CHAPTER 4

RESULTS

(A). Isolation, purification and analysis of antimicrobial protein

4.1 Isolation and purification

Total protein from *N. odorum* fresh leaves was extracted as described in methods section and tested for antimicrobial activity. Crude protein preparation with antimicrobial activity was fractionated by ammonium sulfate fractionation. The fractions (20-50%) saturation having significant antimicrobial activity was pooled, dialyzed, concentrated and was subjected to gel filtration chromatography on Sephadex G-100 column. The protein elution profile of Sephadex G-100 column chromatography is shown in figure 2. Total five protein peaks named G1, G2, G3, G4 and G5 were obtained. Each peak (G1-G5) was tested for antimicrobial activity and protein content. The peak G2 exhibited antimicrobial activity was further purified using ion exchange chromatography on CM-cellulose column. Proteins were eluted with NaCl step gradient of 0 - 1 M NaCl in 50 mM Tris-HCl (pH 8.0). Single peak was obtained with both 50 mM Tris-HCl (pH 8.0) (A) and 150 NaCl (B). The elution patterns are shown in figure 3. The antimicrobial activity and protein content of both peak A and B was tested. The antimicrobial activity was present in 150 mM NaCl eluted peak only (Peak B). This active peak fraction were pooled, dialyzed and concentrated using Amicon 100 kDa cut off membrane and was used for all further studies.

4.2. Analysis of crude and purified protein fraction

4.2.1 SDS-PAGE analysis

To monitor the purification steps and to ensure the purity of the purified protein, the crude, G2 peak of Sephadex G-100 and peak B of CM-cellulose column which showed the antimicrobial activity were analyzed on SDS-PAGE. The coomassie blue

stained 12% SDS-PAGE protein profiles of these fractions are shown in figure 4. As clear from the gel profile that the crude preparation has several proteins ranging from 15-70 kDa (Fig. 4A, lane 2). The G-2 peak after Sephadex G-100 column conferring antimicrobial activity has two protein bands, which were closed in molecular weight. These proteins were separated on CM cellulose column, due to their different affinity. The coomassie blue stained gel profile of peak-B with antimicrobial activity has only single band of 51 kDa without any other contaminating protein. (Figure 4B, Lane 3). Thus finally a 51 kDa protein with antimicrobial activity from *N. odorum* leaves was purified. The 51 kDa purified protein from *N. odorum* leaves having antimicrobial was named as *N. odorum* protein-51 (NOP-51) and the designation will be used for further discussion throughout the thesis.

The purity of the NOP-51 antimicrobial protein was also checked by silver staining. Increasing concentration of purified protein was loaded in each well and the gel after electrophoresis was stained with silver nitrate. Silver stained gel profile of the protein is shown in figure 5. It was clear from the gel that purified protein does not have any other contaminating protein even at higher concentration.

4.2.2 Native- PAGE analysis of NOP-51

The purified antimicrobial NOP-51 was further analyzed on Native-PAGE. The coomassie blue stained 12% native PAGE gel is shown in figure 6 (A). The NOP-51 found to be a monomeric protein as a single 51 kDa band was observed both in Native-PAGE and SDS-PAGE (Fig. 6 A & B).

4.2.3. Reverse-phase high performance liquid chromatography (RP-HPLC)

The purity of the purified protein (NOP-51) was also checked on RP-HPLC. The loading of NOP-51 on reverse phase high performance liquid chromatography on C18 column, resulted in a single sharp elution peak (Fig. 7). This further confirms the purity of NOP-51. Hence it represents purified protein.

4.2.4. Molecular mass determination of NOP-51 using MALDI-TOF

The mass of the purified protein (NOP-51) was determined using Matrix Assisted Laser Desorption Ionization-Time Of Flight (MALDI-TOF). MALDI-TOF further confirmed the purity of the protein as a single peak of mass 51.4 kDa was obtained without any contaminating peak (Fig. 8). This was almost similar as revealed from SDS-PAGE, in which molecular weight of the protein found to be around 51 kDa. Though the MALDI-TOF result seems to be more accurate usually, however, for simplicity mass 51 kDa has been used throughout the thesis.

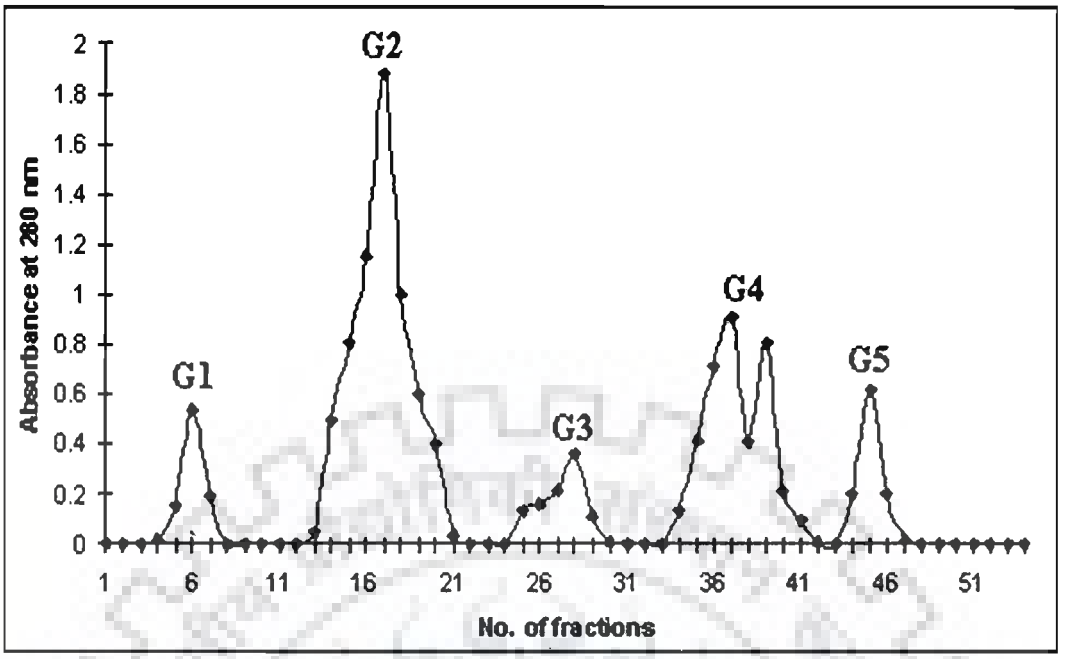

Figure 2. Sephadex G-100 gel filtration chromatography of ammonium sulphate enriched fraction of *Nerium odorum* leaves crude protein preparation. The column was pre-equilibrated and eluted with 50 mM Tris buffer, pH 8.0. Five protein elution peaks G1, G2, G3, G4 and G5 are indicated.

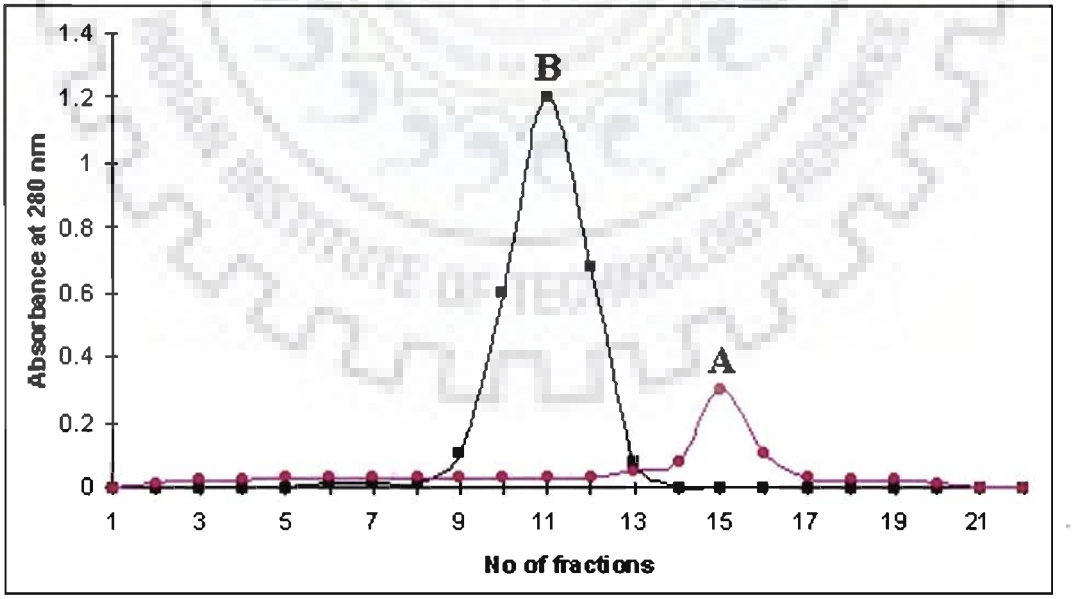

Figure 3. CM-cellulose cation-exchange column chromatography of active peak G2 of Sephadex G-100. The column was pre-equilibrated with 50 mM Tris buffer, pH 8.0, and eluted with step gradient of (0 to 1.0 M) NaCl in 50 mM Tris buffer, pH 8.0. A and B are buffer and 150 mM NaCl eluted peaks, respectively.

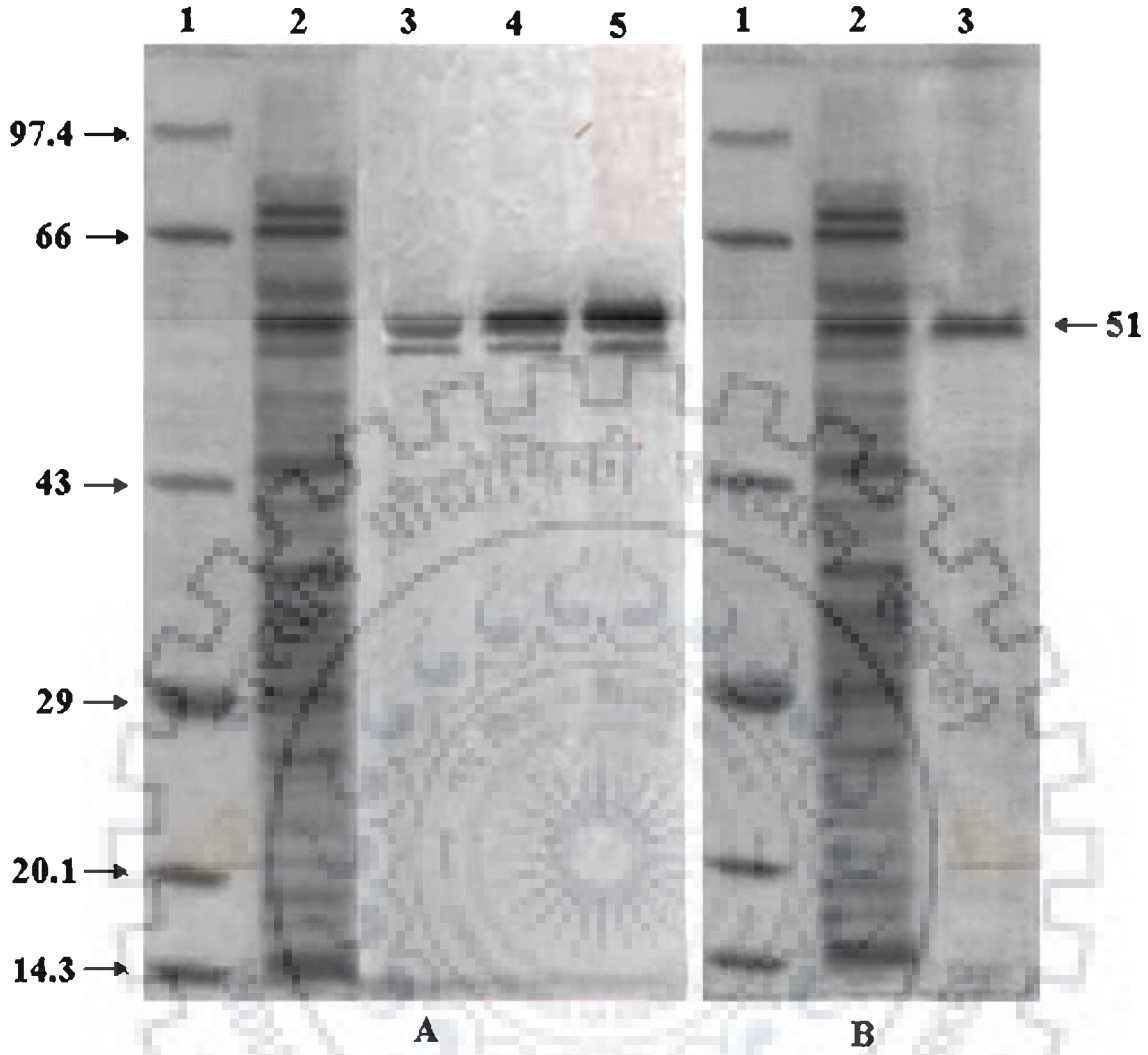

Figure 4. Coomassie blue stained 12% SDS-PAGE protein profiles of crude fraction, G2 peak of Sephadex G-100 and peak B of CM-cellulose column chromatography. Panel A: Lane 1, Molecular weight marker; Lane 2, 30 µg of total protein extract; Lane 3, 4, 5 are the protein after gel filtration chromatography (20, 30, 40 µg respectively). Panel B: Lane 1, Molecular weight marker; Lane 2, 30 µg of total protein extract; Lane 3, 30 µg of peak B after CM-cellulose column chromatography. 51 kDa Protein band is indicated.

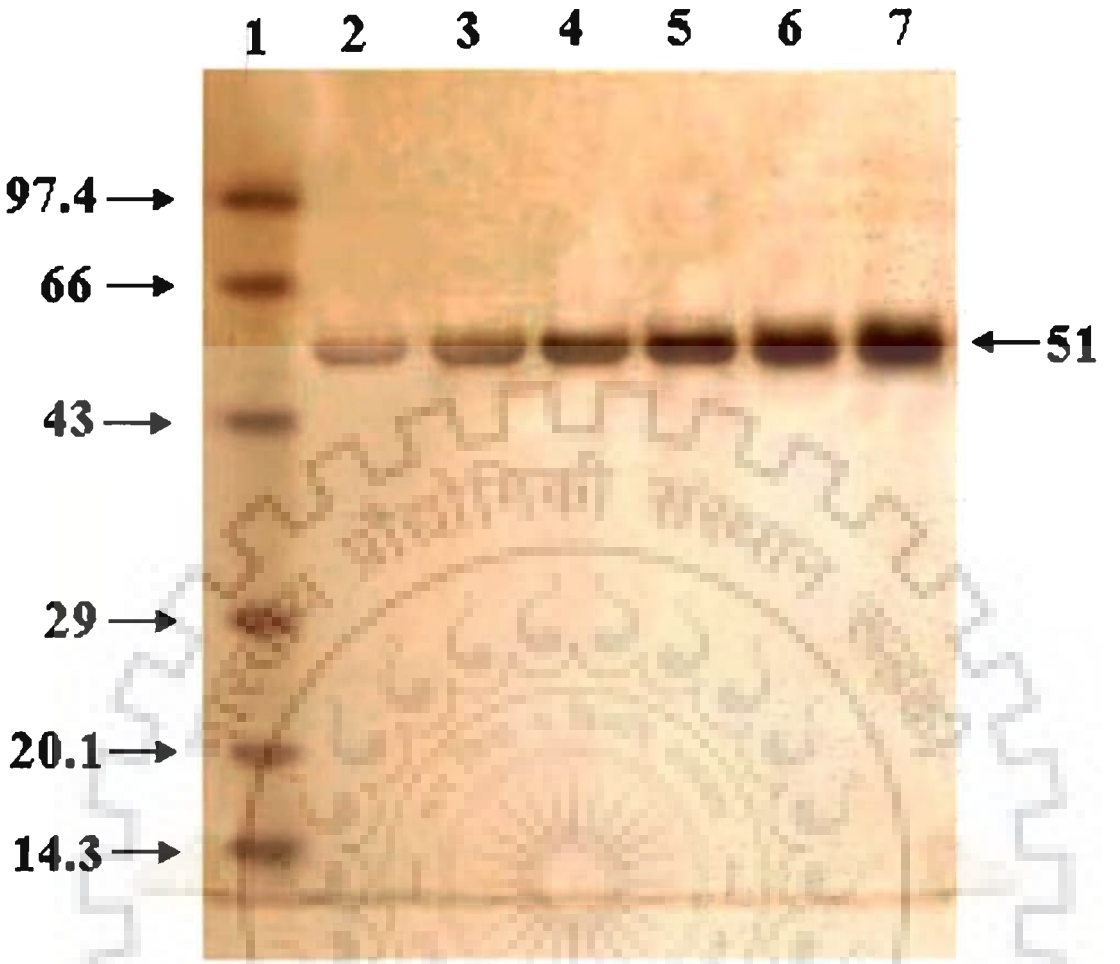

Figure 5. Silver stained 12% SDS PAGE protein profile of peak B of CM-cellulose column chromatography. Lane 1, Molecular weight marker; Lane 2, 3, 4, 5, 6 and 7 are different concentration of peak B eluate (10, 20, 30, 40, 50, and 60 µg, respectively). 51 kDa protein band is indicated.

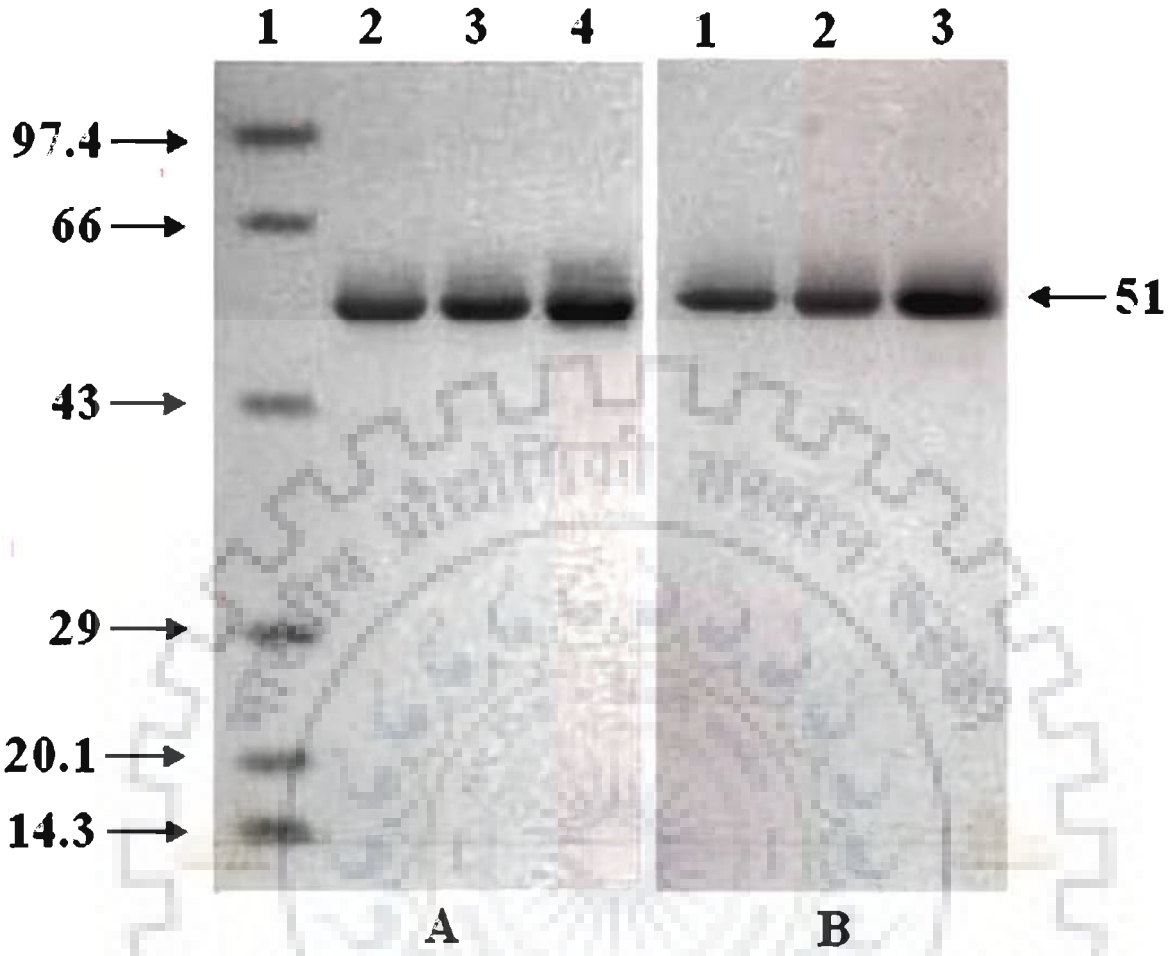

Figure 6. Coomassie blue stained 12% native and SDS-PAGE protein profile of peak B of CM-cellulose column chromatography. A: native-PAGE, Lane 1, Molecular weight marker; Lane 2, 3, 4 are the 20, 30 and 40 µg of peak B eluate. B: SDS-PAGE, Lane 1, 2, 3 are the 20, 30 and 40 µg of peak B eluate.51 kDa protein band is indicated.

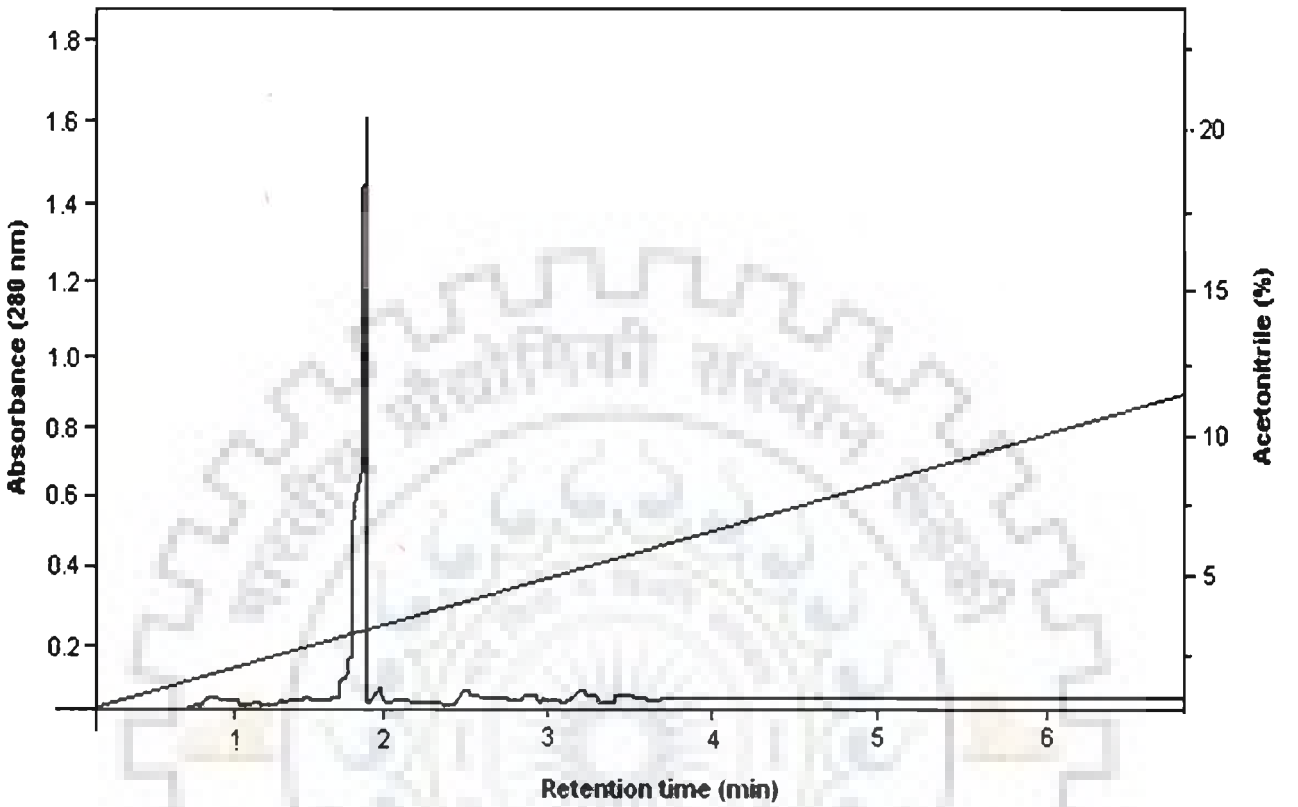

Figure 7: RP-HPLC profile of peak B of CM-cellulose column chromatography. Peak B after cation exchange chromatography was loaded on reverse phase Pharmacia C18 column (25 x 0.4 cm) equilibrated with (0.1% (v/v) TFA). An elution gradient (0 to 99.9% acetonitrile in 0.1% TFA from 0 - 60 min) was employed to elute the protein. Three cycles of reverse phase chromatography of the active protein led to the elution of a single peak containing antimicrobial activity.

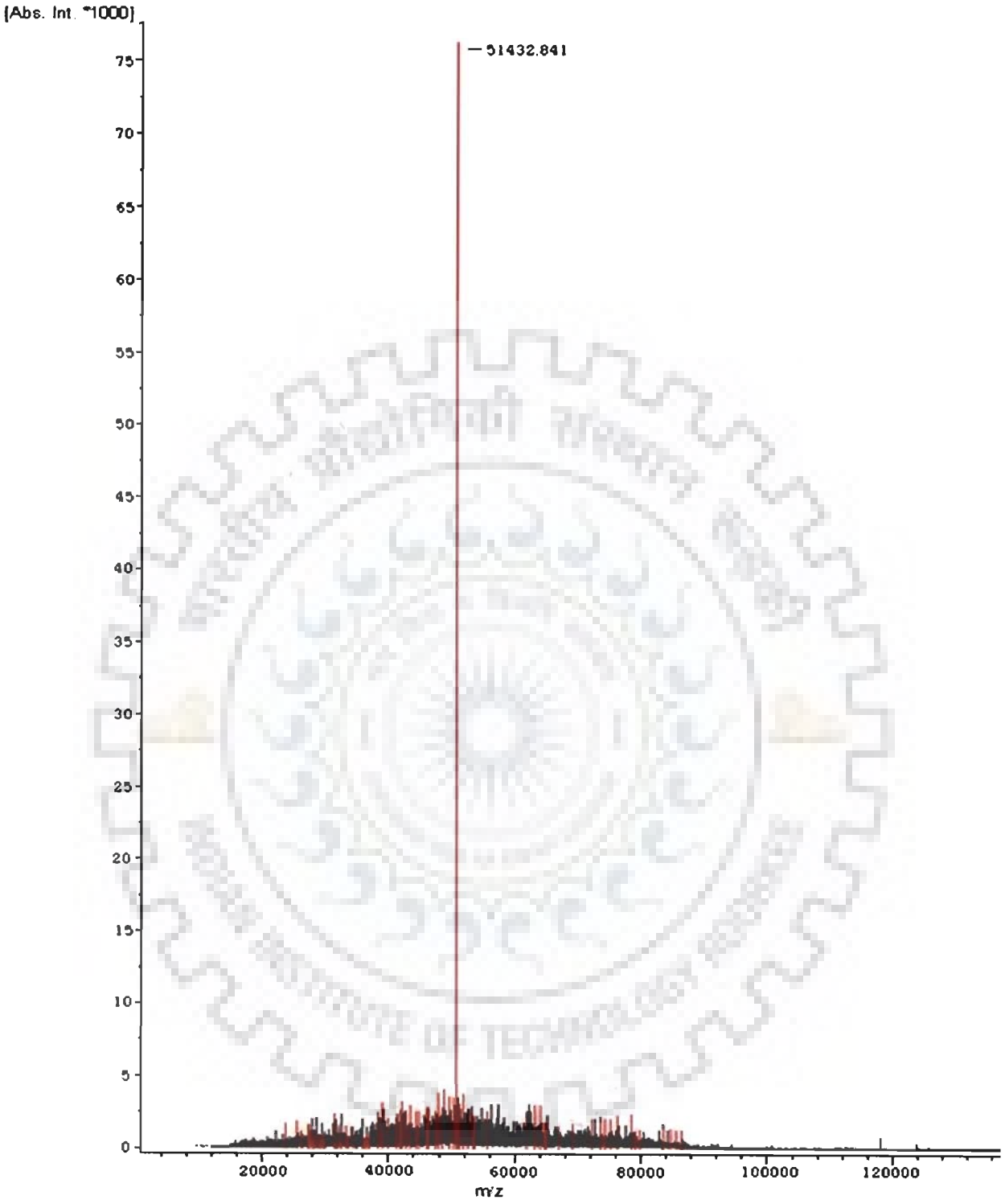

Figure 8. Molecular mass determination of purified protein using mass spectrometery. MALDI-TOF analysis showed the exact molecular mass of purified protein to be 51.4 kDa.

(B) Evaluation of antimicrobial activity of NOP-51

4.3. Evaluation of antibacterial activity

4.3.1. Antibacterial activity assay using disk diffusion and broth dilution methods

In vitro antibacterial susceptibility tests of the crude protein fraction and purified protein (NOP-51) against Gram positive and Gram negative bacteria were performed using disk diffusion method. Both the crude and NOP-51 showed broad spectrum antibacterial activity as acted against all the Gram positive and Gram negative bacteria tested. NOP-51 found to be more effective as significant antibacterial activity was observed against most of the tested bacteria even at much lower concentration compared to crude protein which showed antibacterial activity relatively at higher concentrations. There found to be little differences in the susceptibility among different bacterial strains. The results are summarized in figure 9 and Table 4 & 5.

The MIC and MBC of crude and purified protein (NOP-51) were determined using microdilution method. Gentamicin was used as reference antibiotic for comparisons. The MIC and MBC results are summarized in Table-6. Similar to the observation of inhibition zone formation by disk method, NOP-51 found to be more effective as it has significantly low MIC 12, 25, 25 and 12 µg/ml, for *S. aureus*, *B. subtilis*, *P. putida* and *E. coli,* respectively, compared to crude protein which has 25, 50, 50 and 25 µg/ml against these bacteria. Similarly, the MBC of the NOP-51 found to be significantly lower (25, 50, 50, 50 µg/ml) compared to crude protein (50, 75, 50, 75 µg/ml). MBC of the NOP-51 was much closer to MBC of Gentamicin (31, 31, 31, 15 µg/ml), respectively, against these bacteria. It is clear from the inhibition zone formation, MICs and MBCs values of purified protein (NOP-51) has potent antibacterial activity

against both Gram positive and negative bacteria. IC_{50} of NOP-51 against *S. aureus*, *B. substilis*, *P. putida* and *E. coli,* were determined and shown in figure 10. The IC_{50} found to be 18.4, 19.3, 15.2 and 16.0 μg/ml for *S. aureus*, *B. substilis*, *P. putida* and *E. coli*, respectively.

4.3.2 Time killing curve study

The concentration dependent time killing of NOP-51 against *S. aureus*, *B. substilis*, *P. putida* and *E. coli* was studied. The result of time killing is represented in figure 11. As clear from the figure that at lower concentration (10 μg/ml) NOP-51 was able to cause only 2% decrease in CFU after 2 h in all tested bacteria. However, at higher concentration (50 μg/ml) the bactericidal effect was rapid and 90% decrease in CFU was observed in all four bacteria within 15 min. No viable count was detected after 2 h in each bacterium. These results are in agreement with MIC values results. The concentration dependent time killing curve study was helpful to choose the optimum concentration and time study to observe the morphological changes occurred in bacteria cells after protein treatment using SEM and TEM.

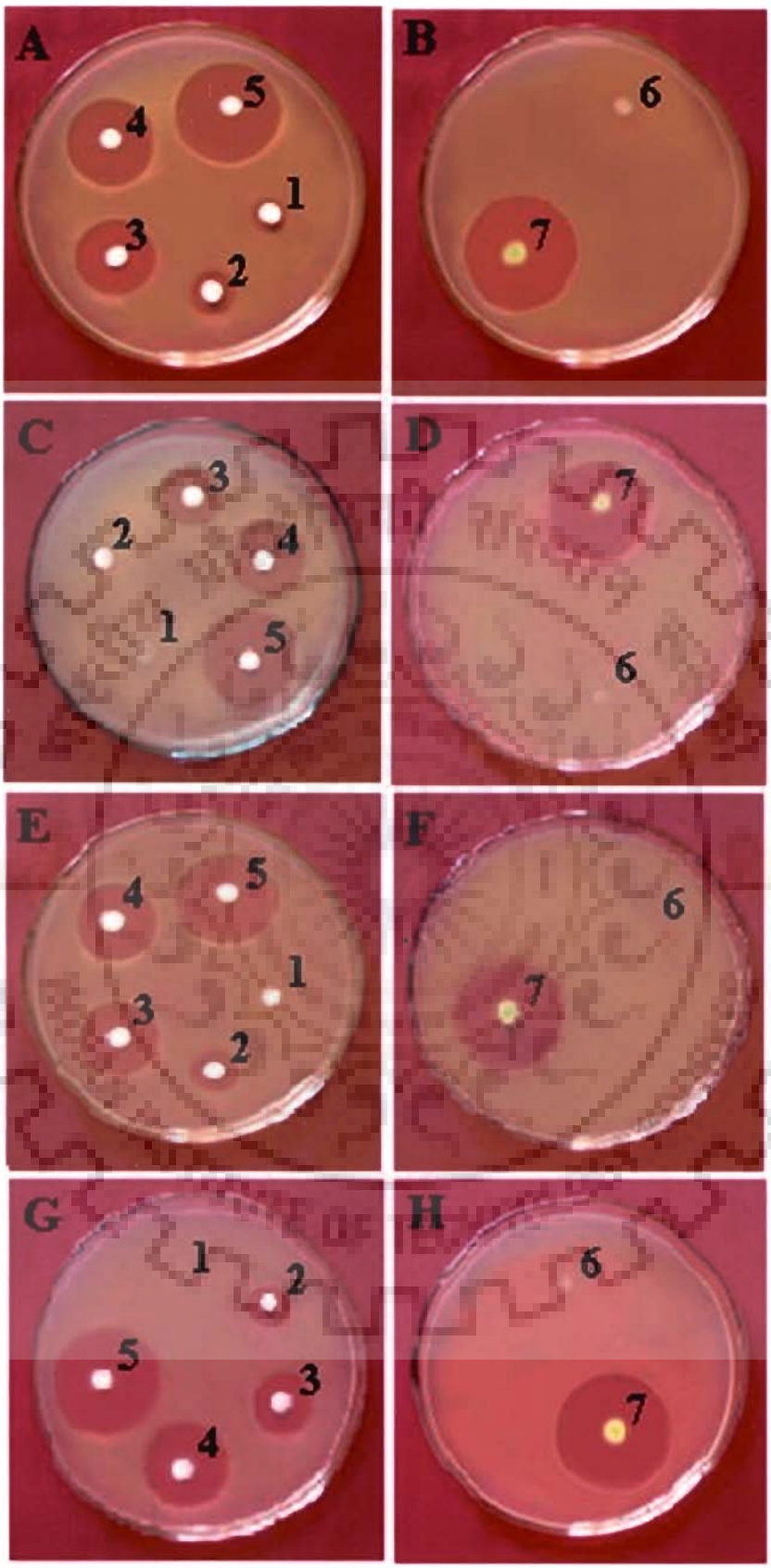

Figure 9. Antibacterial activity by disk diffusion assay. A&B) *S. aureus*; C&D) *B. substilis*; E&F) *P. putida*; G&H) *E. coli*. 1, 2, 3, 4 and 5 are 10, 15, 20, 25, and 30 µg/disk of purified protein (NOP-51); 6- 50 mM Tris-HCl pH 8.0; 7- 30 µg/disk Gentamicin, respectively.

Table 4. Antibacterial activity of crude protein extract by disk diffusion method.

Micro-organisms	Inhibition Zone (mm) [a]					
	Amount of crude protein extract (µg/disk)					Standard antibiotic
	10	20	30	40	50	Gentamicin (30µg/disc)
Staphylococcus aureus (MTCC 2940)	N.D.	6.1±0.12	7.8±0.02	8.7±0.56	12.3±0.43	18.1±0.21
Bacillus substilis (MTCC 2423)	N.D.	N.D.	6.2±0.31	7.9±0.43	14.2±0.32	18.9±0.52
Pseudomonas putida (MTCC 2453)	N.D.	N.D.	6.4±0.16	11.1±0.84	16.1±0.45	19.2±0.28
Escherichia. Coli (MTCC 739)	N.D.	6.5±0.21	7.2±0.76	8.6±0.76	15.5±0.72	18.8±0.92

[a]Values are zone of inhibition diameter and are mean of triplicate ± SE; N.D.= not detectable.

Table 5. Antibacterial activity of NOP-51 by disk diffusion method.

Micro-organisms	Inhibition Zone (mm) [a]					
	Amount of NOP- 51 (µg/disk)					Standard antibiotic
	10	15	20	25	30	Gentamicin (30µg/disc)
Staphylococcus aureus (MTCC 2940)	6.4±0.09	6.9±0.04	8.9±0.12	11.7±0.06	18.7±0.74	18.1±0.21
Bacillus substilis (MTCC 2423)	N.D.	6.7±0.73	7.2±0.12	11.9±0.07	19.2±0.64	18.9±0.52
Pseudomonas putida (MTCC 2453)	N.D.	6.1±0.07	7.4±0.10	10.1±0.04	18.1±0.65	19.2±0.28
Escherichia. Coli (MTCC 739)	N.D.	6.5±0.66	8.4±0.12	11.6±0.06	17.5±0.12	18.8±0.92

[a]Values are zone of inhibition diameter and are mean of triplicate ± SE; N.D. = not detectable.

Table 6. MICs and MBCs determination of crude protein extract and NOP-51 against various bacteria.

Micro-organisms	Minimum inhibitory concentration (MIC) (µg/ml)			Minimum fungicidal concentration (MBC) (µg/ml)		
	Crude protein extract	NOP-51	Gentamicin	Crude protein extract	NOP-51	Gentamicin
Staphylococcus aureus (MTCC 2940)	25	12	31	50	25	31
Bacillus substilis (MTCC 2423)	50	25	31	75	50	31
Pseudomonas putida (MTCC 2453)	50	25	15	50	50	31
Escherichia. Coli (MTCC 739)	25	12	15	75	50	15

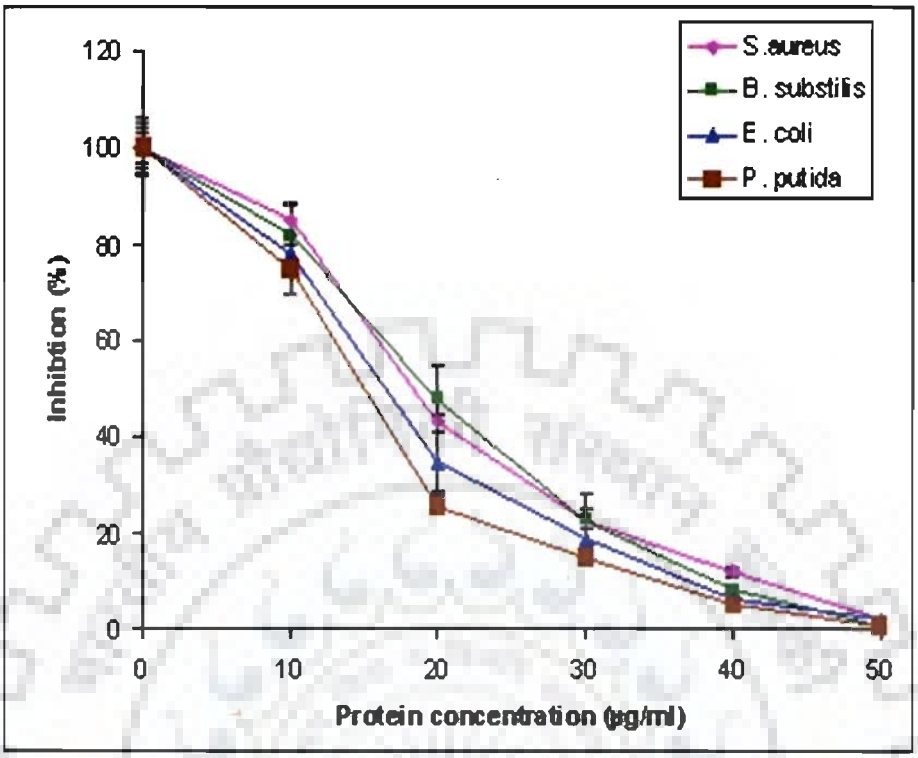

Figure 10. Determination of the IC_{50} value of NOP-51 against *S. aureus*, *B. substilis*. *P. putida*, and *E. coli*, The IC_{50} was calculated to be 18.4, 19.3, 15.2 and 16.0 µg/ml respectively.

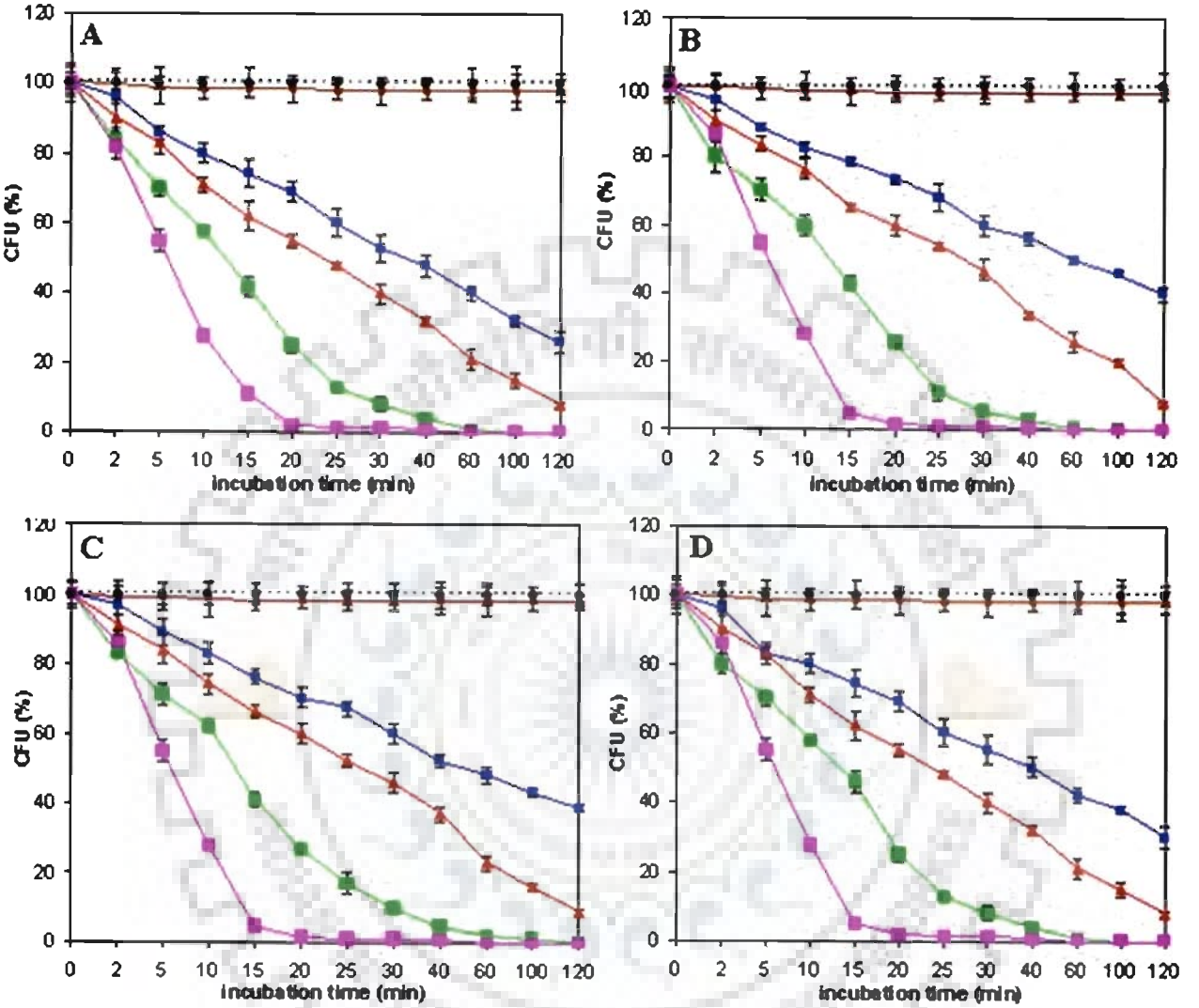

Figure 11. Time kill curve studies of bacteria, *S. aureus* (A), *B. substilis* (B), *P. putida* (C) and *E. coli* (D) cells (1×10^6) were incubated with 10 µg/ml (—), 20 µg/ml (—), 30 µg/ml (—) , 40 µg/ml (—), and 50 µg/ml (—) of 51 kDa protein at 30 °C. The number of surviving cells, at different incubation times, is expressed as the percentage of total cells. The control (·····) is bacteria without protein in the presence of 50 mM Tris HCl pH 8.0. The values are means for three independent measurements.

4.3.3. Effect of NOP-51 on bacterial cell morphology

One of the major modes of antimicrobial proteins and peptides killing of microbes is cell membrane damage which causes changes in the cell morphology and permeability and ultimately results in death. Scanning and transmission electron microscopic study have been the common method to study the cell damage and changes occurring on cell surfaces of bacteria by exposure to antimicrobial agents. Therefore, to see the effects of NOP-51 treatment on bacterial surface morphology SEM and TEM studies were performed.

4.3.3.1. Scanning electron microscopy

The bacterial cells were treated with sub-lethal (10 & 30 µg/ml) and lethal (50 µg/ml) concentration of NOP-51 for 30 min and changes in cell morphology were monitored using SEM. The scanning electron micrographs of *S. aureus*, *B. subtilis*, *P. putida* and *E. coli* are shown in figure 12 & 13, figure 14 &15, figure 16 &17 and figure 18 & 19, respectively. It is clear from the scanning electron micrograph pictures of all bacteria that treatment with NOP-51 found to have remarkable changes in the cell surface morphology and cell shapes of respective bacteria. The untreated cells found to have normal smooth surface and intact shapes of each bacterium (Fig. 12A & 13A, 14A & 15A, 17A & 17A and 18A & 19A). Even the treatment with sub-lethal concentration (10 µg/ml) of NOP-51 found to cause significant changes in cell morphology and shape (Fig. 12B & 13B, 14B &15B, 16B &17B and 18B & 19B). On the other hand 30 min treatment with higher concentration (30 µg/ml) and lethal concentration (50 µg/ml) found to cause more remarkable changes, such as surface roughing (Fig. 12C & 13C, 14C &15C, 16C &17C and 18C & 19C). Cell often showed holes and membrane damage and clumping of

damage cells could be easily seen in each bacterium (Fig 12D &13D, 14D & 15D, 16D & 17D and 18D & 19D). Changes in the bacterial cell surface morphology, cell shape deformity and cell damage was observed in SEM micrographs of all the four tested bacteria provide evidence for the potent antibacterial activity of NOP-51.

4.3.3.2 Transmission electron microscopy

The effect of purified protein (NOP-51) on bacteria was also studied by TEM. The bacterial cells were treated with 50 µg/ml of protein for 30 min. The transmission electron micrographs of control and protein treated cells of *S. aureus*, *B. subtilis*, *P. putida* and *E. coli* are shown in figure 20. The TEM picture of untreated cells of each bacterium showed clear intact cell wall and membrane (Fig 20. A, C, E and G) while the cell wall and membranes of NOP-51 treated bacterial cells were altered and clear deformity could be seen both in cell wall and membrane (Fig 20 B, D, F and H). The shrinkage and damage of cytoplasmic material and membrane could be clearly seen in protein treated cells compared to control. TEM micrographs indicated that the walls of treated bacterial cells were completely irregular in outline, without showing deposition of membranous material in the cell wall with almost complete solubilization. TEM study clearly indicates that the NOP-51 was able to cause damage of the cell wall and membrane in all bacteria which may be resulting in their death.

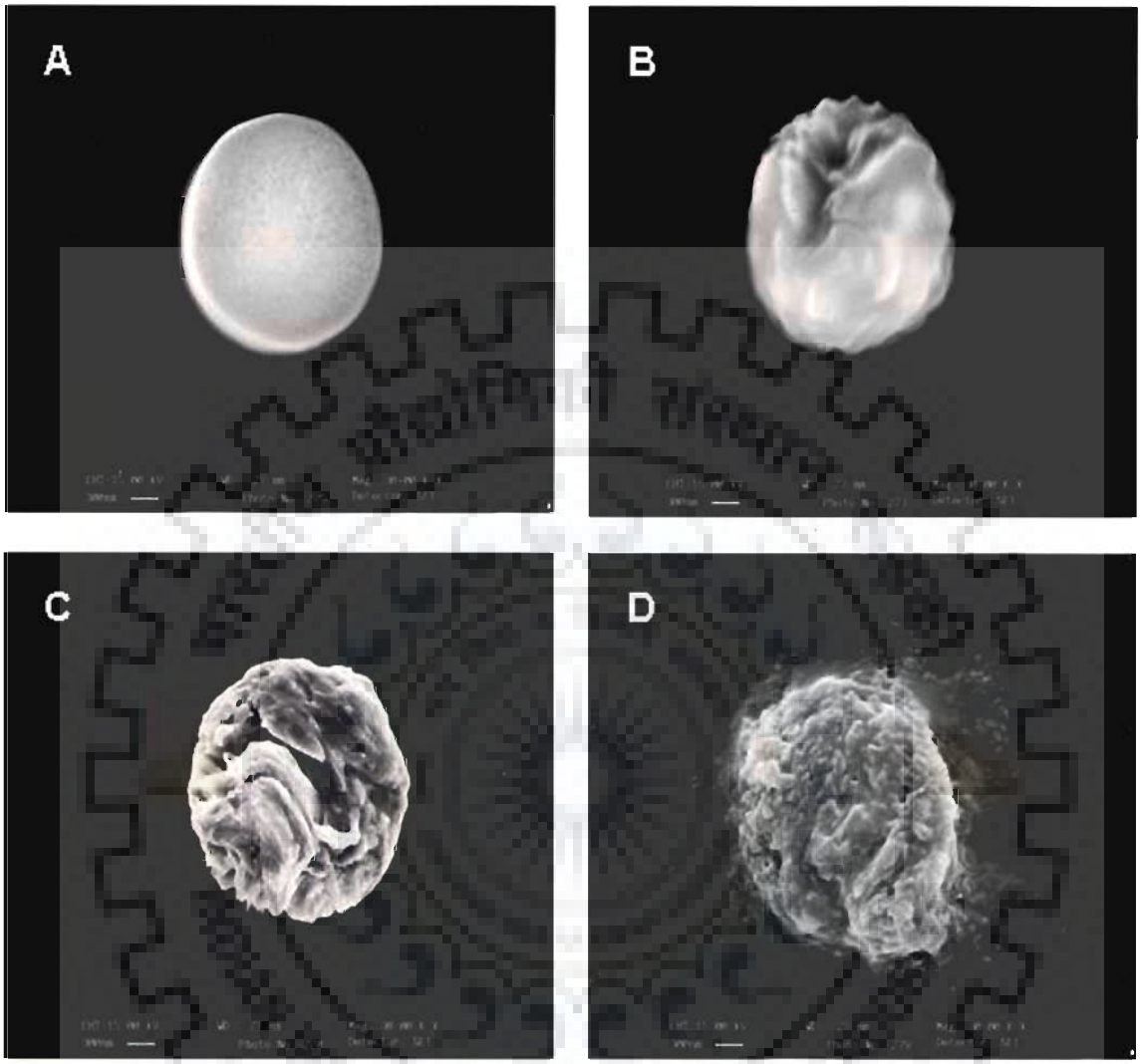

Figure 12. Scanning electron micrograph showing the effect of NOP-51 on *S. aureus* surface morphology after 30 min of incubation. A: Control (50 mM Tris-HCl pH 8.0); B, C & D are 10, 30, and 50 µg/ml concentration of NOP-51, respectively. Magnification: 30, 000 X, Bar: 300 nm.

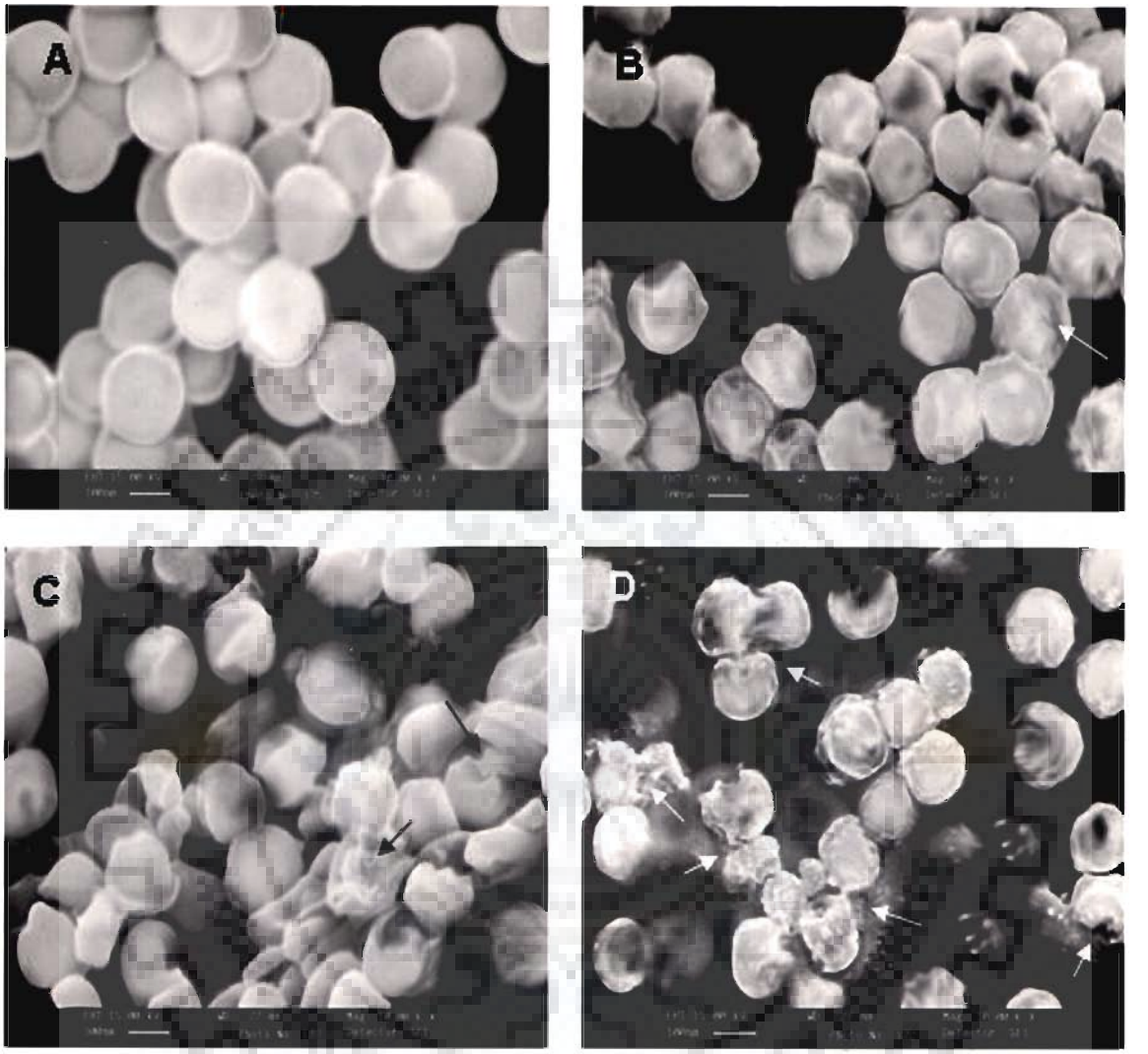

Figure 13. Scanning electron micrograph showing the effect of NOP-51 on *S. aureus* surface morphology after 30 min of incubation. A: Control (50 mM Tris-HCl pH 8.0); B, C & D are 10, 30, and 50 µg/ml concentration of NOP-51, respectively. Magnification: 10, 000 X, Bar : 100 nm. Cells with rough surface and their lysis are indicated.

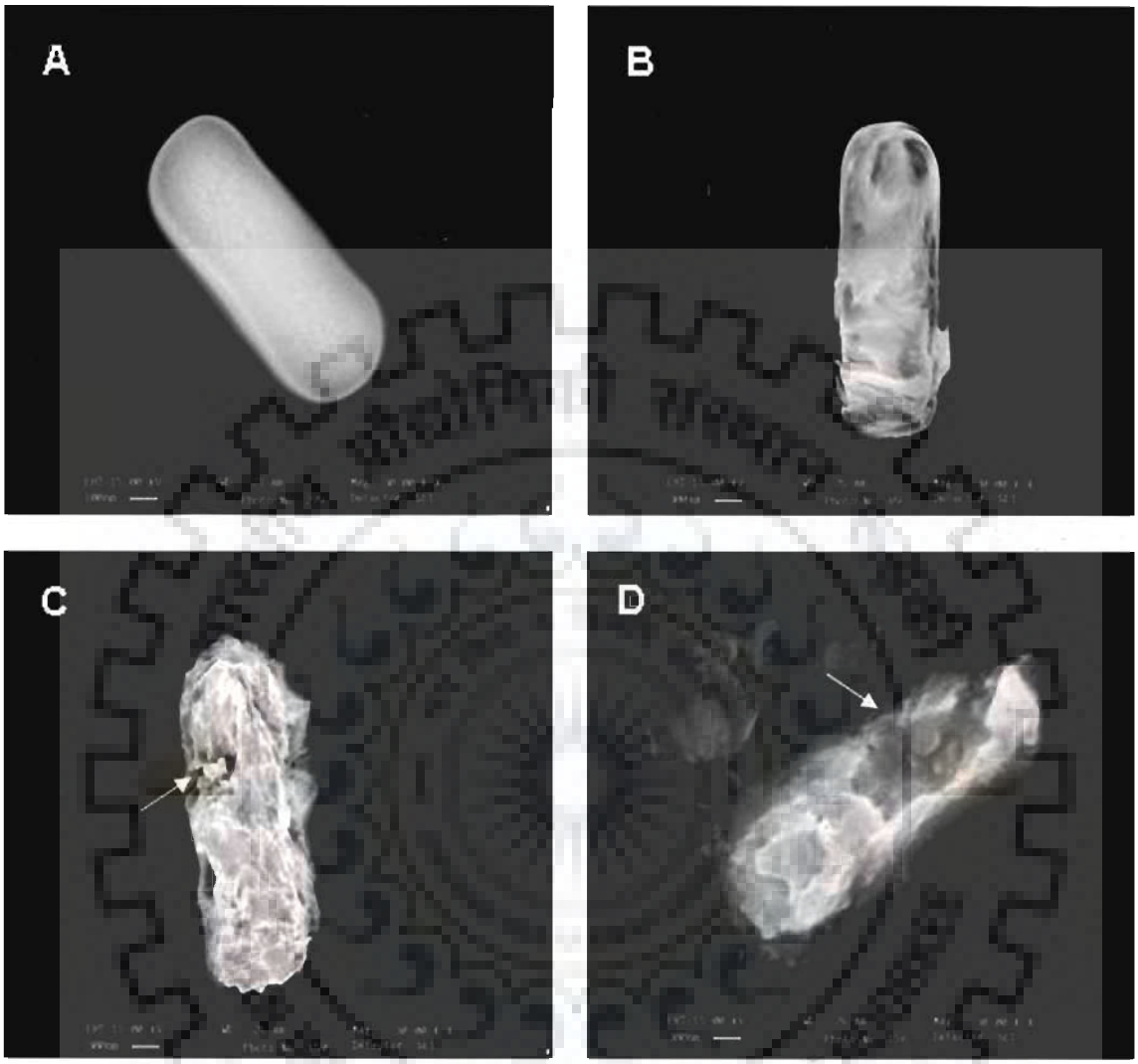

Figure 14. Scanning electron micrograph showing the effect of NOP-51 on *B. substilis* surface morphology after 30 min of incubation. A: Control (50 mM Tris-HCl pH 8.0); B, C & D are 10, 30, and 50 µg/ml concentration of NOP-51, respectively. Magnification: 30, 000 X, Bar: 300 nm. Cell with rough surface and its lysis are indicated.

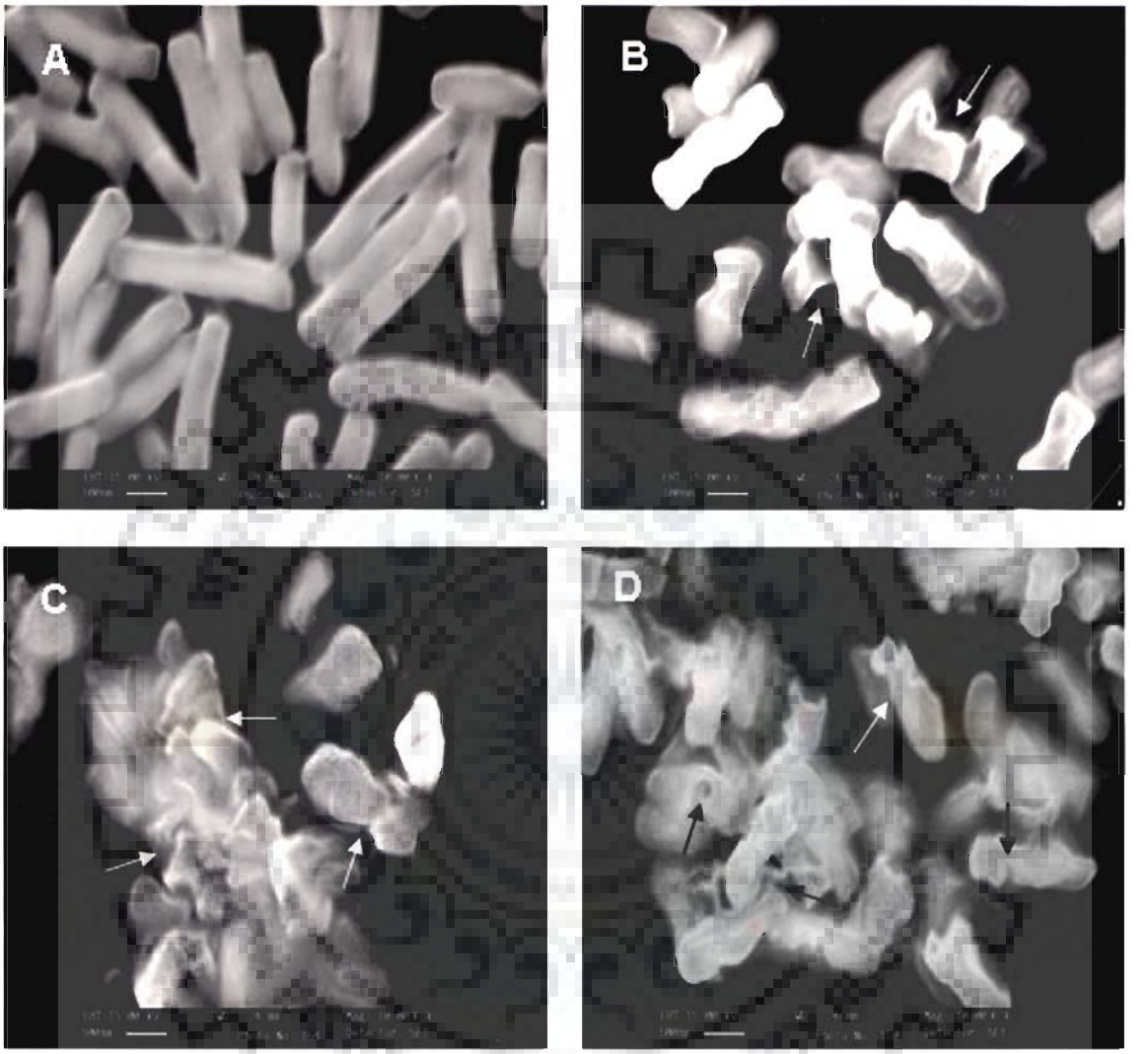

Figure 15. Scanning electron micrograph showing the effect of NOP-51 on *B. substilis* surface morphology after 30 min of incubation. A: Control (50 mM Tris-HCl pH 8.0); B, C & D are 10, 30, and 50 µg/ml concentration of NOP-51, respectively. Cells with rough surface and their clumping are indicated. Magnification: 10, 000 X, Bar: 100 nm.

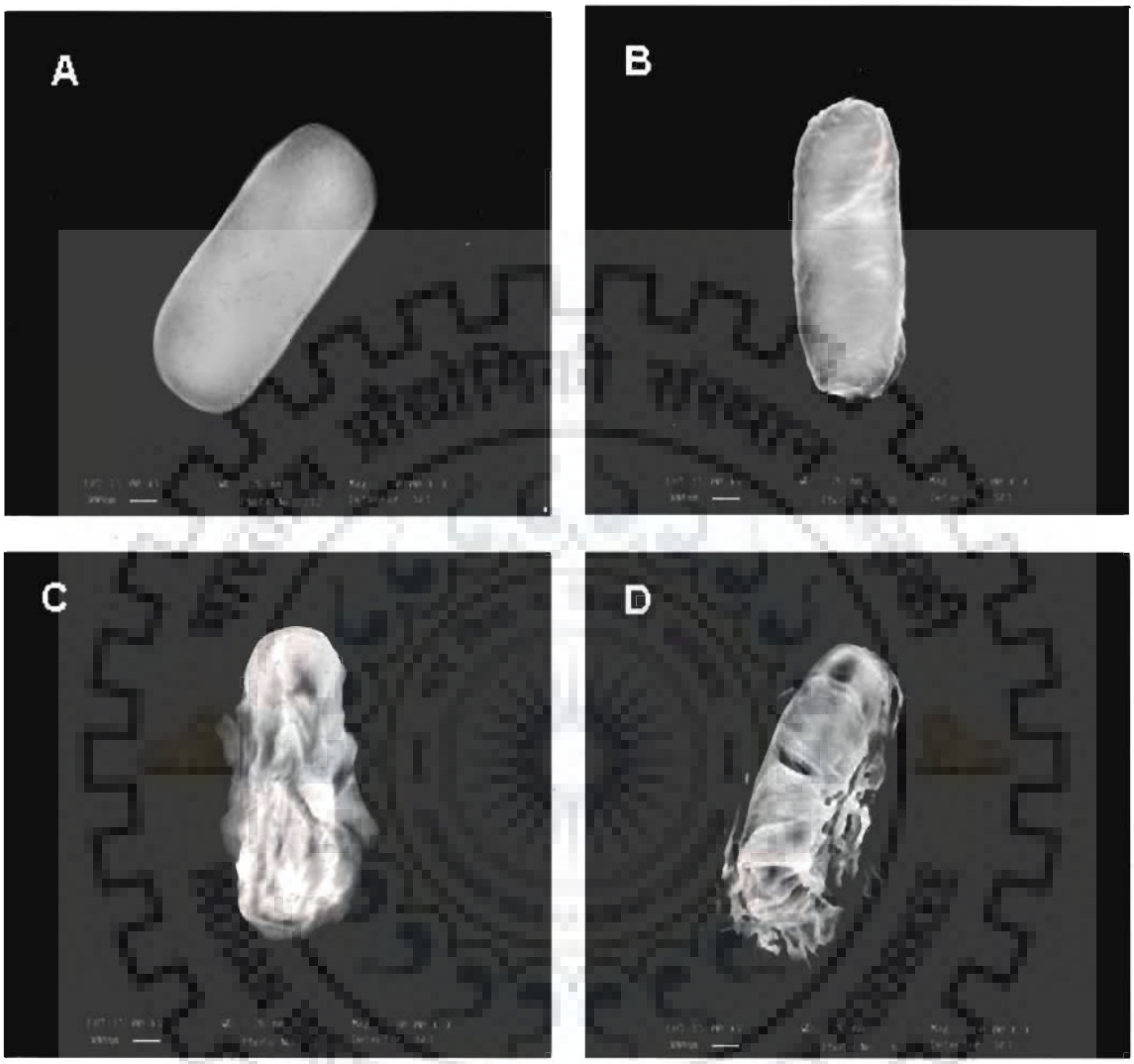

Figure 16. Scanning electron micrograph showing the effect of NOP-51 on *P. putida* surface morphology after 30 min of incubation. A: Control (50 mM Tris-HCl pH 8.0); B, C & D are 10, 30, and 50 µg/ml concentration of NOP-51, respectively. Magnification: 30, 000 X, Bar: 300 nm.

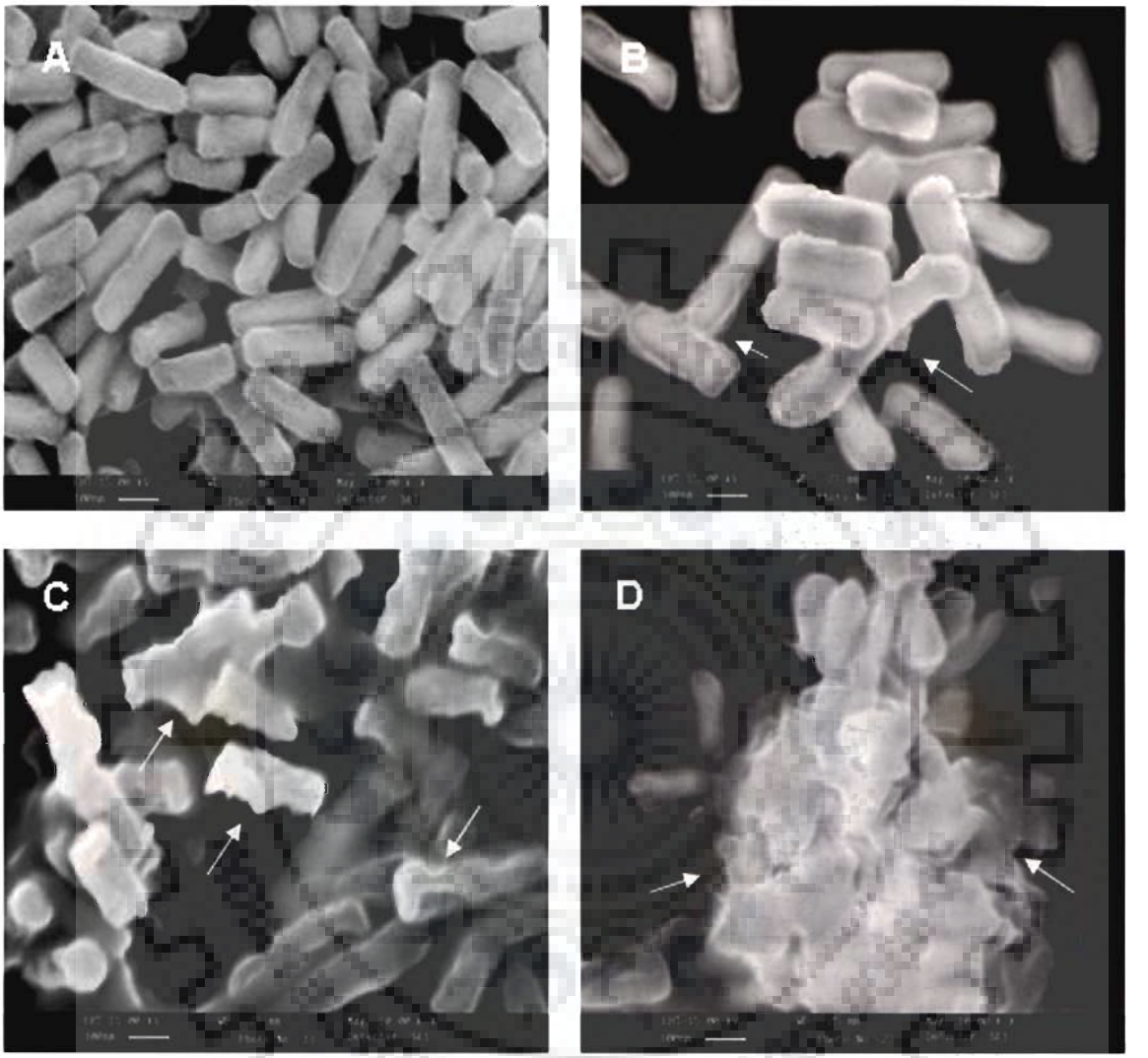

Figure 17. Scanning electron micrograph showing the effect of NOP-51 on *P. putida* surface morphology after 30 min of incubation. A: Control (50 mM Tris-HCl pH 8.0); B, C & D are 10, 30, and 50 µg/ml concentration of NOP-51, respectively. Cells with rough surface and clumping of lysed cells are indicated. Magnification: 10, 000 X, Bar: 100 nm

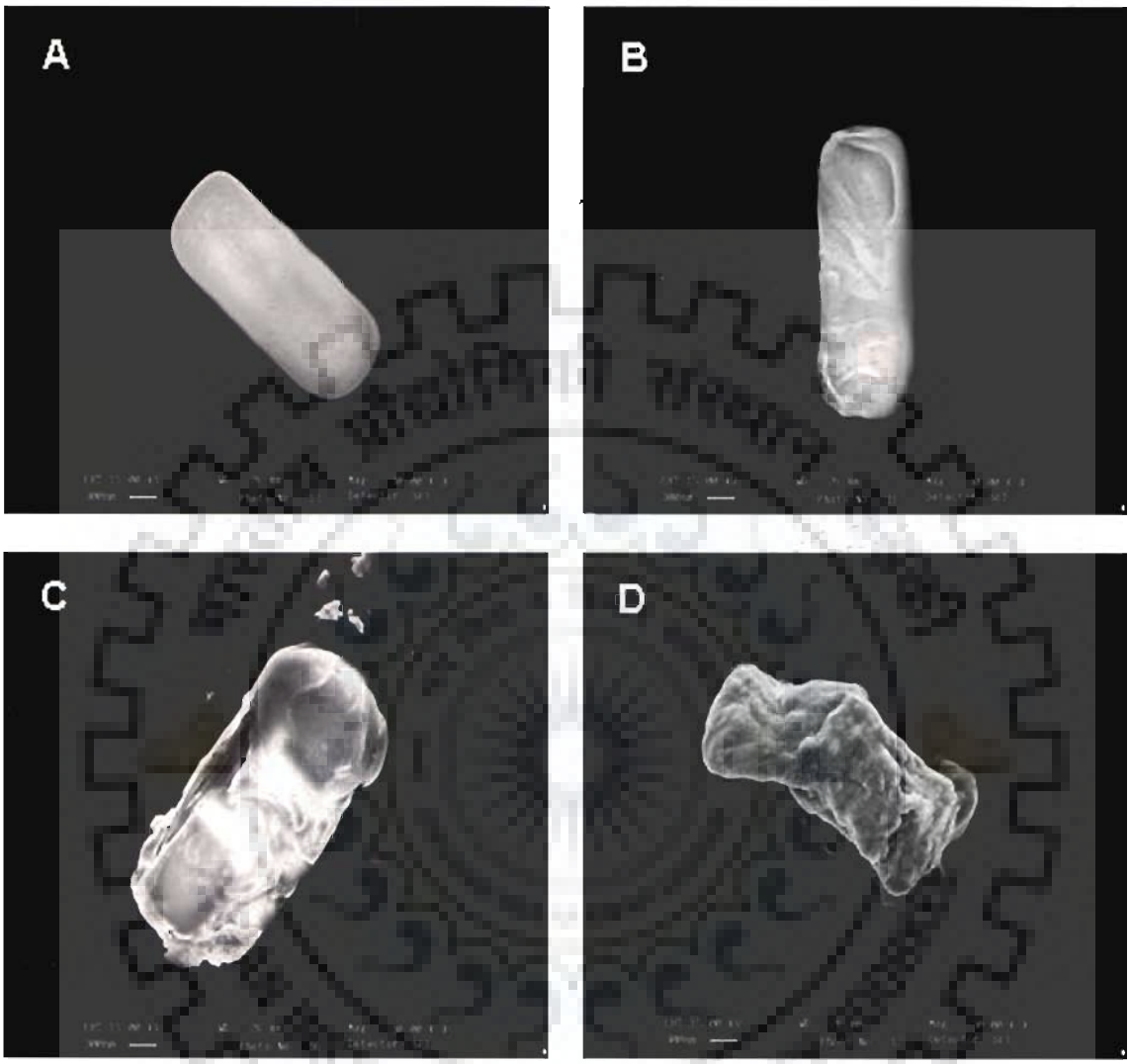

Figure 18. Scanning electron micrograph showing the effect of NOP-51 on *E. coli* surface morphology after 30 min of incubation. A: Control (50 mM Tris-HCl pH 8.0); B, C & D are 10, 30, and 50 µg/ml concentration of NOP-51, respectively. Magnification: 30, 000 X, Bar: 300 nm.

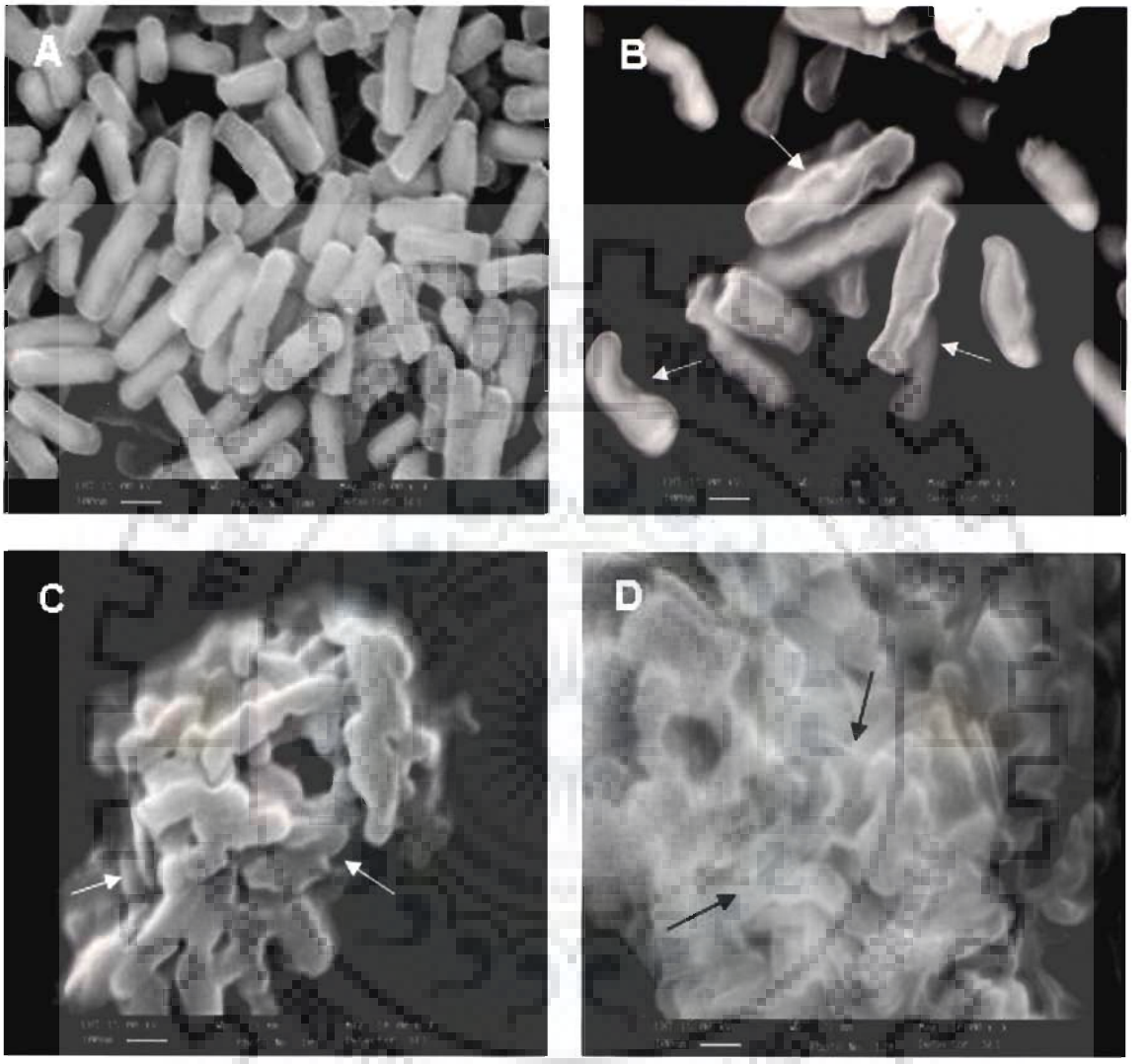

Figure 19. Scanning electron micrograph showing the effect of NOP-51 on *E. coli* surface morphology after 30 min of incubation. A: Control (50 mM Tris-HCl pH 8.0); B, C & D are 10, 30, and 50 µg/ml concentration of NOP-51, respectively. Cells with rough surface and their clumping are indicated. Magnification: 10, 000 X, Bar: 100 nm.

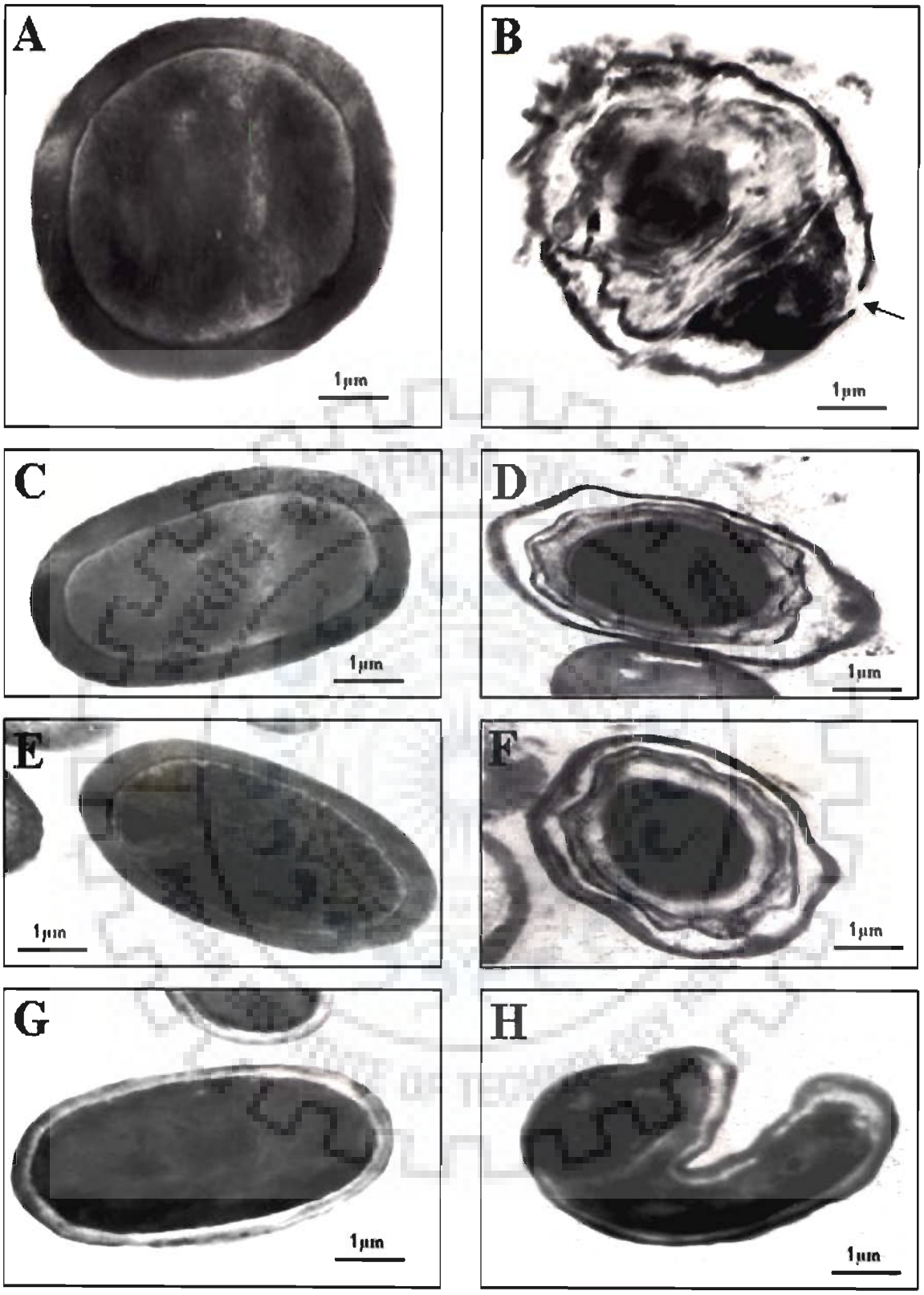

Figure 20: Transmission electron micrograph showing ultrastructural changes in *S. aureus* (A & B); *E. coli* (C & D); *P. putida* (E & F); and *B. substilis* (G & H) after NOP-51 treatment. Cells of *S. aureus* (B), *E. coli* (D), *P. putida* (F) and *B. substilis* (H) were treated with 50 µg/ml of NOP-51 for 30 min. Irregular cell wall in outline, retracting membrane could be seen (D, F & H). Bacterial cell with complete solubilization of cell wall and pore formation are indicated. Control cells of *S. aureus* (A), *E. coli* (C), *P. putida* (E) and *B. substilis* (F) are treated with 50 mM Tris-HCl pH 8.0 (A, C, E & G respectively). Bar: 1 µm.

4.4. Antifungal activity evaluation

4.4.1. Antifungal activity assay using disk diffusion and broth dilution methods

The *in vitro* antifungal activities of crude protein fraction and NOP-51 against several filamentous fungi (*A. fumigatus, A. flavus, A. niger* and *F. oxysporum*) and *C. albicans* were tested using disk diffusion method and the results are shown and summarized in figure 21 & 22 and Table 7 & 8. Similar to the antibacterial activity, crude protein and NOP-51 showed significant antifungal activity against all the fungi tested as could be seen from inhibition zone formation. The NOP-51 was more effective, as significant antifungal activity was observed even at lower concentrations compare to crude fraction. The 25 µg/disk of NOP-51 and 40 µg/disk of crude protein found to be most effective concentrations as significant inhibition zone was observed against all fungi while at lower concentrations inhibition zone could be seen against few fungi only. The zone of inhibition at 30 µg/disk of fluconazole and 30 µg/disk of NOP-51 against these fungi were quite comparable and in some case the NOP-51 found to be even better (Table 8).

MIC and MFC of crude protein and NOP-51 against these fungi were determined and summarized in Table 9. The MICs of NOP-51 against *A. fumigatus, A. flavus, A. niger, F. oxysporum* and *C. albicans*, were 25, 25, 25, 50 and 12 µg/ml, while that of crude protein were 50, 25, 50, 75 and 25 µg/ml, respectively. The MFCs of NOP-51 against most of the fungi found to be 50 µg/ml except for *C. albicans* which had 25 µg/ml. The MFCs of crude protein against these fungi were 50, 50, 75, 75 and 50 µg/ml, respectively. The MIC and MFC of NOP-51 against the above mentioned fungi were almost similar as that of fluconazole and in some cases it was found to be lower than

fluconazole. The IC_{50} of NOP-51 against *A. fumigatus, A. flavus, A. niger, F. oxysporum* and *C. albicans* were found to be 19.3, 15.1, 18.3, 20 and 18.3 µg/ml, respectively (Fig 23).

4.4.2. Time killing curve study

The concentration dependent time killing of NOP-51 against *A. fumigatus, A. flavus, A. niger, F. oxysporum* and *C. albicans* was studied. The time killing curves are shown in figure 24. At a concentration of 10 µg/ml, NOP-51 causes only 4% decrease in CFU within 2 h, however, at higher concentration the fungicidal effect was rapid and at 50 µg/ml almost 90% decrease in CFU was observed with in 15 min. No viable colonies were detected after 2 h.

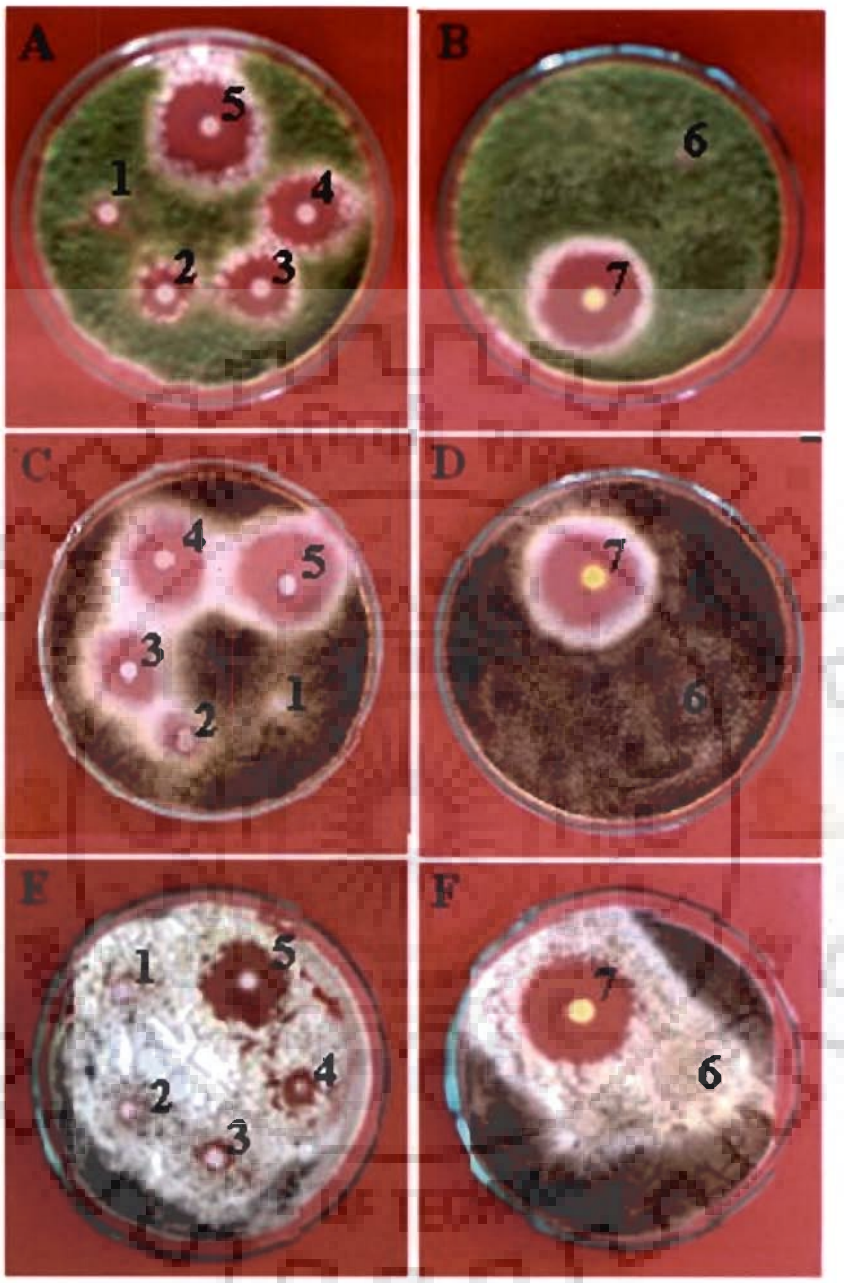

Figure 21: Antifungal activity by disk diffusion method. A & B) *A. flavus*; C & D) *A. fumigatus*; E & F) *A. niger*. 1, 2, 3, 4 and 5 are 10, 15, 20, 25, and 30 µg/disk of purified protein (NOP-51); 6- 50 mM Tris-HCl pH 8.0; 7- 30 µg/disk Gentamicin, respectively.

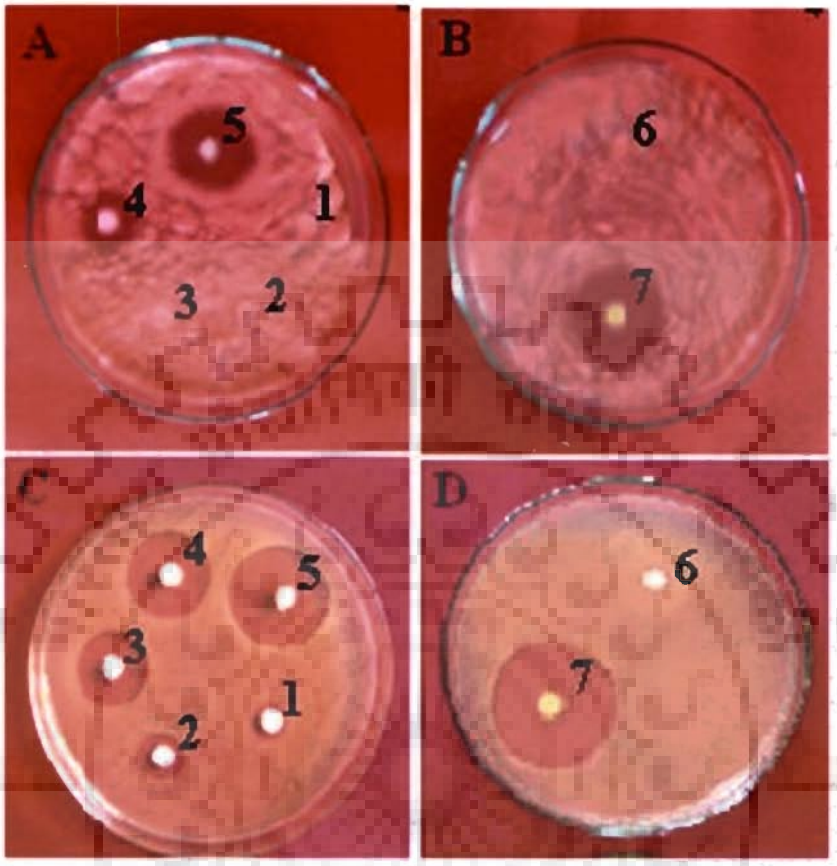

Figure 22: Antifungal activity by disk diffusion method. A & B) *F. oxysporum* C & D) *C. albicans*. 1, 2, 3, 4 and 5 are 10, 15, 20, 25, and 30 µg/disk of purified protein (NOP-51); 6- 50 mM Tris-HCl pH 8.0; 7- 30 µg/disk Gentamicin, respectively.

Table 7. Antifungal activity of crude protein extract by disk diffusion method.

Micro-organisms	**Inhibition Zone (mm)** [a]					
	Amount of crude protein extract (µg/disk)					**Standard antibiotic**
	10	**20**	**30**	**40**	**50**	**Flu (30µg/disc)**
Aspergillus fumigatus (ITCC 4880)	N.D.	N.D.	6.3±0.56	7.7±0.54	13.2±0.52	19.1±1.93
Aspergillus flavus (ITCC 5290)	N.D.	6.1±0.43	7.2±0.47	8.4±0.32	16.3±0.45	19.3±0.55
Aspergillus niger (ITCC 5454)	N.D.	N.D.	N.D.	6.1±0.36	11.2±0.77	20.3±0.32
Fusarium oxysporum (ITCC 4998)	N.D.	N.D.	N.D.	N.D.	6.3±0.41	21.5±0.98
Candida albicans (MTCC 227)	6.1±0.22	6.8±0.81	7.5±0.78	9.1±0.67	20.8±0.11	23.3±1.10

[a]Values are zone of inhibition diameter and are mean of triplicate ± SE; N.D. = not detectable. (Flu- Fluconazole).

Table 8. Antifungal activity of NOP-51 by disk diffusion method.

Micro-organisms	Inhibition Zone (mm) [a]					
	Amount of NOP- 51 (µg/disk)					Standard antibiotic
	10	15	20	25	30	Flu (30µg/disc)
Aspergillus fumigatus (ITCC 4880)	N.D.	6.1±0.12	7.8±0.04	8.7±0.20	21.2±0.02	19.1±1.93
Aspergillus flavus (ITCC 5290)	N.D.	6.2±0.05	7.5±0.47	8.7±0.02	22.5±0.02	19.3±0.55
Aspergillus niger (ITCC 5454)	N.D.	N.D.	6.1±0.21	8.1±0.73	24.2±0.34	20.3±0.32
Fusarium oxysporum (ITCC 4998)	N.D.	N.D.	N.D.	6.4±0.24	15.2±0.41	21.5±0.98
Candida albicans (MTCC 227)	6.3±0.51	6.9±0.83	7.9±0.01	10.1±0.08	20.8±0.01	23.3±1.10

[a]Values are zone of inhibition diameter and are mean of triplicate ± SE; N.D.= not detectable. (Flu- Fluconazole).

Table 9. MICs and MFCs determination of crude protein extract and NOP-51 against various fungi.

Micro-organisms	Minimum inhibitory concentration (MIC) (µg/ml)			Minimum fungicidal concentration (MFC) (µg/ml)		
	Crude protein extract	NOP- 51	Flu	Crude protein extract	NOP- 51	Flu
Aspergillus fumigatus (ITCC 4880)	50	25	31	50	50	62
Aspergillus flavus (ITCC 5290)	25	25	31	50	50	31
Aspergillus niger (ITCC 5454)	50	25	31	75	50	31
Fusarium oxysporum (ITCC 4998)	75	50	15	75	50	15
Candida albicans (MTCC 227)	25	12	7	50	25	7

(Flu- Fluconazole).

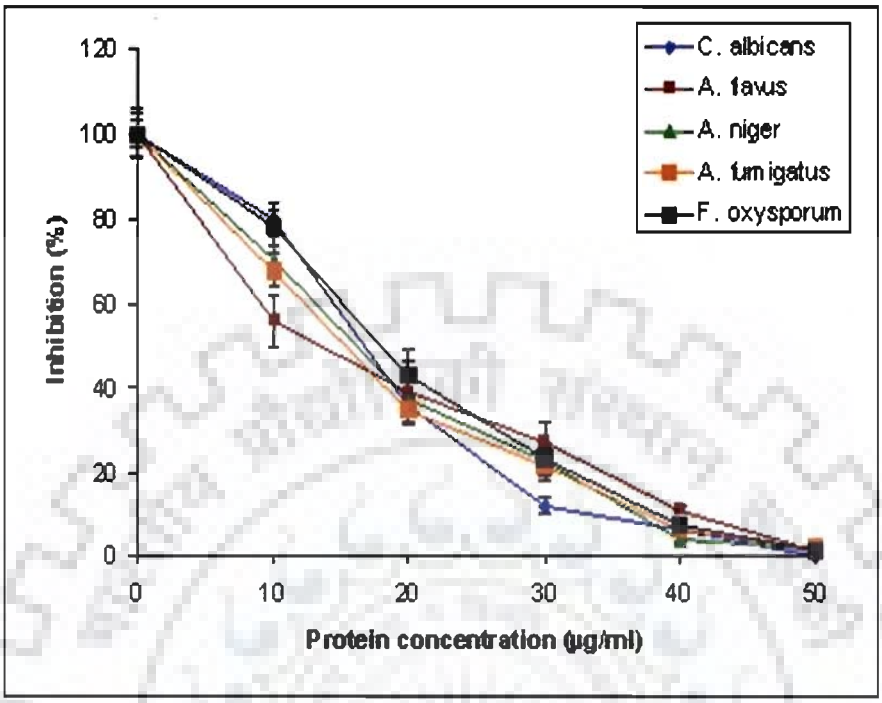

Figure 23. Determination of the IC_{50} value of the antifungal activity of 51 kDa protein from *N. odorum* leaves towards *A. niger, A. flavus, A. fumigatus, F. oxysporum* and *C. albicans*. The IC_{50} was calculated to be 18.3, 15.1, 19.3, 20.0 and 18.3 µg/ml respectively.

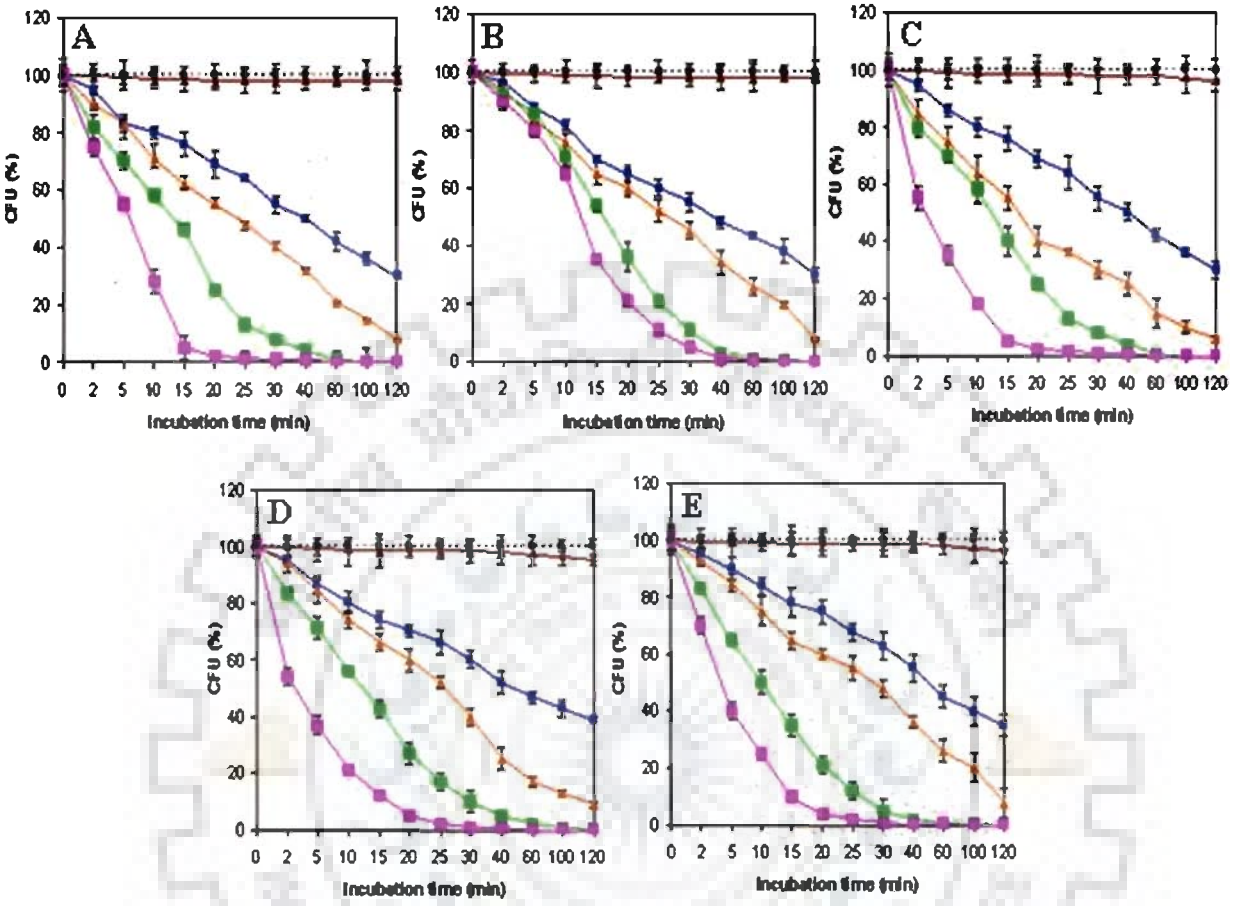

Figure 24. Time kill curve studies of Fungi. *C. albicans* (1×10^6 cells) (A), *A. niger* (B), *A. flavus* (C), *A. fumigatus,* (D) and *F. oxysporum* (E) (1×10^5 spores) were incubated with 10 µg/ml (—), 20 µg/ml (—), 30 µg/ml (—) , 40 µg/ml (—), and 50 µg/ml (—) of 51 kDa protein at 30 °C. The number of surviving cells, at different incubation times, is expressed as the percentage of total cells. The control (·····) is fungi without protein in the presence of 50mM Tris-HCl pH 8.0. The values are means for three independent measurements.

4.4.3. Effect of NOP-51 on fungal morphology

4.4.3.1. Scanning electron microscopy

Since 30 min was found to be the effective time for onset of cell damage. To study the effect of NOP-51 on cell morphology of filamentous fungi (*A. fumigatus, A. flavus, A. niger* and *F. oxysporum*) and *C. albicans* were treated with different concentration of NOP-51 for 30 min and SEM were performed. The SEM micrographs of control and NOP-51 treated samples of *A. fumigatus, A. flavus, A. niger, F. oxysporum* and *C. albicans* are shown in figure 25 26, 27, 28, 29 and 30. In case of filamentous fungi, hyphae of untreated samples were smooth, normal in shape without any alteration in morphology (Fig. 25A, 26A, 27A, & 28A). Protein treated hyphae were altered in shape and abnormal distorted hyphae could be seen in all fungi. Even the lower concentration (10 µg/ml) treatment for 30 min cause significant changes in *A. fumigatus, A. flavus, A. niger, F. oxysporum* (Fig. 25B, 26B, 27B, & 28B). However, drastic changes could be seen at higher concentration (30 and 50 µg/ml), collapsed and damaged hyphae could be easily seen in all fungi (Fig. 25C,D , 26C,D, 27C,D, & 28C,D). Similar observation was also made in *C. albicans* where untreated cells were smooth and normal shape while the protein treated cells surface became rough and irregular shape. The cell with damaged cell wall forming aggregates could be seen at 50 µg/ml protein concentration (Fig. 29 & 30).

4.4.3.2. Transmission electron microscopy

A more detailed analysis of the effect of NOP-51 on fungal cells was performed by transmission electron microscopy. TEM micrographs of control and protein treated *C. albicans* cells indicated that the walls of untreated cells were intact and regular in shape

while those of treated cells were irregular in outline, without showing deposition of membranous material in the wall as observed in the control cultures (Fig 31 A, B, C and D). The treated cell interior looked completely necrotic; internal subcellular organelles were barely identified. Large quantities of fat globules were present near the cell periphery (Fig 31C & D). Hyphae of *A. flavus* and *F. oxysporum* in the absence of purified protein showed a dense cytoplasm adhering to the plasma membrane and cell wall (Fig. 32 and 33). Treatment with 50 µg/ml of NOP-51, caused significant ultrastructural changes in morphology and hyphae with abnormal shape were frequently observed. Fungal cells showed various degrees of cell alteration, including retraction or distortion of the plasma membranes. A pronounced disorganization of the cytoplasm, involution of the vacuole and invaginations of the plasma membranes was detected (Fig. 32 E, F and Fig 33 B). A close examination of the purified protein treated *A. flavus* cells showed that the integrity of plasma membrane of protein treated cells was disrupted. Thus, specific sites of membrane damage and pore formation were observed in *A. flavus* cells treated with protein ((Fig 32 D, G, and H).

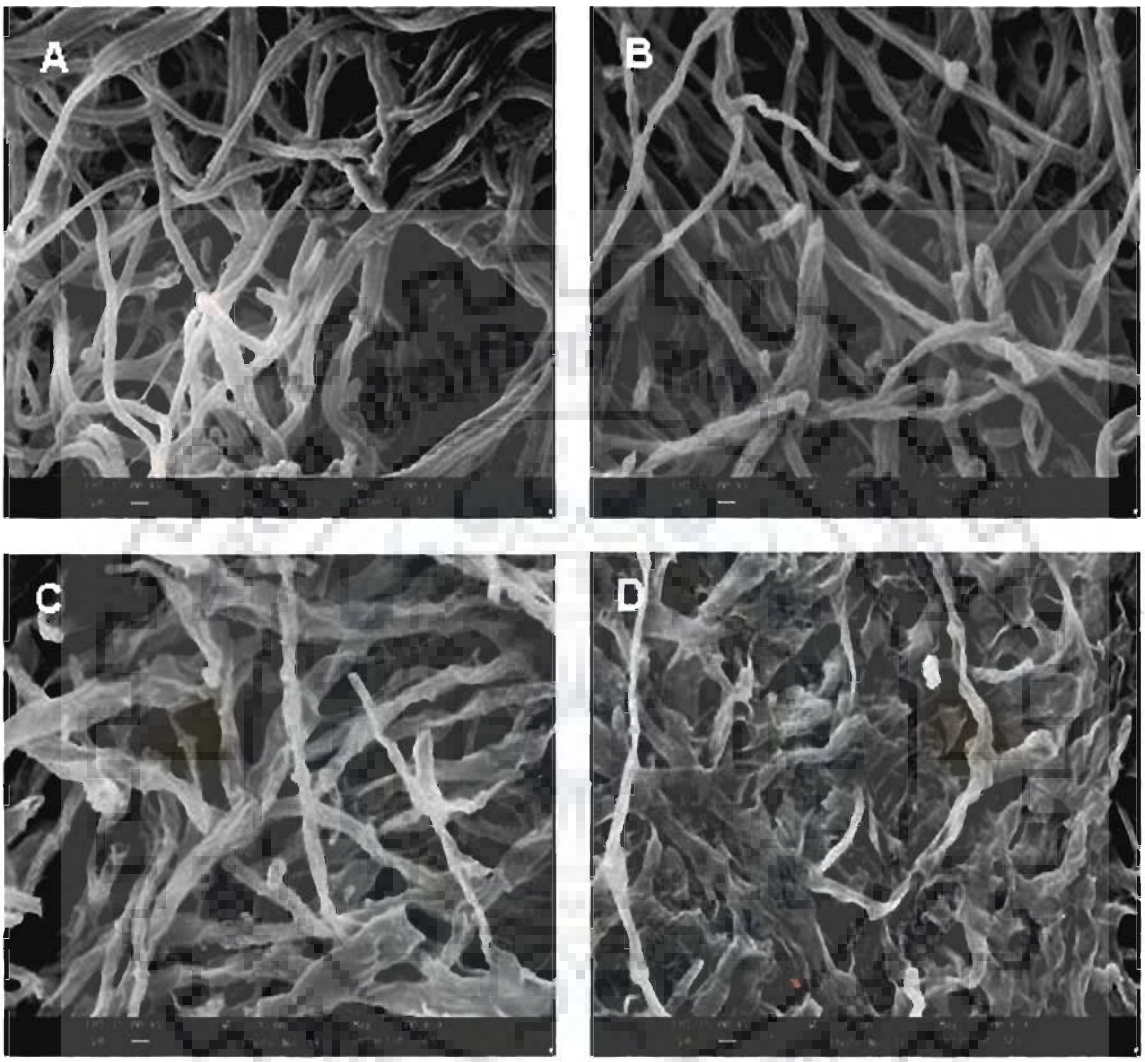

Figure 25. Scanning electron micrograph showing the effect of NOP-51 on *F. oxysporum* surface morphology after 30 min of incubation. A: Control (50 mM Tris-HCl pH 8.0); B, C & D are 10, 30, and 50 µg/ml concentration of NOP-51, respectively. Magnification: 2, 000 X, Bar: 2 µm.

Figure 26. Scanning electron micrograph showing the effect of NOP-51 on *A. niger* surface morphology after 30 min of incubation. A: Control (50 mM Tris-HCl pH 8.0); B, C & D are 10, 30, and 50 µg/ml concentration of NOP-51, respectively. Magnification: 2, 000 X, Bar: 2 µm

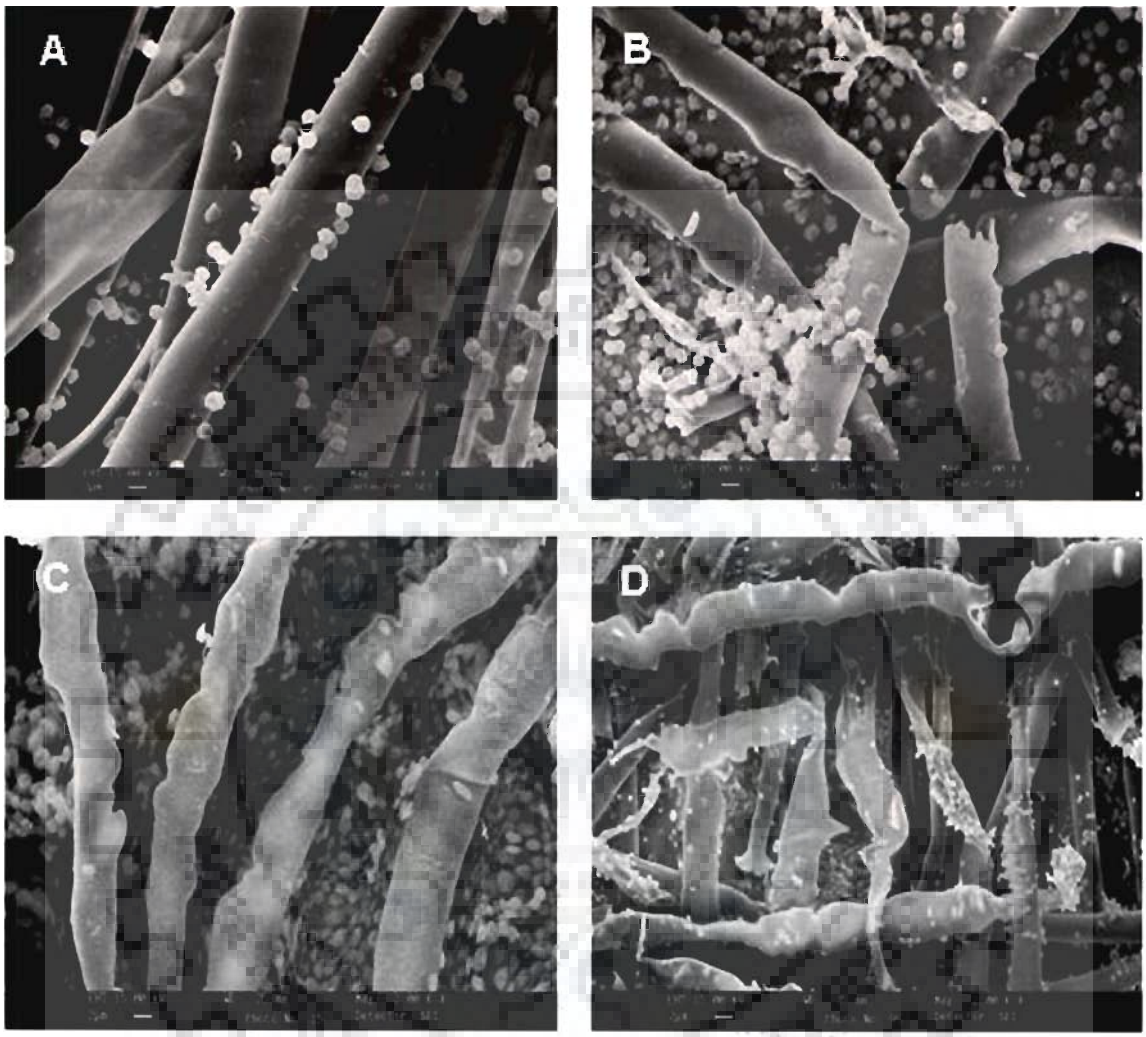

Figure 27. Scanning electron micrograph showing the effect of NOP-51 on *A. fumigatus* surface morphology after 30 min of incubation. A: Control (50 mM Tris-HCl pH 8.0); B, C & D are 10, 30, and 50 µg/ml concentration of NOP-51, respectively. Magnification: 2, 000 X, Bar: 2 µm

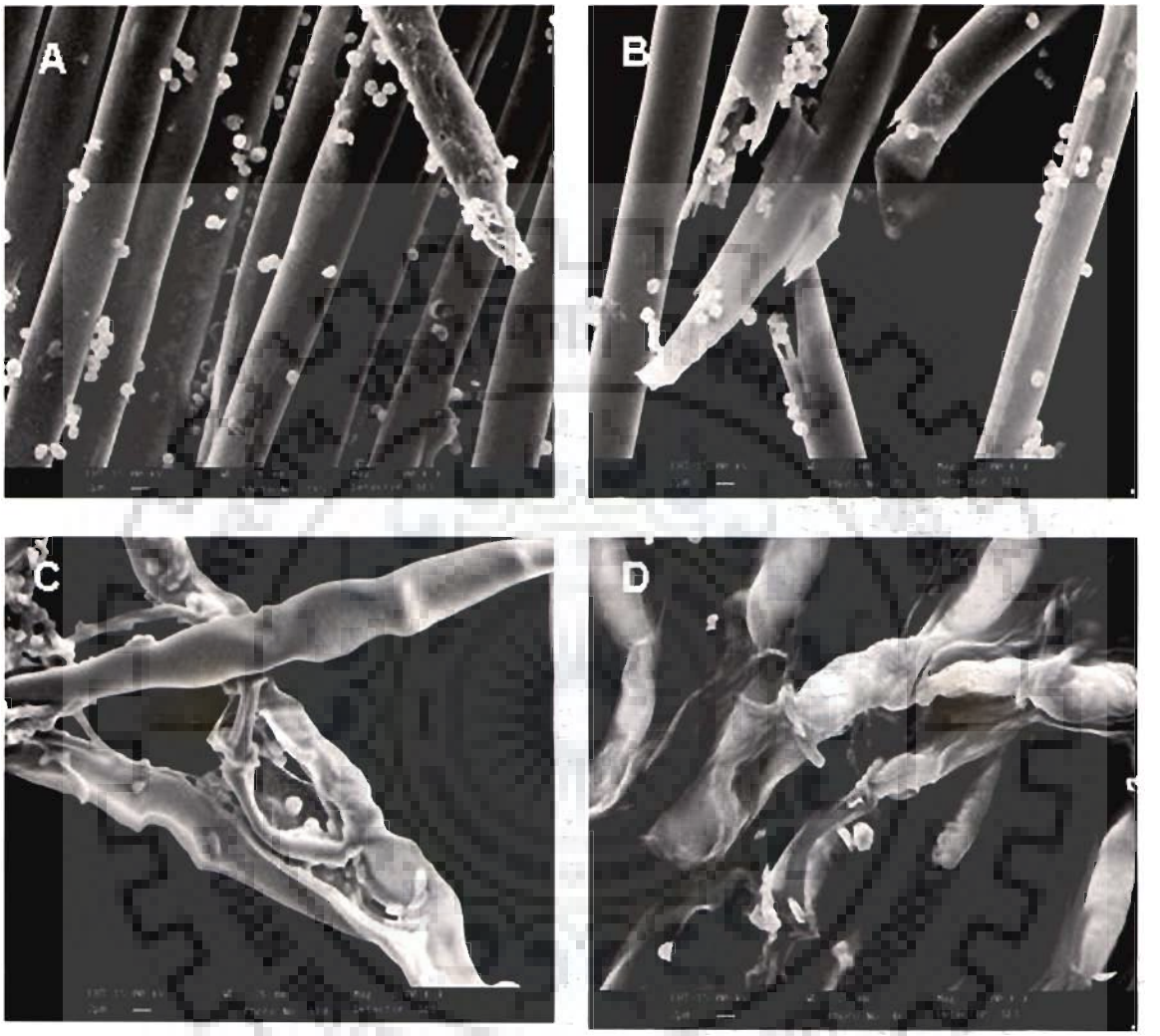

Figure 28. Scanning electron micrograph showing the effect of NOP-51 on *A. fumigatus* surface morphology after 30 min of incubation. A: Control (50 mM Tris-HCl pH 8.0); B, C & D are 10, 30, and 50 µg/ml concentration of NOP-51, respectively. Magnification: 2, 000 X, Bar: 2 µm.

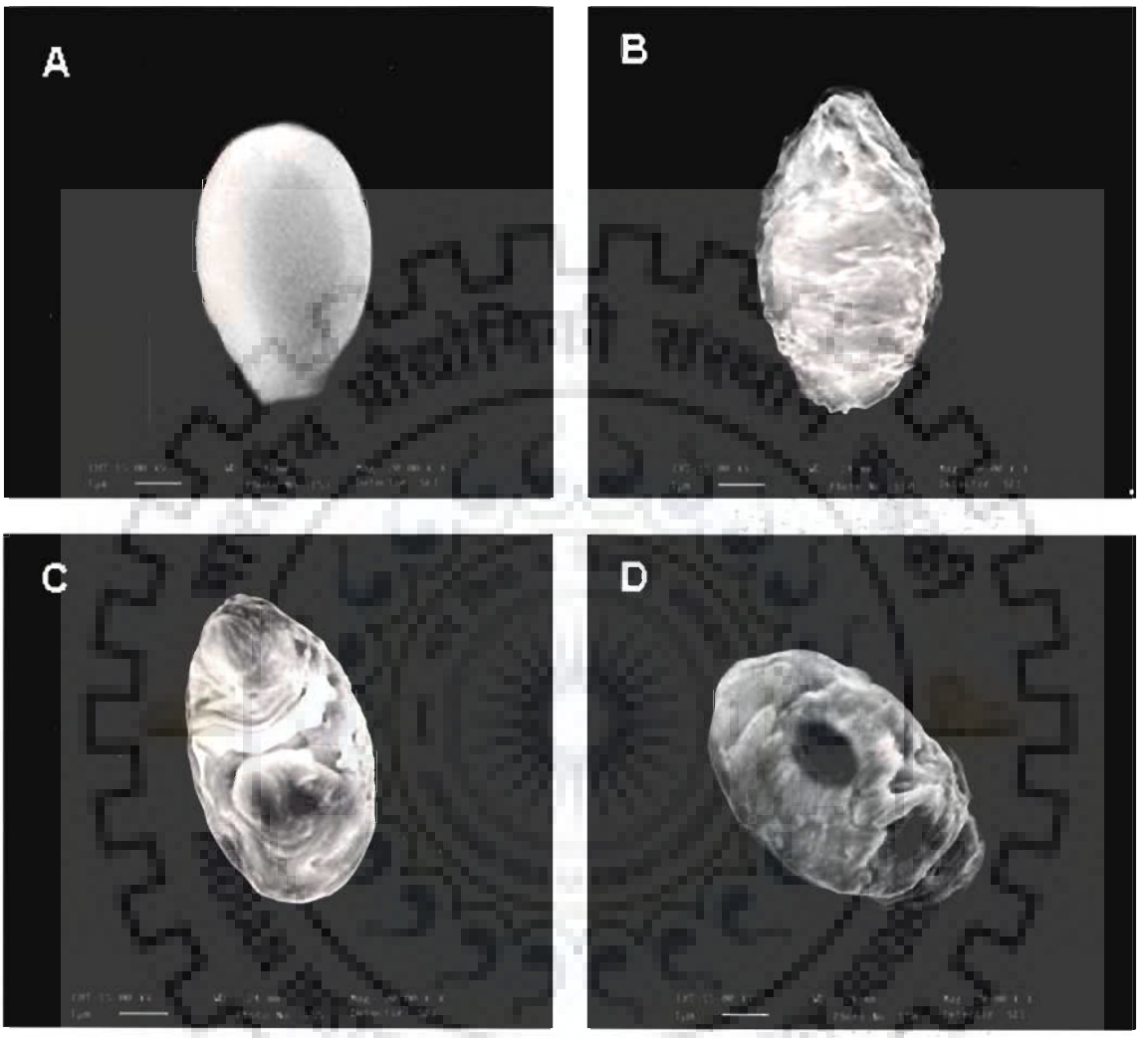

Figure 29. Scanning electron micrograph showing the effect of NOP-51 on *C. albicans* surface morphology after 30 min of incubation. A: Control (50 mM Tris-HCl pH 8.0); B, C & D are 10, 30, and 50 µg/ml concentration of NOP-51, respectively. Magnification: 20, 000 X, Bar: 1 µm.

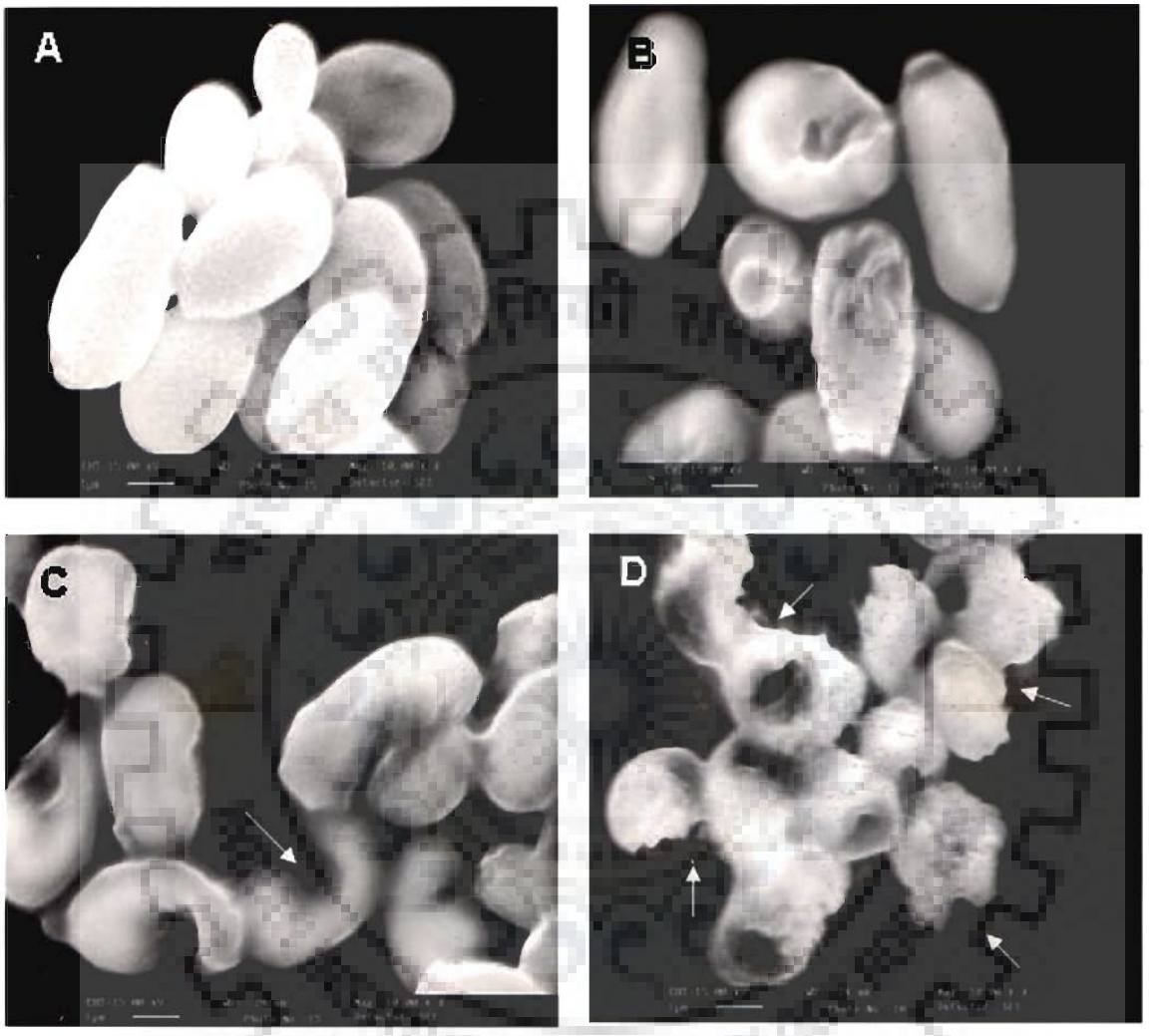

Figure 30. Scanning electron micrograph showing the effect of NOP-51 on *C. albicans* surface morphology after 30 min of incubation. A: Control (50 mM Tris-HCl pH 8.0); B, C & D are 10, 30, and 50 µg/ml concentration of NOP-51, respectively. Cells with rough surface and their clumping are indicated. Magnification: 10, 000 X, Bar: 1 µm.

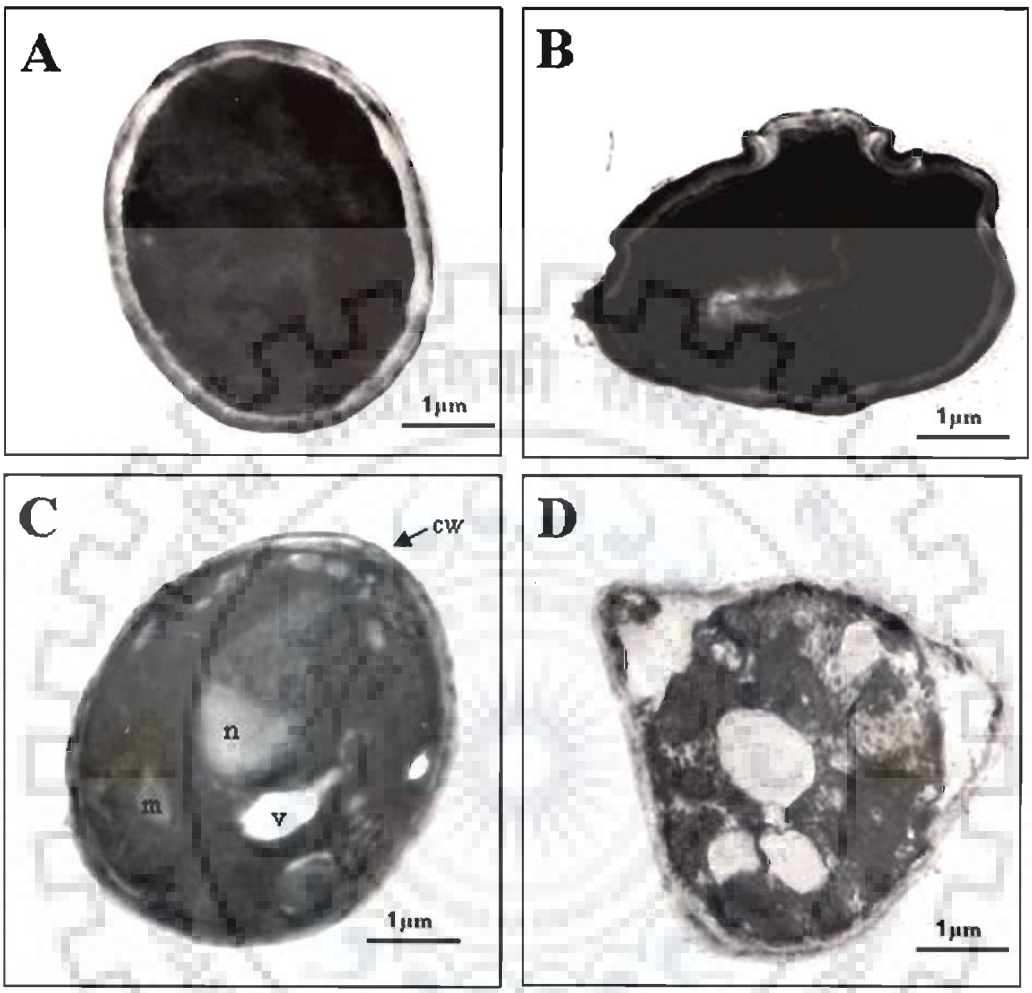

Figure 31: Transmission electron micrograph showing the effect NOP-51 in *C. albicans*. Cell treated with 50 µg/ml of NOP-51 (B & D) for 30 min. Cells showing completely irregular cell wall in outline with retracting membrane (C). Cell interior looked completely necrotic; internal subcellular organelles were barely identified. Large quantities of fat globules were present near the cell periphery (D). Control cells were treated with 50 mM Tris-HCl pH 8.0 (A & C). (m- mitochondria; n- nucleus; v- vacuole; cw- cell wall) (Bar: 1µm).

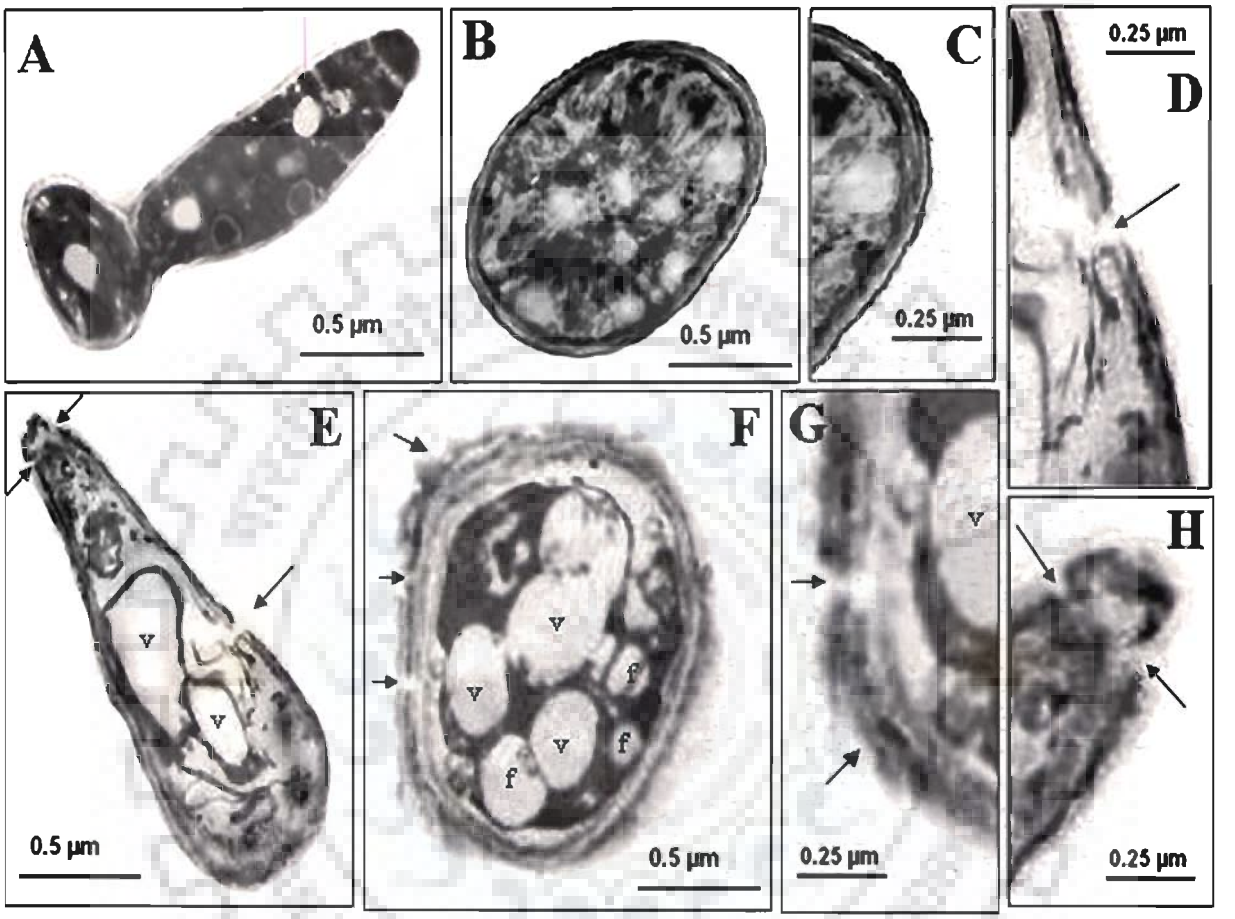

Figure 32: Transmission electron micrograph showing the effect of 50 µg/ml of NOP-51 in *A. flavus* (D, E, F, G & H) for 30 min. Fungal cells exhibited significant morphological and ultrastructural changes, such as increased vacuolation, fat globules and retraction of membranes. Membrane shedding and pore formation are indicated. Control cells were treated with 50 mM Tris-HCl pH 8.0 (A, B & C) Bar: 0.5 µm and 0. 25 µm). (v- vacuole; f- fat globule)

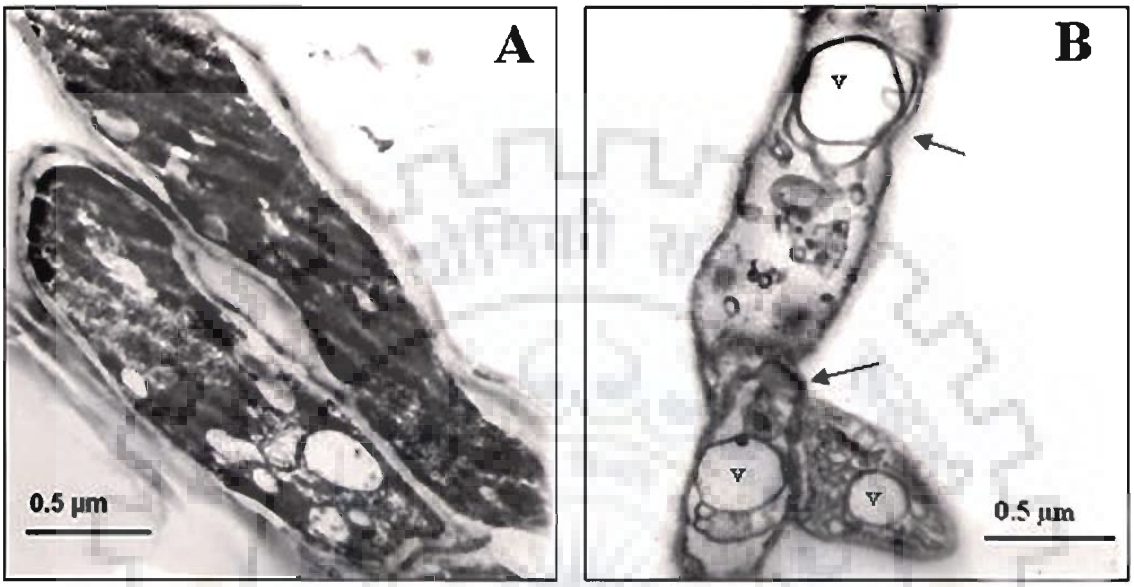

Figure 33: Transmission electron micrograph showing the effect of 50 µg/ml of NOP-51 in *F. oxysporum* for 30 min. Cells exhibited significant morphological and ultrastructural changes. Cells with increased vacuolation and membrane shedding are indicated. Control cells were treated with 50 mM Tris-HCl pH 8.0 (A) (Bar: 0.5 µm) (v- vacuole).

4.5. Effect of pH and temperature on NOP-51 antimicrobial activity

Antimicrobial activity of the NOP-51 was tested by treating protein at different temperature up to 100°C for 20 min. The antibacterial activity retained up to 75% and 50 % at 70°C and 80°C, respectively. However, it was drastically reduced at 100°C (Fig. 34). The antimicrobial activity was stable between pH 5 and 9, but remarkably reduced at pH 10 (Fig. 35). Antimicrobial activity of NOP-51 was observed for one month at room temperature and 100%, 50% and 5% activity was retained on 5^{th}, 20^{th} and 30^{th} day, respectively (Fig. 36).

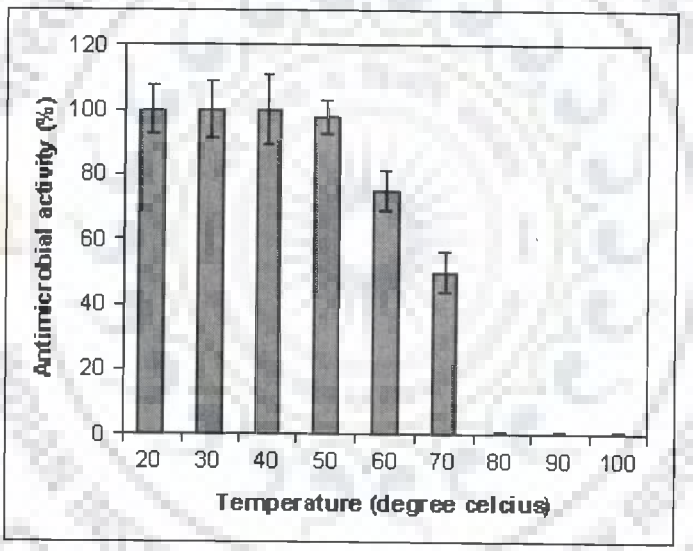

Figure 34. Effect of temperature on antimicrobial activity. Aliquots of NOP-51 were heated at different temperature for 20 min. Antimicrobial activity of NOP-51 was decreased to 75% and 50% of the initial activity by heating at 60°C and 70°C respectively and was completely lost by heating at 80°C.

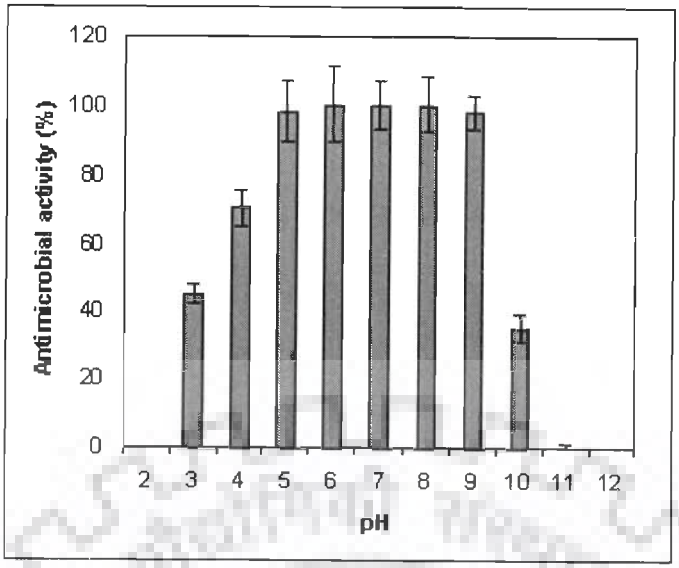

Figure 35. Effect of pH on antimicrobial activity. Aliquots of NOP-51 were stored in various buffers (pH 2.2 - 12) for 1 h. Antimicrobial activity of each sample was determined. The activity was stable in between pH 5 and 9.

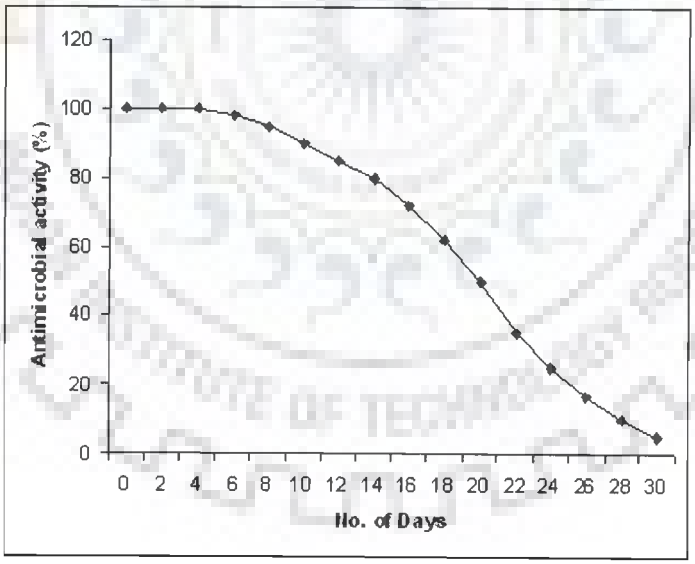

Figure 36. Effect of one month storage of NOP-51 at room temperature on antimicrobial activity. 100% activity was observed on 4th day, 50% and 5 % activity was retained on 20th and 30th day respectively.

(C) Immunomodulatory activity of purified protein (NOP-51):

N. odorum is well known for its medicinal properties from ancient time. It has been used in leprosy and other diseases of a scaly nature. A polyphenolic cardiac glycoside called Oleandrin derived from the leaves of *N. oleander* has been shown to possess anti-inflammatory and tumor cell growth-inhibitory effects. However, there is no report on the study of therapeutically active proteins from this plant so far. Therefore, an attempt was made to evaluate the immunomodulatory potential of the purified protein (NOP-51) in mouse peritoneal macrophages and splenocytes both in *in vitro* and *in vivo* conditions.

4.6. *In vitro* immunomodulatory activity of NOP-51

The levels and persistence of pro-inflammatory cytokines and NO play an important role in determining the extent of inflammation. The effect of NOP-51 on the release of inflammatory mediators like NO and TNF-α in elicited mouse peritoneal macrophages and splenocytes proliferation was investigated.

4.6.1. Effect on Nitric oxide production in peritoneal macrophages

The effect of the purified protein, NOP-51 on LPS-stimulated Nitric oxide release were studied using thioglycollate elicited peritoneal macrophages. The effect of NOP-51 on NO production in unstimulated and LPS-stimulated peritoneal macrophages is shown in figure 37. It is clear from the result that unstimulated macrophages produced a low level of nitrite (1.05 ± 0.8 μM). Treatment of macrophages with protein (25-150 μg/ml) alone did enhance nitrite production in unstimulated peritoneal macrophages to a low level but significantly at 150 μg/ml (6.2 ± 0.9 μM). A substantial increase in nitrite production was observed when cells were activated with LPS (5 μg/ml) for 24 h (60.6 ± 3.0 μM). However, the co-treatment of peritoneal macrophages with LPS (5 μg/ml) and

different concentrations of protein (25-150 μg/ml) evoked a significant inhibition of nitrite release in a concentration dependant manner.

4.6.2. Effect on TNF-α production in peritoneal macrophages

The effect of the purified protein NOP-51 on LPS-stimulated TNF-α release was also studied in thioglycollate elicited peritoneal macrophages. The effects of NOP-51 on TNF-α release in unstimulated and LPS-stimulated peritoneal macrophages are shown in shown in figure 38. The unstimulated macrophages produced a low level of TNF-α (25 ± 6 pg/ml). Similar to the treatment of macrophages with protein (25-150 μg/ml) alone did not have much change in the TNF-α production in unstimulated peritoneal macrophages except for a little enhancement at higher concentrations (40 ± 6.0 pg/ml) compared to control (31 ± 4.0 pg/ml). On the other hand stimulation of cells with LPS (5 μg/ml) induced elevated release of TNF-α (712 ± 17.0 pg/ml) but a concentration dependent inhibition of TNF-α production was observed when peritoneal macrophages were co-treated for 24 h with LPS (5 μg/ml) and different concentrations of protein (25-150 μg/ml). Nearly 50% inhibition was observed at protein concentration of 100-150 μg/ml. The cell viability was found to be greater than 95% at these protein concentrations as confirmed by trypan blue. Thus, the observed inhibition of TNF-α and NO production was not due to cell death.

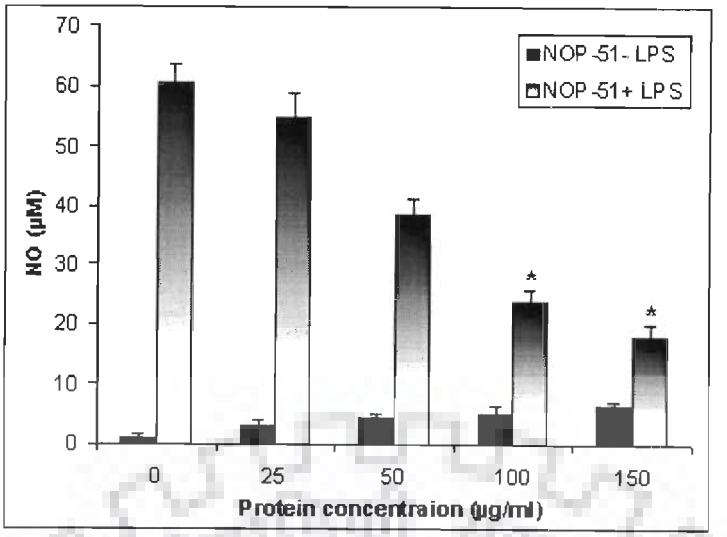

Figure 37. Effect of NOP-51 on the NO production in thioglycollate-elicited mouse peritoneal macrophages. The cells were treated with protein buffer or indicated concentrations of NOP-51 (25-150 µg/ml) and grown for 24 h in the presence or absence of LPS (5 µg/ml). The culture supernatants were used to measure the nitrite level using Griess reagent. Results are expressed as mean ± S.E. of six replicate comparing to the control; * $p < 0.05$.

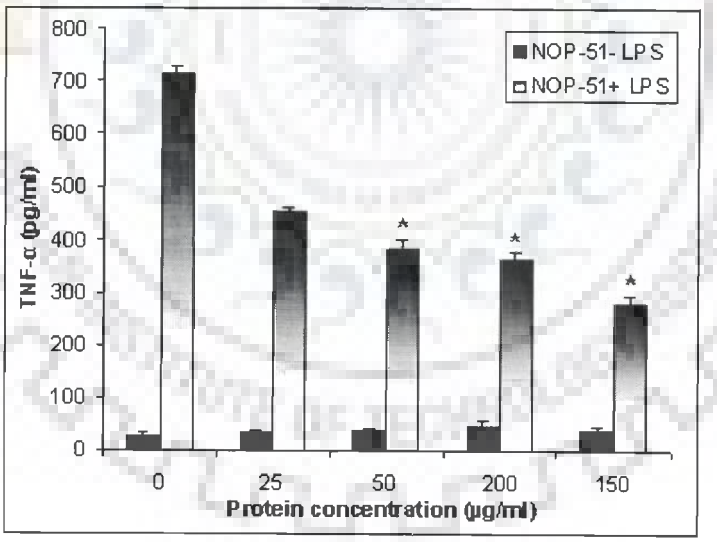

Figure 38. Effect of NOP-51 on the TNF-α production in thioglycollate-elicited mouse peritoneal macrophages. The cells were treated with protein buffer or indicated concentrations of NOP-51 (25-150 µg/ml) and grown for 24 h in the presence or absence of LPS (5 µg/ml). The culture supernatants were used to measure the TNF-α level using ELISA kit. Results are expressed as mean ± S.E. of six replicate comparing to the control; * $p < 0.05$.

4.6.3. Effect of *in vitro* treatment of NOP-51 on Splenocyte proliferation

The effect of purified protein (NOP-51) on splenocytes proliferation in presence or absence of mitogens Con-A and LPS was evaluated (Fig. 39). Protein treatment alone demonstrated a strong stimulation of splenocytes proliferation as compared to untreated cells and a dose response relationship was observed. Con-A and LPS treatment of untreated cells caused the stimulation of splenocytes proliferation by activating T and B lymphocytes, respectively. However, cells co-treated with Con A and NOP-51 or LPS and NOP-51, there were augmentation of Con-A and LPS stimulatory effects in a dose dependent manner and it was highest at 150 µg/ml concentration. Thus, NOP-51 has stimulatory effects on splenocytes proliferation alone and has synergistic effects on Con-A and LPS induced proliferations.

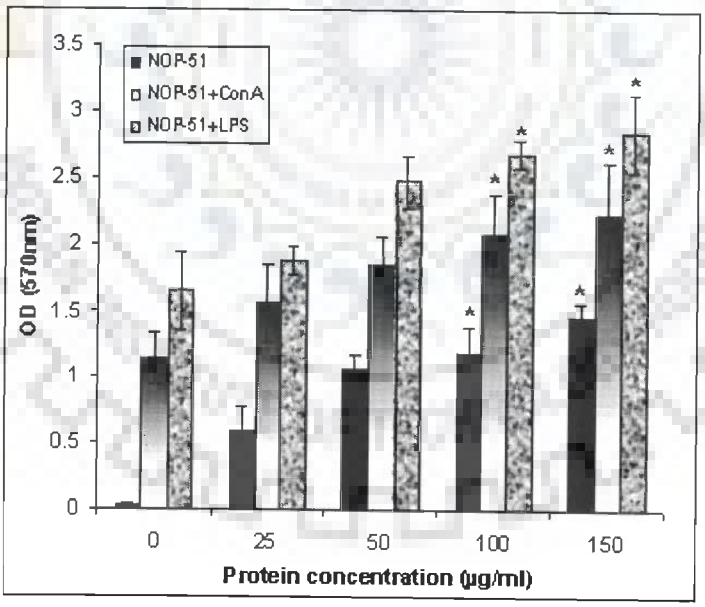

Figure 39. Effect of NOP-51 on the splenocytes proliferation. The cells untreated (control treated with protein buffer) or treated with indicated concentrations of NOP-51 (25-150 µg/ml) were grown for 24 h in the presence or absence of LPS (5 µg/ml) or Con-A, respectively and proliferation assayed using MTT. Results are expressed as mean ± S.E. of six replicate comparing to the control; * $p < 0.05$.

4.7.4. *In vitro* Cytotoxicity assay

During experimentation in the 24 h treatment of peritoneal macrophage and splenocytes cultures with different concentration of protein (25-150 µg/ml), no cytotoxicity was observed at the 150 µg/ml concentration in Trypan blue dye exclusion method. All protein concentration did not cause any cell death with the percentage viability of macrophages and splenocytes of more than 97% and 96%, respectively.

4.7. *In vivo* immunomodulatory activity of NOP-51

Animals treated either with buffer (Group-I) or different concentrations of NOP-51 (Group II: 25 µg/ml; Group III: 50 µg/ml; Group IV: 100 µg/ml; and Group V: 150 µg/ml) in a volume dose of 200 µl i.p. for 15 consecutive days, were sacrificed after 24 h of last dose and the immunomodulatory effects were studied.

4.7.1. Effect of NOP-51 on body weight, lymphoid organ weight

The effect of NOP-51 on mice body weight and various organs are summarized in Table-10. None of the protein doses showed any type of toxicity or mortality in the treated animals. Very little differences were observed in animal's body weight among protein treated groups and also compared control animals. No changes in the spleens weight were observed at any dose when compared with control (50 mM Tris-HCl pH 8.0 treated) except for dose 25 µg/ml, where a slight increase in relative organ weight of spleen was observed. As regard relative organ weight of thymus, liver and kidney no significant changes were observed.

Table 10. Effect of NOP-51 on the body and organ weight of mice.

Treatment group	Body weight	Spleen	Thymus	Liver	Kidney
(I) Control	31.2 ± 1.21	0.64 ± 0.03	0.25 ± 0.02	4.62 ± 0.12	1.54 ± 0.04
(II) 25 µg/ml	34.5 ± 0.91	0.69 ± 0.12	0.25 ± 0.01	4.63 ± 0.15	1.55 ± 0.03
(III) 50 µg/ml	33.0 ± 1.12	0.66 ± 0.04	0.26 ± 0.10	4.51 ± 0.03	1.58 ± 0.19
(IV) 100 µg/ml	33.4 ± 1.56	0.66 ± 0.06	0.27 ± 0.05	4.62 ± 0.21	1.67 ± 0.05
(V) 150 µg/ml	33.4 ± 0.78	0.65 ± 0.02	0.26 ± 0.02	4.60 ± 0.13	1.62 ± 0.13

Results are expressed as mean ± S.E. of six replicate.

4.7.2. Effect of NOP-51 on macrophage function

Peritoneal macrophages from each treatment groups of animals were removed and single cell suspension of peritoneal macrophages was prepared. Viability of the each treated groups was tested by Trypan blue dye exclusion assay and nearly 96% cell viability was found to be in each case. The cell suspension was used in macrophages function assay.

4.7.2.1. Effect on cellular lysosomal enzyme activity

The cellular lysosomal enzyme activity was used to determine the phagocytic activity of peritoneal macrophages. The NOP-51 treatment enhanced the cellular lysosomal acid phosphatase activity in dose dependent manner and almost 80% lysosomal enzyme activity was observed at higher concentrations, 100 and 150 µg/ml (Fig. 40). Thus, the i.p NOP-51 treatment of mice at a dose 25 -150 µg/ml for 15 days found to have stimulatory effect on the peritoneal macrophages phagocytic activity assessed after 24 h of the last dose.

4.7.2.2. Effect on macrophages candidacidal activity

Peritoneal macrophages were collected from mice administered with different NOP-51 concentrations i.p. showed marked increase in candidacidal activity in a dose

dependent manner as compared to control (Fig. 41). Therefore, enhanced candidacidal activity of protein treated peritoneal macrophages may be due to augmented functions of macrophages including its phagocytosis.

4.7.2.3. Effect on nitrite production in peritoneal macrophages

Peritoneal macrophages from the *in vivo* treated control animals (treated with protein buffer only, Group-I) and treated with different NOP-51 concentrations (Group II: 25 μg/ml; Group III: 50 μg/ml; Group IV: 100 μg/ml; and Group V: 150 μg/ml) were collected and checked for its viability. More than 97% cell viability was observed in each group and these cells were used for NO release assay. Cells of each group were grown in absence or presence of LPS (5μg/ml) for 24 h and the level of NO in supernatant were assayed. The effect of *in vivo* treatment of NOP-51 on NO production in unstimulated and LPS-stimulated peritoneal macrophages is shown in figure 42. It is clear from the result that unstimulated macrophages produced a low level of nitrite (1.05 ± 0.2 μM). Treatment of macrophages with protein (25-150 μg/ml) alone did enhance nitrite production in unstimulated peritoneal macrophages to a low level but significant at 150 μg/ml (4.2 ± 0.9 μM). A substantial increase was observed in NO production when cells were activated with LPS (5 μg/ml) for 24 h (67.6 ± 3.0 μM). However, the co-treatment of peritoneal macrophages with LPS (5 μg/ml) and different concentrations of protein (25-150 μg/ml) evoked a significant inhibition of nitrite release in a concentration dependant manner.

4.7.2.4. Effect on TNF-α production in peritoneal macrophages

Similarly, the effect of NOP-51 *in vivo* treatment was also studied. The cells of each treated group were grown in absence or presence of LPS (5μg/ml) for 24 h and the

level of TNF-α in supernatant was assayed. The effect of NOP-51 *in vivo* treatment on TNF-α release in unstimulated and LPS-stimulated peritoneal macrophages is shown in figure 43. The unstimulated macrophages produced a low level of TNF-α (30 ± 7.0 pg/ml). Similar to *in vitro* study, the *in vivo* treatment of macrophages with protein (25-150 μg/ml) alone did not have much change in the TNF-α production in unstimulated peritoneal macrophages except for a little enhancement at higher concentrations (42 ± 11 pg/ml) as compared to control (30 ± 7 pg/ml). On the other hand stimulation of cells with LPS (5 μg/ml) induced elevated release of TNF-α (763 ± 12 pg/ml) but a concentration dependent inhibition of TNF-α production was observed when peritoneal macrophages were co-treated for 24 h with LPS (5 μg/ml) and different concentrations of protein (25-150 μg/ml). Nearly 45% inhibition was observed at 50 μg/ml protein concentration. The cells viability was found to be greater than 95% at these protein concentrations as checked by trypan blue. Thus the observed inhibition of TNF-α and NO production was not due to cell death.

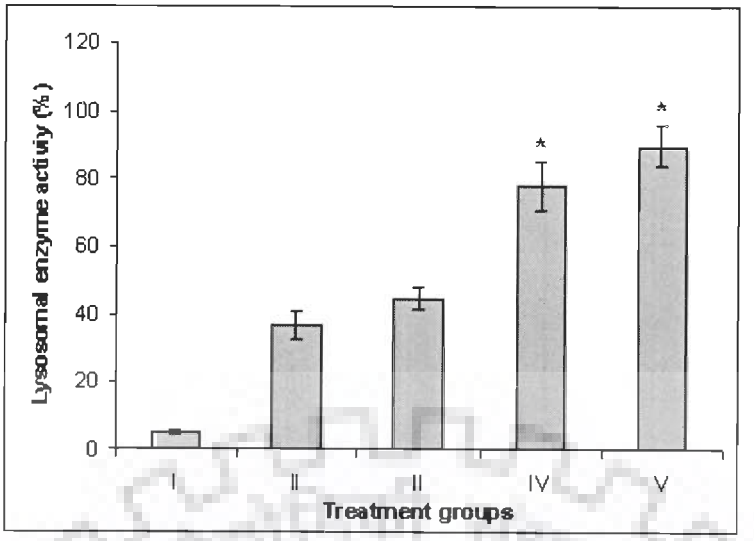

Figure 40. Effect of *in vivo* treatment of NOP-51 on peritoneal macrophages cellular lysosomal activity. Group I (control treated with buffer), II, III, IV and V are different NOP-51 concentrations (25, 50, 100 and 150 µg/ml of protein respectively). A volume dose 200 µl was administered daily i.p. for 15 consecutive days. Peritoneal macrophages collected 24 h after last dose and acid phosphatase activity assayed. Results are expressed as mean ± S.E. of six replicate comparing to the control; * $p < 0.05$.

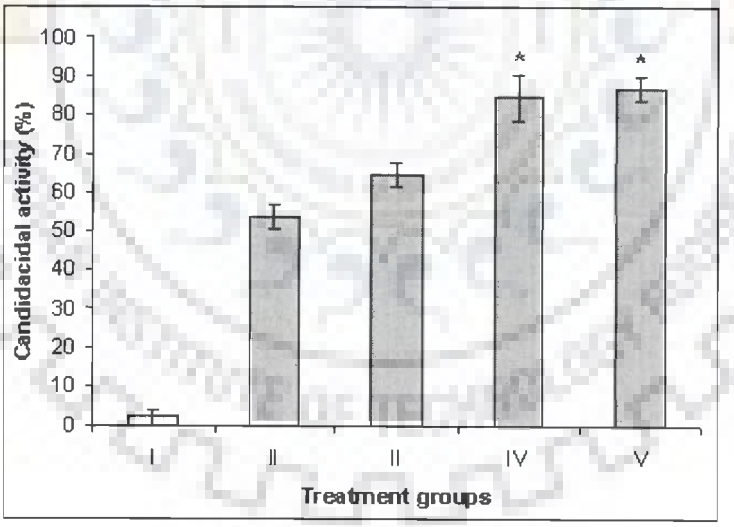

Figure 41. Effect of *in vivo* treatment of NOP-51 on peritoneal macrophages candidacidal activity. Group I (buffer alone), II, III, IV and V are animals treated with different NOP-51 (25, 50, 100 and 150 µg/ml respectively). Peritoneal macrophages from each treated groups were collected 24 h after last dose and used for candidacidal activity assay. Results are expressed as mean ± S.E. of six replicate comparing to the control; * $p < 0.05$.

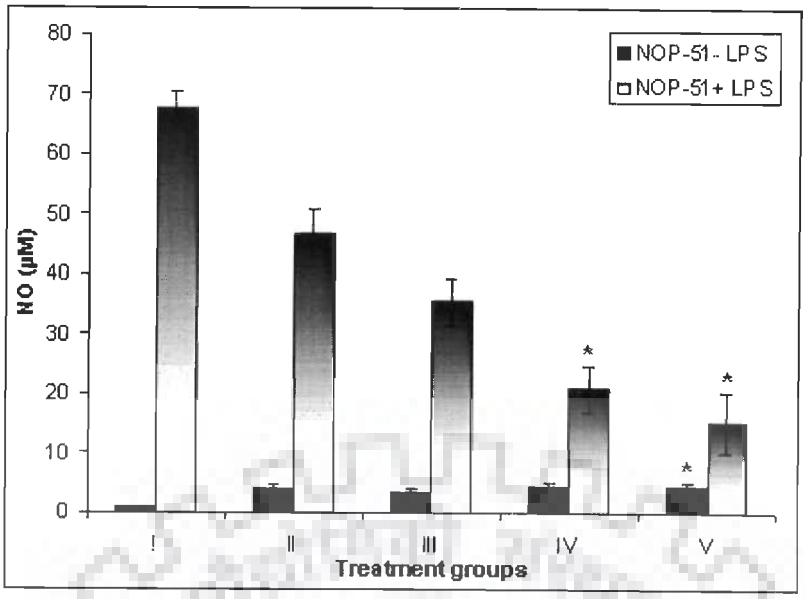

Figure 42. Effect of *in vivo* treatment of NOP-51 on peritoneal macrophages NO production. Peritoneal macrophages were collected from each treatment group I (control treated with buffer alone), groups II, III, IV and V treated with 25, 50, 100 and 150 μg/ml of NOP-51, respectively, and were grown for 24 h in absence or presence of LPS (5 μg/ml). NO level in respective groups cell culture supernatants were measured using Griess reagent. Results are expressed as mean ± S.E. of six replicate comparing to the control; * $p< 0.05$.

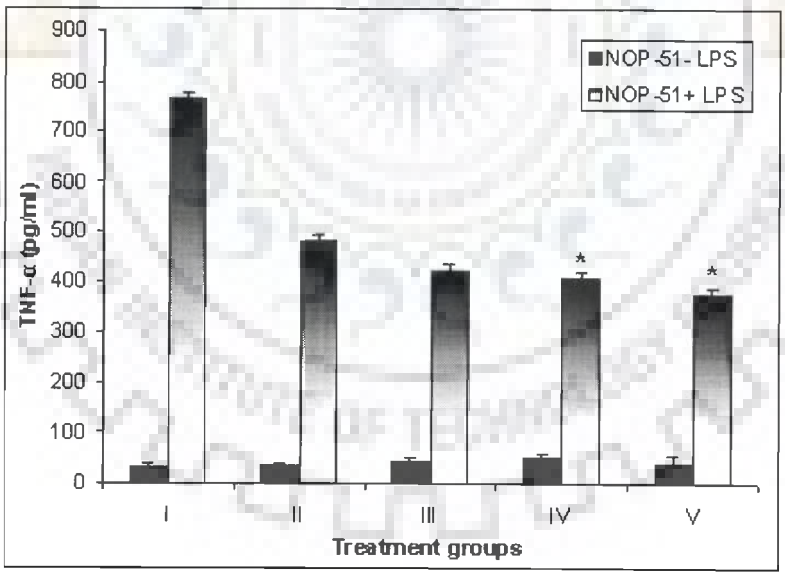

Figure 43. Effect of NOP-51 *in vivo* treatment on peritoneal macrophages TNF-α production. Peritoneal macrophages were collected from each treatment group I (control treated with buffer alone), groups II, III, IV and V treated with 25, 50, 100 and 150 μg/ml of NOP-51, respectively, and were grown for 24 h in absence or presence of LPS (5 μg/ml). TNF-α released in respective culture supernatants were determined. Results are expressed as mean ± S.E. of six replicate comparing to the control; * $p< 0.05$.

4.7.3. Splenocyte functions assay

Spleen from each treatment group was removed aseptically and single cell suspension of splenocytes was prepared. About 97% viability was found to be in each treated group by Trypan blue dye exclusion assay and these cells were used for further study

4.7.3.1. Effect of *in vivo* treatment of NOP-51 on Splenocyte proliferation

Immunomodulatory effect of NOP-51 on splenocytes proliferation was also studied. The effect of NOP-51 *in vivo* treatment on splenocytes proliferation is shown in figure 44. Similar to the *in vitro* treatment, all *in vivo* treated animal groups showed stimulatory effect on splenocytes proliferation in a dose dependent manner as compared to control even in absence of mitogens. The predetermined optimum dose of lipopolysaccharide (LPS) and concavalin A (Con-A) at 5 µg/ml were used as mitogen for stimulating B and T lymphocytes respectively. Compared to LPS and Con-A stimulated splenocytes proliferation in untreated groups, the proliferation of splenocytes was significantly enhanced in a dose related manner and it was highest at 150 µg/ml of dose (the highest tested dose in present study). Thus, the *in vivo* treatment of animals with NOP-51 alone also has stimulatory effect on splenocytes proliferation and it further augmented the Con-A and LPS activated response.

4.7.3.2. Effect on IL-2 production

Since the NOP-51 has stimulatory effect on splenocytes proliferation and it augmented the Con-A activated proliferation. The IL-2 release by splenocytes in control and protein treated animal groups in presence and absence of Con-A was assayed. The effect on IL-2 production is summarized in figure 45. The i.p. administration of NOP-51

had a stimulatory effect on IL-2 release as compared to control (untreated animals group) but the overall stimulation was low. Con-A treatment of splenocytes of control group has pronounced effect on IL-2 release. Similar to the effect on splenocytes proliferation the co-cultivation of cells with Con-A and NOP-51 augmented the stimulation on IL-2 release by splenocytes in dose dependent manner. Thus it has synergistic effect on IL-2 production

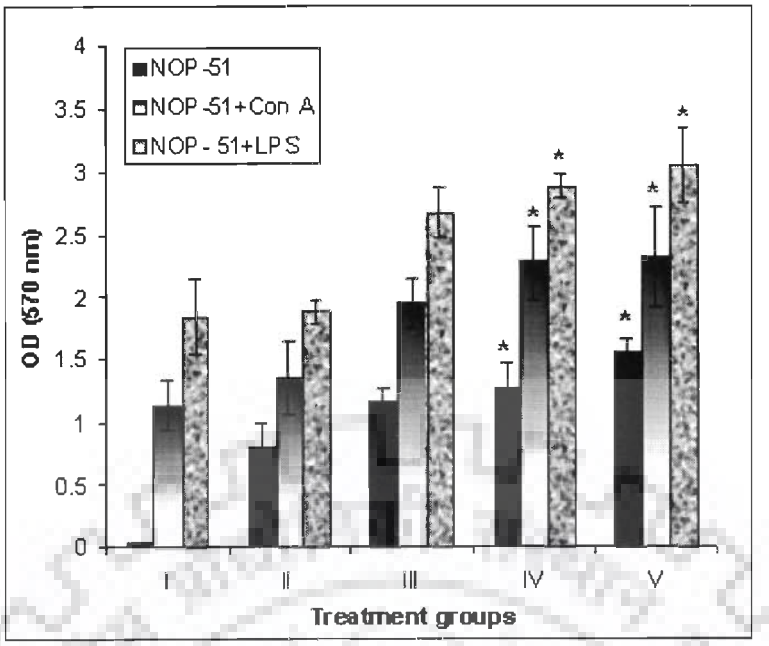

Figure 44. Effects of *in vivo* treatment of NOP-51 on splenocytes proliferation. Group I, II, III, IV and V was administered with 200 µl of 50 mM Tris-HCl pH 8.0, 25, 50, 100 and 150 µg/ml of protein respectively. Results are expressed as mean ± S.E. of six replicate comparing to the control; * $p< 0.05$.

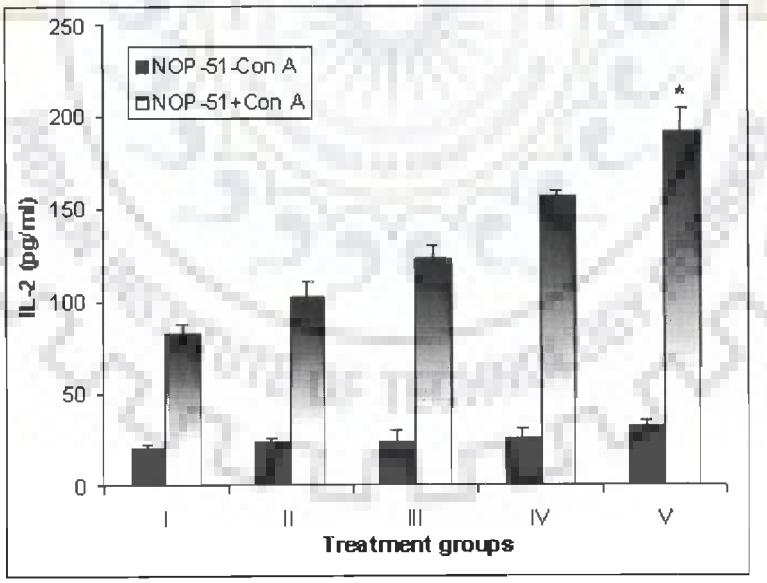

Figure 45. Effects of *in vivo* NOP-51 on IL-2 production in splenocytes cell culture supernatant. Group I, II, III, IV and V was administered with 200 µl of 50 mM Tris-HCl pH 8.0, 25, 50 100, and 150 µg/ml of protein respectively. Results are expressed as mean ± S.E. of six replicate comparing to the control; * $p< 0.05$

(E) Toxicological investigations

In order to see NOP-51 as potential antimicrobial and immunomodulatory agent, its toxicological effect was also evaluated in mice.

4.8. Lactate dehydrogenase cytotoxicity

Although more than 97% cell viability was obtained at all tested protein doses which ensure that it does not have toxic effect. However it was further confirmed by LDH estimation in the culture supernatant of all doses treated cultures of macrophages and splenocytes. No differences were observed in the LDH level in the control and different protein concentration treated culture supernatants macrophages and splenocytes. Thus, no toxicity was observed in macrophages and splenocytes culture supernatant.

Table 11. Determination of cytotoxicity of NOP-51 by lactate dehydrogenase (LDH) leakage assay of splenocytes and macrophages cell culture supernatant.

Treatment*	LDH leakage (IU/ml) in splenocytes culture supernatant	LDH leakage (IU/ml) in macrophage culture supernatant
(I) Control	5.12 ± 0.12	5.02 ± 0.21
(II) 25 µg/ml	5.34 ± 0.22	5.22 ± 0.01
(III) 50 µg/ml	5.35 ± 0.34	5.11 ± 0.14
(IV) 100 µg/ml	5.12 ± 0.02	5.13 ± 0.17
(V) 150 µg/ml	5.25 ± 0.12	5.21 ± 0.42

*Group I, II, III, IV, and V are control, 25, 50, 100 and 150 µg/ml NOP-51 treated animals, respectively. Results are expressed as mean ± S.E. of six replicate.

4.9. Hematological parameter

The effect of NOP-51 treatment on various blood cells and hemoglobin are shown in Table-9. Total RBC, WBC and hemoglobin level did not show any appreciable change.

Table 12: Hematological investigation in male swiss albino mice treated with NOP-51.

Treatment*	Total RBC (10^6/mm^3)	Total WBC (10^3/mm^3)	Total hemoglobin (g/100ml)
(I) Control	4.0 ± 0.4	5.9 ± 0.3	11.0 ± 1.0
(II) 25 µg/ml	4.0 ± 0.2	5.0 ± 0.1	11.1 ± 0.5
(III) 50 µg/ml	4.1 ± 0.3	5.0 ± 0.2	11.1 ± 1.2
(IV) 100/ml µg	4.0 ± 0.6	5.0 ± 0.7	11.1 ± 1.1
(V) 150 µg/ml	4.4 ± 0.2	5.0 ± 0.2	11.1 ± 1.3

*Group I, II, III, IV and V are control, 25, 50, 100 and 150 µg/ml NOP-51 treated animals, respectively. Results are expressed as mean ± S.E. of six replicate.

4.10. Effect of NOP-51 on liver function

Effect of NOP-51 on various liver function parameters SGPT, SGOT, urea, bilirubin, and serum protein was evaluated and shown in table-10. There was no significant elevation in the level of SGPT, SGOT, urea, bilirubin, and serum protein at any of the doses used in this study.

Table 13: Blood serum clinical biochemistry of male swiss albino mice treated NOP-51.

Treatment*	Urea (mg/dl)	SGPT (U/ml)	SGOT (U/ml)	Bilirubin (mg/dl)	Serum protein (mg/dl)
(I) Control	35.2 ± 1.7	25.3 ± 1.2	36.0 ± 1.6	0.79 ± 0.1	4.4 ± 0.7
(II) 25 µg/ml	35.2 ± 1.5	25.3 ± 0.8	36.2 ± 0.7	0.79 ± 0.1	4.5 ± 0.2
(III) 50 µg/ml	35.2 ± 0.9	25.2 ± 1.0	36.4 ± 0.5	0.79 ± 0.2	4.4 ± 0.4
(IV) 100 µg/ml	35.3 ± 1.1	25.5 ± 1.5	36.4 ± 1.7	0.79 ± 0.1	4.4 ± 0.2
(V) 150 µg/ml	34.8 ± 1.8	25.3 ± 1.4	36.5 ± 1.8	0.79 ± 0.2	4.2 ± 0.1

*Group I, II, III, IV and V are control, 25, 50, 100 and 150 µg/ml NOP-51 treated animals, respectively. Results are expressed as mean ± S.E. of six replicate.

(E) Identification of NOP-51 protein

4.11. N-terminal sequence determination

The first 12 amino acid residue of NOP-51 was determined by Edman degradation. The 12 N-terminal amino acid sequence of the purified protein were Ala-Pro-Ala-Tyr-Asn-Tyr-Gly-Val-Ala-Val-Tyr-Glu. Since the protein showed potent antimicrobial activity, the amino acid sequence was searched against the NCBI BLAST short sequence search protein data base. The N-terminal sequence of first 12 amino acids did not show significant similarity with any of the antimicrobial proteins; however, showed 33.3% resemblance with antifungal protein from *Arabidopsis thaliana* and *Triticum aestivum* (Table 14). Furthermore, since the NOP-51 has strong inhibition on LPS induced NO production by macrophages and it was also found to have antioxidant activity in preliminary study (data not shown here). The N-terminal sequence of first 12 amino acids was subjected to homology search with antioxidant proteins present in NCBI database. No significant homology could be found with any of the protein available in database except for a low 33.3 % resemblance with the superoxide dismutase in first N-terminal 12 amino acids (Table 15.). The N-terminus sequence homology search result was in conclusive as 12 amino acid residues from a 51 kDa protein is too small and 33% homology shown with mentioned protein is very low. This needs further investigation using complete *de novo* sequence of protein.

Table 14. The N-terminal sequence homology search of NOP-51 with other antimicrobial protein available in NCBI protein sequence database.

Accession no.	Species	Protein	Sequence	Identity %
--	*Nerium odorum*		1 APAYNYGVAVYE 12	-
Q9FI23	*Arabidopsis thaliana*	Low-molecular cysteine-rich antifungal protein	12 APAMVEAQKLCE 24	33.3
O64392	*Triticum aestivum*	Wheatwin-1 precursor, Pathogenesis-related protein	44 APAVSAYCATWD 56	33.3
O64393	*Triticum aestivum*	Wheatwin-2 precursor, Pathogenesis-related protein	46 APAVSAYCATWD 58	33.3
P83139	*Malva parviflora*	Antifungal protein 5	12 APAPXXXXGIRN 24	25

Table 15. The N-terminal sequence homology search of NOP-51 protein with other antioxidant protein available in NCBI protein sequence database.

Accession no.	Plant	Sequence	Identity %
-	*Nerium odorum*	1 APAYNYGVAVYE--- 12	-
NP_001077494	*Arabidopsis thaliana*	1 MAKGVAVLNSSE 12	33.3
AAT66935	*Malus xiaojinensis*	1 MVKGVAVLGSSE 12	33.3
AAN60796	*Brassica juncea*	1 MGKGVAVLNSGE 12	33.3
CAA43270	*Arabidopsis thaliana*	1 MAKGVAVLNSSE 12	33.3
ABI81470	*Thlaspi caerulescens*	1 MAKGVAVLNSSE 12	33.3
CAA39444	*Nicotiana plumbaginifolia*	1 MVKAVAVLSSSE 12	25
AAV88605	*Pennisetum glaucum*	5 TAGADGVANIN--- 16	25
AAC15842	*Raphanus sativus*	5 AVTANYVLKPPP-- 17	25
CAE54085	*Fagus sylvatica*	12 RTMAKGVAVLS--- 23	33.3
ABL85055	*Brachypodium sylvaticum*	12 APAPPSLPALRR-- 24	25

4.12. Identification by LC MS/MS

The internal amino acid sequencing of NOP-51 was performed by LC-MS/MS after tryptic digestion and alkyl reduction. LC-MS/MS analysis provides many significant new insights into the nature of NOP-51 protein and also supported some of the N-terminus sequence homology search observations. In total, twenty eight peptides were obtained from the protein. The search under MSDB for all twenty eight peptides showed significant homology to Manganese superoxide dismutase (*Citrullus lanatus* and *Zantedeschia aethiopica)*, Membrane protein MP27, Glutathione S-tranferease (*Cucurbita maxima,* Pumpkin and *Lycopersicon esculentum*), Protein PV100 (*Cucurbita maxima*), Caleosin (*Sesamum indicum*), and Heat shock protein (Soyabean and sunflower) (Table 13). Since the highest score was shown with *Citrullus lanatus* Manganese superoxide dismutase, the detail homology match of NOP-51 peptides fragment with complete amino acid sequence of *Citrullus lanatus* is shown in figure 47.

The protein showing high score match with NOP-51 (mentioned in table-16) were further analyzed by short sequence homology search using BLAST. The homology search under NCBI BLAST short sequence search for each fragment showed significant homology to antimicrobial seed protein vicilin, Manganese superoxide dismutase and glutathione S-tranferase. Peptide RLSEGGVLVIPAGHPIAIMASPNENLRL showed 72% homology to Vicilin, an antimicrobial seed protein isolated from *Vicia faba, Pisum sativum, Lupinus albus, Gossypium hirsutum and Macadamia integrifolia* (Fig. 46). The peptide KKLSVETTANQDPLVTKG showed 100% homology to Mn superoxide dismutase isolated from *Oryza sativa, Zea mays, Cucumis sativus, Citrullus lanatus, Triticum aestivum, Musa acuminata, Marchantia paleacea, evea brasiliensis, Vitis*

vinifera, Camellia sinensis, Fagus sylvatica and *Prunus persica* and peptide KALEQLHEAINKG also showed more than 100% homology to Mn superoxide dismutase from *Citrullus lanatus, Cucumis sativus, Gossypium hirsutum* and *Nicotiana tabacum,* (Fig. 48). Peptide KDKAPLLPSDPYQRA showed more than 90% homology to Gluthione S-tranferase from *Cucurbita maxima, Carica papaya, Lycopersicon esculentum* and *Vitis vinifera.* Peptide KYLGGDSFSLADLHHLPVLGYLLATPSKKL showed 66% homology to other Glutathione S-tranferase from *Arabidodpsis thaliana* (Fig. 49).

Table 16. Sequence homology studies of twenty eight peptides generated by LC-MS/MS by mass spectrometery database.

Accession no.	Description	Score	Queries matched	Peptides
Q6QGY4	Mn superoxide dismutase *Citrullus lanatus*	224	4	KLQSAIKF KGHTSTVVKL KALEQLHEAINKG KKLSVETTANQDPLVTKG
T10443	Probable major protein body membrane protein MP27	140	4	KGGDVYRI RTGPFEFFGFTTSSRR RIPAGSVFYMVNVGEGQRL KSLFVPQYLDSSLILFVRR
Q8H9E7	Glutathione S - transferase *Cucurbita maxima*	117	3	RIALAEKG RFWVDFIDKKL KDKAPLLPSDPYQRA
T44430	Protein PV100 *Cucurbita maxima*	96	3	RNFLAGRE KELAFNVEGKQ RLSEGGVLVIPAGHPIAIMASPNENLRL
T50831	Superoxide dismutase *Zantedeschia aethiopica*	96	1	KKVTVETTANQDPLVTKG
Q9SQ57	Caleosin *Sesamum indicum*	79	2	KEAIRRC RCYDGSLFEYCAKM
HHSY17	Heat shock protein 17 soybean	72	3	RVLQISGERN RFRLPENAKV KKPDVKAIDISG
JC7899	Glutathione S - transferase Pumpkin	67	3	KLHEGEHKK RVLAALYEKG KYLGGDSFSLADLHHLPVLGYLLATPSKKL
Q8GVD1	Glutathione S - transferase *Lycopersicon esculentum*	67	2	RIALAEKE KDKAPLLPSDPYERA
S23529	Heat shock protein, 17.6 common sunflower	63	3	RVLQISGERC RFRLPENAKM KAMMENGVLTVVVPKE

Accession no		Sequence		Identity %	Score	E- value
NOP-51	1	RLSEGGVLVIPAGHPIAIMASPNENLRL	27	-	-	-
P08438	354	YKAKKLSPGDVLVIPAGYPVAIKASSNLNLVGFG	379	72	46.9	1e-05
P13918	355	QNYKALSSGDVFVIPAGHPVAVKASSNLDLLG--	376	68	43.1	1e-04
P02854	338	LYRAKLSPGDVFVIPAGHPVAINASSDLNLGLGI	362	68	42.2	3e-04
P02855	167	SYKAKLTPGDVFVIPAGHPVAVRASSNLNLLLGF	191	64	41.8	3e-04
P09799	491	RVRAQLSTGNLFVVPAGHPVTFVASQNEDLGLLG	525	56	38.4	0.004
Q9SPL4	551	QVKARLSKREAIVVPVGHPVVFVSSGNENLLLFA	576	50	32.9	0.16

Figure 46. Sequence homology of peptide RLSEGGVLVIPAGHPIAIMASPNENLRL by NCBI BLAST short sequence search. Peptide showed significant homology to Vicilin, an antimicrobial protein

QKHHQTYITNYNKALEQLHEAINKGHTSTVVKLQSAIKFNGGGH
INHSIFWNNLAPIHEGGGEPPKGSLGWAIDSQFGSLEALIQRVN
AEGAALQGSGWVWLALDKELKKLSVETTANQDPLVTKGSALVPL
LGIDVWEHAYYF

Figure 47. Homology match of NOP-51 peptide fragments generated by LC-MS/MS with complete amino acid sequence of highest scorer Manganese superoxide dismutase from *Citrullus lanatus* (accession no. Q6QGY4).

Accession no		Sequence		Identity %	Score	E- value
NOP-51	1	KKLSVETTANQDPLVTKG	18	-	-	-
AAA57131	159	LDKEAKKLSVETTANQDPLVTKGANLVP	187	100	57.9	7e-08
P41978	160	LDKEPKKLSVETTANQDPLVTKGASLVP	188	100	57.9	7e-08
AAA57130	159	LDKEAKKLSVETTANQDPLVTKGANLVP	187	100	57.9	7e-08
ABK55698	103	LNKELKKLSVETTANQDPLVTKGSALVP	131	100	57.9	7e-08
AAS48178	103	LDKELKKLSVETTANQDPLVTKGSALVP	131	100	57.9	8e-08
AAB68036	159	LDKEAKKLSVETTPNQDPLVTKGSNLYP	187	100	57.1	1e-07
AAM34758	96	LDKGSKKLCVETTANQDPLVTKGANLVP	124	94	54.5	8e-07
BAD13494	169	LNKDLKKLVVETTANQDPLVTKGLIPLL	181	94	52.4	3e-06
CAB53458	131	LDKELKKLVVETTANQDPLVTKGPTLVP	159	94	52.4	3e-06
ABX79342	156	LDKDLKKLVVETTANQDPLVTKGPNLVP	184	94	52.0	5e-06
AAT68778	158	VDKELKKLVVETTANQDPLVTKGPSLVP	186	94	52.0	5e-06
ABI26729	110	LDKELKKLVVETTANQDPLVTKGANLVP	138	94	52.0	5e-06
CAC19487	8	LDKELKKLVVETTANQDPLVTKGPTLVP	36	94	49.8	2e-05
AAM62550	159	LDKELKKLVVDTTANQDPLVTKGGSLVP	187	88	49.8	2e-05
NP_191194	159	LDKELKKLVVDTTANQDPLVTKGGSLVP	187	88	49.8	2e-05
AAN15216	153	VDKEFKRLVVETTANQDPLVTKGPSLVP	180	88	49.8	2e-05
AAQ20004	173	DKEFNKRLVVETTANQDPLVTKGSSLVP	201	88	49.8	2e-05
ABA10482	157	DKESQKRLVVETTANQDPLVTKGPNLVP	184	88	49.8	2e-05
CAC69403	105	VDKELKRLVVETTANQDPLVTKGATLVP	133	88	49.8	2e-05
ABQ52658	169	LDKELKRLVVETTANQDPLVTKGPNLVP	197	88	49.4	2e-05
AAL07333	159	LDKELKKLVVDTTANQDPLVTKGGSLVP	187	88	49.4	2e-05
ABB83613	29	LDKELKRLVVETTANQDPLVTKGSHLVP	57	88	49.4	2e-05
CAA42737	168	LDKDLKRLVVETTANQDPLVTKGASLVP	197	88	49.4	2e-05
ABL75952	158	LDKELKRLVVDTTANQDPLVTKGASLVP	187	88	49.0	3e-05
AAL24044	96	VDKELKRLVVETTANQDPLVTKGASLVP	124	88	49.0	3e-05
CAD29434	103	LDKELKRLVVETTANQDPLVTKGPNLVP	131	88	48.6	5e-05
CAC05259	151	VDKELKRLVIETTANQDPLVTKGPSLAP	179	83	47.3	1e-04
AAC78469	116	VNKELKKLVIETTPNQDPLVTKGPHLVP	154	83	46.9	1e-04
ABK32075	152	LDKESKHLVVETTANQDPLVTKGPSLVP	180	83	46.9	1e-04
AAF65768	164	LEKESKRLVVETTSNQDPLVTKGPLVPL	192	83	46.9	2e-04
AAS77885	158	VDKESKKLVIETTANQDPLMTKGPNLVP	186	83	46.0	3e-04
CAC69402	103	VDKELKRLVVETTSNQDPLVTKGPSLVP	131	83	46.0	3e-04
ABR23341	155	LNKDLKRLVVETTQNQDPLVTKGLLPLP	183	83	45.2	5e-04
ABI35908	157	LDKEFKRLVIETTPNQDPLVTKGASLLP	185	77	44.3	0.001
CAC05260	144	VDKQLKRLVVETTANQDVLATKGASLVP	172	77	40.1	0.09
CAJ01676	155	VDKELKRLVIETTADQEPLVSKGANLVP	183	66	39.7	0.02
BAA86881	155	YNKELKRLQVETTQNQDPLSSKGLIPLL	184	72	37.1	0.12

Figure 48 a. Sequence homology studies of peptide KKLSVETTANQDPLVTKG by NCBI BLAST short sequence search. Peptide showed significant homology to Mn superoxide dismutase enzyme.

Accession no		Sequence		Identity %	Score	E- value
NOP-51	1	KALEQLHEAINKG	13	-	-	-
ABK55698	7	ITNYNKALEQLHEAINKGHTST	30	100	39.7	0.023
AAS4817 8	7	ITNYNKALEQLHEAINKGHTSTV	30	100	39.7	0.035
AAC78469	30	ITNYNKALEQLHEAIQKGDSSTV	53	92	36.7	0.18
AAB02052	64	VTNYNKALEQLHEAISKGDASAA	82	92	36.3	0.24
ABA00455	63	ITNYNKALEQLHEAIQKGDSSTV	86	92	35.4	0.43

Figure 48 b. Sequence homology studies of peptide KALEQLHEAINKG by NCBI BLAST short sequence search. Peptide showed significant homology to Mn superoxide dismutase enzyme.

Accession no		Sequence		Identity %	Score	E- value
NOP-51	1	KDKAPLLPSDPYQRA	15	-	-	-
BAC21261	73	IDEVWKDKAPLLPSDPYQRAQARFW	98	100	50.7	1e-05
CAA04391	73	IDEVWSDKAPLLPSDPYQRAQARFW	98	100	46.9	2e-04
AAL92873	74	IDEVWKDKAPLLPSDPYERAQARFW	99	93	46.4	2e-04
ABL84692	73	IDEVWCDKSPLLPSDPYQRAQARFW	100	92	45.2	4e-04
CAI48072	74	IDEVWKDKAPLLPTDPYERAQARFW	99	86	44.8	6e-04
ABQ95990	73	IDEVWKDKAPLLPSDRYQRAQARFW	98	93	44.3	9e-04
AAP30740	73	IDEVWCDKSPLLPSDPYQRAQARFW	98	92	42.6	0.003
NP_173161	73	IDEVWPSKTPLLPSDPYQRAQAKFW	98	92	42.2	0.005
AAP04396	20	IEEVWKDKAPLLPSDPYDRAQARFW	45	87	41.4	0.007
AAG34799	74	IDEVWNDKAP-LPSDPYERAQARFW	99	85	41.4	0.007
ABG73420	73	IDEVWNDKSPLLPSDPYQKRSSQVW	98	84	39.2	0.03
AAO61854	73	IDEVWHDKSPLLPSDPHQRAVARFW	98	85	38.8	0.04

Figure 49 a. Sequence homology studies of peptide KDKAPLLPSDPYQRA by NCBI BLAST short sequence search. Peptide showed significant homology to Glutathione S-transferase

Accession no.		Sequence		Identity %	Score	E-value
NOP-51	1	KYLGGDSFSLADLHHLPVLGYLLATPSKKL	30	-	-	-
JC7899	156	KYLGGDSFSLADLHHLPVLGYLLATPSKKL	186	100	90.6	1e-17
BAF01093	150	KYLAGETFTLTDLHHIPVIQYLLGTPTKKL	180	66	63.4	1e-09
NP_178394	238	KYLAGETFTLTDLHHIPVIQYLLGTPTKKL	268	66	63.4	1e-09
AAP58391	158	KYLAGETFTLTDLHHIPVIQYLLGTPTKKL	188	66	63.0	2e-09
ABK81651	155	KYLGGDCFGLADLHHLPTLHYLLGSSAKKL	185	73	62.6	3e-09
ABQ96853	154	KYLGGDSFTLADLHHAPVLHYLIGTKMKSL	184	80	58.3	6e-08
AAY86772	157	KYCAGDTFTLTDLHHIPVIQYLLQTPSKKL	187	73	57.9	7e-08
AAF65767	154	KYLGGDSFTLADLHHLPNLQLLLNHQSKKV	184	75	55.8	3e-07
CAI51314	154	KYLGGDSFTLADLHHAPGLHYLFGTKVKSL	184	76	52.8	2e-06
BAB70616	155	KYMGGECFTLVDLHHLPSLHYLMKSQSKKL	185	63	52.8	3e-06
AAO61853	106	KYLACDYFTLADLNHLPNIQCLLATPSKKL	136	70	52.4	3e-06
ABQ96852	154	KYLGGESFTLADLHHAPSLHYLMGSKVKSL	184	67	49.4	3e-05
CAA96431	154	KYLGGDSFTLVDLHHIPNIYYLMSTKVKEV	184	68	47.3	1e-04
ABK32074	155	KYLGGDDFTLADLHHIPIVNNLMHTDKVKP	185	77	45.6	4e-04
ABI79308	153	KYLAGDFFSLADLSHLPFTHYLANSMGKEY	183	82	40.8	0.016
AAD56395	153	KYLAGDSLSLADLNHVSVTLCLAATPYASL	183	66	39.7	0.024
CAA68993	154	KYLAGEFFSLADLSHLPSLRFLMNEGGFSH	184	73	38.4	0.053

Figure 49 b. Sequence homology studies of peptide KYLGGDSFSLADLHHLPVLGYL-LATPSKKL by NCBI BLAST short sequence search. Peptide showed significant homology to Glutathione S-transferase

CHAPTER 5

DISCUSSION

There has always been a continual battle between humans and the multitude of microorganisms that cause infection and disease. A number of major disease caused by various bacteria, fungi, viruses, protozoan parasites, like plague, tuberculosis, malaria, and more recently, acquired immunodeficiency syndrome (AIDS), have affected substantial portions of the human population, causing significant morbidity and mortality. But the discovery of penicillin in middle of the 20th century opened the way for development of several important antibiotics and other chemical agents for all kinds of infections from bacteria, fungi, virus and protozoa and scale seems to be tilted in favor of humans. However, the so called potential conquest of infectious diseases was short lived. Prolong and indiscriminate use of these drugs manifested in emergence of multidrug resistant pathogens. As antimicrobial and other drug usage increased, so did the level and complexity of the resistance mechanisms exhibited by pathogens. Multidrug resistant strains have emerged in all important pathogenic bacteria (Tenover, 2006; Poole and Srikumar, 2001), fungi (Fostel and Lartey, 2000; Sanglard *et al.*, 1997) and in other microbes (Kurome, 1998). The struggle to gain the upper hand against infections continues even today. Control and treatment of the infectious diseases caused by these multidrug resistant pathogens is one the major concern presently (McGowan, 2001). There are several reasons for its concern. First, infections caused by drug-resistant/multidrug resistant pathogenic strains, particularly bacteria (Tenover, 2006; Poole and Srikumar, 2001) and fungi (Fostel and Lartey, 2000; Sanglard *et al.*, 1997), are becoming common problem in healthcare institutions. There has been sudden alarming rise in antimicrobial infections (Chambers, 2001; Karlowsky, 2003) due to multidrug resistant and opportunistic pathogens predominantly in immunocompromised hosts. Drug

resistance often results in treatment failure, which can have serious consequences, especially in critically ill patients (Kang, 2005; Ibrahim, 2000). Second, the resistant pathogenic strains may also spread not only within healthcare institutions, but in communities as well (Francis *et al.*, 2005; Herold *et al.*, 1998). Third, the spread of resistant pathogens within the community poses obvious additional problems for infection control. Finally, with respect to the cost containment pressures of today's healthcare environment, antimicrobial drug resistance places an additional burden on healthcare costs (McGowan, 2001).

Antibiotic resistance has become a global public-health problem, thus it is imperative that new antibiotics continue to be developed. Consequently, in recent few years the attention of scientist have been focused in the development of alternative safer drugs and/or the recovery of natural molecules that would allow the consistent and proper control of pathogen caused diseases. Ideally, these molecules should be as natural as possible, with a wide range of action over several pathogens, easy to produce, and not prone to induce resistance. One such group of compounds are explored for such novel properties are antimicrobial peptides/proteins (AMPs). They represent a new family of antibiotics that have stimulated research and clinical interest (Wu *et al.*, 1999) as new therapeutic options for infections caused by multidrug resistant bacteria. They have been termed "natural antibiotics", because they are active against a large spectrum of microorganisms, including bacteria and filamentous fungi in addition to protozoan and metazoan parasites (Liu et al. 2000; Vizioli and Salzet, 2003). The effect of AMPs can go beyond isolated cells, as shown by the inhibition they can exert their effect over clusters of pathogenic bacteria, such as in biofilm development (Singh *et al.* 2002). Additionally,

these antimicrobial proteins also have other biological activities such as antitumor, and immunomodulatory, which further increase their potential as therapeutic agents. A large numbers of antimicrobial proteins/peptides have been isolated, characterized and their potential antimicrobial activity has been established from wide diverse sources from lower invertebrates to vertebrates including human and plants (Marshall and Arenas, 2003). AMPs have many of the desirable features of a novel antibiotic class and can complement conventional antibiotic therapy.

Recently, much attention has been focused towards plants for therapeutics proteins for two main reasons. First, the large scale production of therapeutics proteins/peptides (commonly known as biologics) of heterologous origin in plants. In recent few years, transgenic plant expression systems were developed for the production of biologics (Suslow *et al.,* 2002), known as plant made pharmaceuticals (PMPs). Second, the isolation of natural therapeutic proteins/peptides present in various plants. Several therapeutic proteins/peptides have been isolated from various plants and attempts are on to identify new therapeutic proteins. The present study was focused on the second aspects. It describes the isolation, purification and characterization of a potential antimicrobial and immunomodulatory protein from *N. odorum* which showed potent antimicrobial activities against a broad range of bacteria and fungi. The *N. odorum* was chosen for the present study due to its known applications in anti-inflammatory and other diseases, and easy availability throughout the year. A 51 kDa protein from *N. odorum* fresh leaves was isolated, purified and characterized using various biochemical and biophysical techniques. The purified protein was found to be a monomeric protein as same molecular weight band was observed in both SDS and Native-PAGE. Antimicrobial

activity of the crude protein and purified protein (NOP-51) was tested against various bacteria and fungi. However, to ensure that the antimicrobial activity was due to the purified protein (NOP-51) but not of any contaminating proteins, the purity of the purified protein was checked by silver staining, RP-HPLC and MALDI-TOF. The absence of any contaminating protein bands in the silver stained gel even at even at higher protein concentrations and the presence of single elution peak in HPLC and MALDI-TOF confirms the purity of protein and whatever activity were observed it was due to NOP-51 only.

The crude protein preparation and purified protein (NOP-51) was found to have broad spectrum antibacterial activity as it showed action against both gram positive (*S. aureus and B. substilis*) and gram negative bacteria (*P. putida and E. coli*). NOP-51 was supposed to be the main antibacterial protein in the crude protein fraction as the activity of the purified protein increased compared to crude protein fraction against all tested bacteria. The MIC values of the crude protein and NOP-51 was found to be significant as compared to reference (gentamicin) and even lower in most of the cases particularly in *S. aureus*. The values were much lower than the MIC (25-100 mg/l) of the various other antibacterial and antifungal proteins/ peptides reported in earlier studies (Ye *et al.,* 2000; Silphaduang *et al*., 2006; Bindschedlera *et al*., 2006 Mangoni *et al*., 2004; Xia and Ng, 2005; Lee *et al*., 2007). Compared to some earlier antimicrobial proteins/peptides report, where antimicrobial activity was found to be either against Gram positive or Gram negative bacteria, NOP-51 showed antimicrobial activity against both Gram positive and Gram negative bacteria. Although protein has significant antibacterial activity against all bacteria used in the present study, but it found to be more effective against *S. aureus*.

This was a significant observation as *S. aureus* is one of the major pathogenic bacteria. The antibacterial potential of the NOP-51 was reflected from disk diffusion, MIC, MBC and concentration dependent time killing curve. MICs and MBCs of NOP-51 against all the four bacteria (*S. aureus*, *B. subtilis*, *E. coli* and *P. putida)* often coincide (less than a two-fold difference), indicating that NOP-51 is not only bacteriostatic but also bactericidal and killing is generally the highly desirable bactericidal mode of action.

The crude protein and NOP-51 also found to have potential antifungal activity. Similar to the antibacterial activity, both the crude and NOP-51 was found to have broad spectrum antifungal activity against various filamentous fungi (Thies *et al.*, 2005; Ng, 2004; Chu et al., 2005; Xia and Ng, 2005) and *C. albicans*. Protein was more effective against *A. fumigatus, A. flavus* and *C. albicans* compared to *A. niger* and *F. oxysporum*. Antifungal activity against these fungi was obtained only at relative higher concentrations. The *F. oxysporum* found to be less susceptible while the *C. albicans* most, compared to others. Similar to the antibacterial activity, the antifungal potential of the NOP-51 was reflected from disk diffusion assay, MIC, MFC and concentration dependent time killing curve. MICs and MFCs of NOP-51 were much closer to that of flucanozole (reference antifungal drug). MICs and MFCs of NOP-51 against all the four filamentous fungi *A. fumigatus, A. flavus, A. niger* and *F. oxysporum* and *C. albicans,* often coincide (less than a two-fold difference), indicating that it is not only fungistatic but also fungicidal and the killing is generally the highly desirable fungicidal mode of action. Based on its broad spectrum antifungal activity and MICs and MFCs values, NOP-51 was found to be better antifungal protein as compared to earlier reported antifungal proteins (Thies *et al.*, 2005; Ng, 2004; Chu *et al.*, 2005).

Based on the MICs for each bacterium, time killing curve was constructed in order to evaluate the effect of different concentrations of NOP-51. Colony count assays were performed to determine the time required for NOP-51 to kill the various bacteria. The present study showed that NOP-51 at 50 µg/ml concentration killed all tested bacteria within 15 min, as no viable count could be seen. This indicates that NOP-51 was very effective in killing of broad range of bacteria (*S. aureus, B. substilis, P. putida and E. coli*). Earlier studies regarding time dependent killing of antimicrobial proteins/peptides showed to be very rapid and most of them exert their antimicrobial effects within minutes of exposure to microbes. The peptides such as magainin 2 (Zasloff, 1987), cecropin P1 (Boman *et al.*, 1993), PR-39 (Boman *et al.*, 1993) and SMAP29 (Kalfa, 2001), kill bacteria in 15-90 minutes. Regardless of the time required, or the specific antimicrobial mechanism, specific steps must occur to induce bacterial killing (Zhao *et al.*, 2001) and the rapid killing process has been most plausibly attributed to the action of these peptides on the bacteria and fungi membrane (Otvos, 2002; Lehrer *et al.*, 1993). Our observation is well in agreement with earlier observation of time killing by various antimicrobial proteins/peptides. Both the antibacterial and antifungal activity assay confirmed the antimicrobial potential of NOP-51.

Although hundreds of antimicrobial peptides/proteins have been isolated from various sources so far, only a limited number of them have been investigated extensively for their mode of action. A variety of techniques such as microscopy, model membranes, ion channel formation, fluorescent dye, NMR and others, have been used to assess the mechanisms of antimicrobial peptide activity. However, each method provides a slightly different view of peptide activity and no single technique is capable of adequately

determining the mechanism of action of the peptides. Although the exact mechanism of action of antimicrobial proteins/peptides remains a matter of controversy, there is a consensus that they selectively disrupt the cell membranes (Kalfa, 2001; Boman *et al.*, 1993; Zhao *et al.*, 2001). Several reports regarding the study and understanding of antimicrobial proteins/peptides mode of action against various bacteria (Kalfa, 2001; Boman *et al.*, 1993) and fungi (Lee *et al.*, 2002) have appeared in last few years.

To get insight into the possible mechanism of NOP-51 antimicrobial action, the effects of NOP-51 on bacterial surface morphology and anatomical changes, was investigated using SEM and TEM. The SEM and TEM observation of NOP-51 treated bacteria presented in this study clearly confirm its potent bactericidal effect. The protein found to have remarkable changes in the cell surface morphology in all tested bacteria. SEM micrographs results suggest that biochemical activities occurring at cell wall and membrane. It indicated that NOP-51 certainly had some activity on the cell surface that resulted in morphological abnormalities. The exposure to sub-lethal and lethal doses of NOP-51 make the cell surface irregular, rough and clustering of damaged cells took place in all bacteria used in the present study. The clustering of the damaged cells may be due to release of cell surface polysaccharides. Scanning and transmission electron microscopy have been used to demonstrate the damaging effects of antimicrobial peptides on the ultra structure of microbial cells (Lee *et al.*, 2002; Mangoni *et al.*, 2004). Microscopic analyses have shown that different antimicrobial peptides have different effects on microbial cells, which indicates that different peptides have different target sites or mechanisms of activity. Alteration in surface morphology of bacterial cells after exposure to antimicrobial proteins/peptides such as sarcotoxin I (Kanai and Natori, 1990), melitin

(Matsuzaki *et al.*, 1997), SMAP-29 (Kalfa, 2001), magainin (Zasloff, 1987) and LL-37 (Agarbath *et al.*, 1995), have been observed and almost in all these cases blebbing and blistering of the outer membrane was seen. However, in contrast to the above mentioned antimicrobial proteins/peptides blebs formation on the bacterial surface was not observed in present study. The NOP-51 effects were more similar to temporin-L action reported earlier on *E. coli* (Mangoni *et al.*, 2004). TEM micrographs of NOP-51 treated bacterial cells (*S. aureus, B. substilis, P. putida and E. coli*) indicated that ultra structural changes took place after exposure to NOP-51 and thinning and deformity in cell wall, retraction and irregularity in membranes and its detachment from cell wall could be seen in all bacteria. These observations were similar to the earlier reports (van Dijk *et al.*, 2007; Park *et al.*, 2006; Sahl *et al.*, 2004; Agarbath *et al.*, 1995; Matsuzaki *et al.*, 1997). All these study demonstrate that NOP-51 has different mode of action than the above mentioned antimicrobial peptides. Though the exact mechanism of action of NOP-51 is not clear from the SEM and TEM but it is clearly indicate that its mode of action is cell wall and membrane damage and disruption. Our study supports the earlier SEM and TEM studies observations where the antimicrobial peptides causes disruption of cell walls and membranes which resulted in the release of cell content and ultimately death of cell (Sung and Lee, 2008; Park *et al.*, 2006; Matsuzaki *et al.*, 1997; Agarbath *et al.*, 1995). However, it is not clear yet whether the actual killing of bacteria is the result of one of the followings factors. First due to protein induced membrane permeation either via pore formation or membrane disintegration, or the protein binding to DNA and interferes with DNA and protein synthesis or interact with other intra cellular targets and thus inhibits various vital functions which lead to death.

Almost similar effects were also observed with fungi. As in the case of many other protein/peptides, including cecropin (Silvestro *et al.*, 2000), maganinin II (Zasloff, 1987), all of which exhibited antifungal and antibacterial effects. NOP-51 was anticipated to exhibit antifungal property, hence, its antifungal activity was also tested against broad range of fungi. NOP-51 was found to be a potent antifungal protein as it showed action against various filamentous fungi (*A. fumigatus, A. flavus, A. niger* and *F. oxysporum*) and *C. albicans*. Based on the MICs for each fungi, time killing curve were constructed in order to evaluate the effect of different concentrations of NOP-51. Colony count assays were performed to determine the time required for NOP-51 to kill the various fungi. Like the bacterial studies, NOP-51 at 50 μg/ml concentration killed all fungi within 20-30 min, as no viable counts were seen. This indicates that NOP-51 was effective fungicidal against broad range of fungi (*A. fumigatus, A. flavus, A. niger, F. oxysporum* and *C. albicans*). SEM and TEM studies also clearly showed the fungicidal activity of NOP-51. Although there are many studies of antifungal activity of proteins (Thies *et al.*, 2005; Ng, 2004; Chu et al., 2005; Skerlavaj, 1999; Ye and Ng, 2003), yet there are only limited studied demonstrating their effect on the morphology and ultra structure (Skerlavaj, 1999; Ye and Ng, 2003; Chu *et al.*, 2005). In the present study, the effects of NOP-51 on the cell morphology and ultra structure of (*A. fumigatus, A. flavus, A. niger, F. oxysporum* and *C. albicans*), were investigated using SEM and TEM. Compared to untreated filamentous fungi (*A. fumigatus, A. flavus, A. niger* and *F. oxysporum),* drastic change were observed in surface morphology of hyphae of these fungi treated with different concentration of NOP-51. Completely squashed, severely collapsed, flattening and ultimately dying hyphae were observed in all filamentous fungi (*A. fumigatus, A. flavus,*

A. niger, and *F. oxysporum)* at 30 and 50 µg/ml concentration after 30 min incubation. The hyphae showed lack of cytoplasm, damage, loss of integrity and rigidity of cell wall. These observations indicate that the mode of action of antifungal activity of NOP-51 is a result of its attack on the cell wall and retraction of cytoplasm in the hyphae which ultimately result in hyphal death. Such modification induced by NOP-51 may be related to the interference of NOP-51 with enzymatic reactions of cell wall synthesis, which affect fungal cell wall morphogenesis and growth. *A. fumigatus* and *A. flavus* were found to be more susceptible as deformity in hyphae could be seen even at lower concentration of NOP-51 (10 mg/ml) compared to *F. oxysporum* in which damaged hyphae could be seen only at higher doses. TEM also showed disruption of cell wall and membrane, pore formation can be seen in each case (Fig. 32 and 33). Cell interior look completely necrotic, internal organelles were barely identified and large number of fat globules and vacuoles could be seen. Our observation was similar to previous study where damage of hyphae has been reported (Theis *et al.*, 2005). NOP-51 treatment also caused major changes in *C. albicans* cell surface. SEM micrographs showed that surface of the untreated cell was smooth while those of NOP-51 treated cells were ruffled and became rugged. (Fig. 29 and 30). The NOP-51 caused deformity of cell wall, necrosis of the cytoplasm and accumulation of fat globules on the periphery with few big vacuoles could be seen in TEM. (Fig. 31). A number of antifungal protein were identified and their possible mode of action has been studied and it has been reviewed in detail in a recent article (Theis *et al.*, 2005; Theis and Stahl, 2004; Selitrennikoff, 2001). Cell wall, plasma membrane, ribosome and other intracellular targets have been reported to be the site of action of antifungal proteins. Plasma membrane is the target for majority of the proteins

(Sung and Lee, 2008; Brown *et al.*, 2008; Morsy *et al.*, 2008). The observations were similar to the earlier reports (Sung and Lee, 2008; Brown *et al.*, 2008; Morsy *et al.*, 2008). Though the exact mechanism was not clear from the SEM and TEM studies but it was clearly revealed that cell wall and membrane is the site of action. Thus, all these result and observations confirm that NOP-51 is a potent bactericidal and fungicidal protein.

In addition to their antimicrobial activity, antimicrobial proteins found to have other biological activities like anti-tumor (Ghosh and Maiti, 2007), mitogenic (Fisher and Yang, 2002), cell signaling (Sheu *et al.*, 2004) and immunomodulatory (Liu *et al.*, 2007; Hsu *et al.*, 1997). There are excellent review articles describing their various possible functions and its therapeutic potentials (Otvos *et al.*, 2005; Gordon *et al.*, 2005; Zhang and Falla, 2006). In the present study, besides the evaluation of direct antimicrobial activity against various bacteria and fungi, Immunomodulatory potential of NOP-51 was also evaluated. Efficacy of an antimicrobial drug depends on its both antimicrobial potential as well as the host immune system. It has been suggested long back that at the bottom there is only one genuine scientific treatment for all diseases, and that is to stimulate the phagocytes (Meer, 2003). On the other hand, the response of the host to infection may be deleterious; pro-inflammatory cytokines (e.g. TNF, IL-1, IL-8) and secretary products of WBC (reactive oxygen metabolites, chloramines) are able to produce serious tissue damage. Lysis of microorganisms by host factors (complement, lysosomal enzymes, perforins etc) as well as by antibiotics may increase these deleterious effects. Therefore to cope up with these effects, drug that had immunomodulatory activity

have been searched. In general, immunomodulatory effects of drugs fall into one of the following categories or combinations thereof;

1. Stimulation of the inflammatory response either by increasing the pro-inflammatory cytokines (TNF, IL-1, IL-8) or by augmenting the phagocytes or T-cell function.
2. Inhibition of the inflammatory response either by decreasing the pro-inflammatory cytokines or by inhibiting phagocytes or T-cell function or by inducing apoptosis of inflammatory cells.
3. Stimulation of the counter regulatory ant-inflammatory responses by increasing anti-inflammatory cytokines such as IL-4, IL-10 and TGF-β.
4. Inhibition of the counter anti-inflammatory response by inhibiting an anti-inflammatory cytokines (eg. IL-10, IL-4, TGF-β).

In the present work, the immunomodulatory activity of NOP-51 was evaluated by studying its effect on mice peritoneal macrophage and splenocytes function both under *in vitro* and *in vivo* conditions. Its effect on peritoneal macrophages phagocytic activity, production of TNF-α and NO, and splenocytes proliferation were studied. In mammals, phagocytosis is crucial defense mechanism which protect against pathogen invasions, and apoptotic cell scavenging is performed by phagocytes like macrophages, dendritic cells, and granulocytes (Stuart and Ezekowitz, 2005). Besides, its role in phagocytosis, macrophages play pivotal role in cell mediated immunity due to its antigen processing and presentation ability. Macrophages are known as major immune cells and synthesize a variety of immunomodulatory mediators, including reactive oxygen species and cytokines. The reactive free radical nitric oxide (NO) synthesized by inducible NO

synthase (iNOS), the pro-inflammatory cytokines TNF-α and IL-12, are the major macrophage derived inflammatory mediators and play an important role in immune system (Freman and Natanson, 2000). However, exacerbated production of these mediators have been associated with a range of inflammatory diseases including atherosclerosis (Toutouzas *et al.*, 1998), trauma (Szabo and Thiemermann, 1994), sepsis (Kilbourn *et al.*, 1990), multiple sclerosis (Parkinson *et al.*, 1997) and arthritis (Miyasaka and Hirata,. 1997.). Therefore, to avoid NO overproduction, the use of exogenous modulators becomes essential. A number of plant derived materials have been investigated as inhibitors of NO production (Rimbach *et al.*, 2000; Wang *et al.*, 2000; Ryu *et al.*, 2003).

The anti-inflammatory potency of NOP-51 protein on LPS-induced thioglycollate elicited mouse peritoneal macrophages was investigated in *in vitro* conditions. The NOP-51 showed potent dose dependent inhibitory effect on LPS induced NO production by macrophages. NOP-51 was also found to have dose dependent inhibitory effect on TNF-α production which is a main pro-inflammatory cytokines. TNF-α stimulates the NO production by macrophages. Strong inhibitory effect of NOP-51 in *in vivo* treatment on LPS induced NO and TNF-α by peritoneal macrophages were also observed. Thus NOP-51 was found to be a potential anti-inflammatory agent. Since TNF-α stimulates the NO production and known as main pro-inflammatory cytokine secreted during the early phase of acute and chronic inflammatory diseases, such as asthma, rheumatoid arthritis, septic shock (Tracey and Cerami, 1994), NOP-51 could be a potential anti-inflammatory agent against treatment of these diseases.

Phagocytic activity of NOP-51 was studied by estimating the candidacidal and lysosomal enzyme activities in peritoneal macrophages. Peritoneal macrophages collected from mice administered i.p. with different concentrations of NOP-51 showed marked increase in candidacidal and lysosomal enzyme activities in a dose dependent manner compared to untreated animals. Therefore, NOP-51 showed stimulatory effect on macrophages phagocytosis. The enhanced candidacidal and enhanced transformation of p-NPP to a coloured compound by the membrane associated acid phosphatase activity of the treated macrophages has been used to study the phagotytosis index in earlier studies (Suzuki *et al.*, 1990).

The effect of *in vitro* and *in vivo* treatment of NOP-51 on mouse splenocytes proliferation were also studied in absence or presence of mitogen LPS and Con-A. The NOP-51 had stimulatory effect on splenocytes proliferation alone without mitogens in dose dependent manner both in *in vitro* and *in vivo* conditions. However, co-treatment of NOP-51 either with LPS or Con-A was found to augment the LPS and Con-A induced proliferation. The result clearly indicated that it has stimulatory effect on both T and B-Cell population. Augmentation of Con-A induced IL-2 production by splenocytes of NOP-51 treated cells was also observed. Our observations are in agreement with earlier studies where plant derived components or plant extract showed to have stimulatory effect on splenocytes proliferation (Ha *et al.*, 2006). As cytokines secreted by various immune cells have antagonist or stimulatory effect on each other, it is not clear whether or to what extent the anti-inflammatory response of macrophages and splenocytes proliferation effect on each other has, and to understand this further detail study need to be performed.

It is well known that a large number of stimuli induce NF-kB binding activity, including mitogens such as PHA, Con-A and LPS, cytokines likes TNF-α (Baeuerle and Henkel, 1994). It has been demonstrated in an earlier study that ethanol extract of Glossogyne *tenuifolia* (Taiwanese folk medicine) interfere with LPS and PHA signaling and elicited peritoneal macrophages and splenocytes, respectively, by reducing kB binding activity and it was suggested that the inhibition of inflammatory mediators synthesis may be in part involved in the attenuation of NF-kB activation (Ha *et al.*, 2006). It may be possible that similar thing was happening in NOP-51 anti-inflammatory activity. However, there is no study which confirms the present study, this is merely a speculation.

The *in vitro* and *in vivo* toxicity study demonstrated that NOP-51 did not showed any toxicity as revealed from the cell viability assay, LDH assay and various serum and liver parameters. It further confirms the potential of NOP-51 as a potent antimicrobial and immunomodulatory agent. Although *N. odorum* is well known for its various medicinal values, Oleandrin, a polyphenolic cardiac glycoside from its leaves possessing anti-inflammatory and tumor cell growth-inhibitory effects have been reported (Manna *et al.*, 2000; Stenkvist, 1999). However, this is the first report showing purification of a protein with potent antimicrobial and immunomodulatory activity.

Antimicrobial peptides/proteins (AMP) are considered as attractive, alternative therapies and are basic element of novel new generation of drug for treatment of bacterial, fungal, viral infections and the treatment of cancer. Till now more than 900 different antimicrobial peptides have been identified or predicted from nucleic acid sequences (http:/www.bbcm.univ.trieste.it/ossi/search.htm). Great diversity in

antimicrobial proteins, with large variations in molecular mass, N-terminus sequence and antimicrobial specificity have been observed. Several antimicrobial proteins with a variety of molecular masses have been reported. They can be divided into thaumatin like proteins (Koiwa *et al.*, 1997), glucanases (Beffa and Meins, 1996), chitinases (Watanabe *et al.*, 1999), lipid transfer proteins (Nielsen *et al.*, 1996), ribosome inactivating proteins (Lam *et al.*, 1998; Vivanco *et al.*, 1999), trypsin inhibitors (Joshi *et al.*, 2000; Kouzuma *et al.*, 2000; Park *et al.*, 2000), cycophilin like proteins (Ostoa-Saloma *et al.*, 2000; Lee *et al.*, 2007) and others. The molecular mass of antimicrobial protein NOP-51 isolated in the present study from *N. odorum*, found to be 51 kDa which is much larger than the majority of the antimicrobial protein mass. However it is much closer to the mass value of 52 kDa antimicrobial proteins panaxagin and quinqueginsin isolated from ginseng (Tzi and Wang, 2001). Like the majority of antibacterial and antifungal proteins, NOP-51 also found to be monomeric. However some antimicrobial proteins like those from ginseng (Tzi and Wang, 2001) are reported to be dimeric.

In order to identify the nature of NOP-51, the N-terminal sequence of first 12 amino acids was searched for homology in NCBI databases using BLAST. N-terminal sequence of first 12 amino acids did not show significant similarity with any of the antimicrobial proteins, however showed 33.3% resemblance with antifungal protein from *Arabidopsis thaliana* and *Triticum aestivum.* Besides, the N-terminal sequence of first 12 amino acids was also showed 33.3% resemblance with the superoxide dismutase. The N-terminus sequence homology search result was inconclusive as 12 amino acid residues from a 51 kDa protein was too small and 33% homology shown with mentioned protein is very low. Further identification of NOP-51 with LC-MS/MS analysis provides many

significant new insights into the nature of NOP-51. In total, 28 major peptide fragment were obtained and the search under MSDB for all twenty eight peptides showed 10 hits with different proteins described in results with significant score. The homology search under NCBI BLAST short sequence search for selected peptide fragments showed significant homology to antimicrobial seed protein vicilin, manganese superoxide dismutase and glutathione S-tranferase. Specific peptides sequence homology search showed 72% homology to vicilin, an antimicrobial seed protein isolated from various plants such as *Vicia faba, Pisum sativum, Lupinus albus, Gossypium hirsutum, and Macadamia integrifolia;* 100% homology to Mn superoxide dismutase isolated from *Oryza sativa, Zea mays, Cucumis sativus, Citrullus lanatus, Triticum aestivum, Musa acuminata, Marchantia paleacea, evea brasiliensis, Vitis vinifera, Camellia sinensis, Fagus sylvatica* and more than 90% homology to Gluthione S-tranferase from *Cucurbita maxima, Carica papaya, Lycopersicon esculentum,* and *Vitis vinifera.* However, the high sequence homology match at specific peptide sequence level with vicilin (72%), Mn-superoxide dismutase (100%) and Gluthione S-tranferase (more than 90%), clearly indicates that NOP-51 share domain level homology with these proteins. It is logical that the observed antimicrobial and immunomodulatory activity of NOP-51 in the present study may be due to the presence of these domains. A number of proteins with multifunctional domains have been reported from various sources and these domains attributed to their various biological activities (Ghosh and Maiti, 2007; Wasser, 2002; Winder *et al.*, 1998; Wong and Ng, 2005). Though the present data clearly indicates the basis of the multifunctional activities of the NOP-51, but the exact nature of NOP-51

could not be confirmed with certainty from the present observation and this has to be further analyzed and confirmed using complete *de novo* amino acids sequence data.

Thus a 51 kDa protein named NOP-51 with multi biological activities such as antibacterial, antifungal, anti-inflammatory and immunostimulatory, has been purified from *N. odorum* leaves. This is the first report of purification and identification of an active protein from *N. odorum.* The NOP-51 could be a potential antibacterial, antifungal and immunomodulatory agent and may be very useful therapeutic protein. As it has significantly high activity compared to previously reported antimicrobial proteins and do not have any toxicity both *in vitro* and *in vivo* even at highest tested dose in the present study. Recently, much emphasis has been focused on multifunction therapeutic proteins and a number of pharmaceutical firms globally are developing such therapeutic drugs for various applications and many of them are under clinical trials (De Clercq, 2004). Inimex Pharmaceuticals (Canada) is developing antimicrobial proteins that not only directly kill bacteria but selectively trigger the body's innate defenses without causing inflammation at sub therapeutic doses when compared to conventional drugs. Preliminary results demonstrated that combination therapy of these immunostimulating peptides significantly lowered bacterial load *in vivo* in a murine *S. aureus* infection model (Gordon *et al.*, 2005). The broad spectrum antimicrobial proteins have been used for the development of 'chemical condom' to control the spread of sexually transmitted diseases or imaging probes for bacterial and fungal infections, by exploiting their specificity for microbial membranes (Welling *et al.*, 2004). Though the immunomodulatory potential of NOP-51 in present study has been confirmed in both *in vitro* and *in vivo* conditions, but its various antibacterial and antifungal potential needs to be tested in suitable *in vivo* bacterial and fungal disease model.

CHAPTER 6

SUMMARY

Nerium odorum is well known for its various medicinal values such as anticancer, anti-inflammatory, immunomodulatory and it has been used in leprosy and other diseases of scaly nature. Not much study has been done on the identification of its active constituents except an immunomodulatory active acidic pectic polysaccharide and oleandrin, a polyphenolic cardiac glycoside from the leaves possess anti-inflammatory and tumor cell growth-inhibitory effects. No studies on biological active protein/peptides have been carried so far. In the present work, an attempt has been made to purify a potent antimicrobial and immunomodulatory active protein from *N. odorum* leaves. The work is summarized under following sections

Purification and characterization

Total protein from the fresh leaves of *N. odorum* was extracted using extraction buffer and the protein with antimicrobial activity was purified by ammonium sulfate fractionation, followed by Sephadex G-100 gel filtration and CM-cellulose cationic exchange chromatography. Finally, a 51 kDa protein having potent antibacterial and antifungal activity was obtained. In order to check the purity, purified protein was separated on 12% SDS-PAGE and native-PAGE. A single band of 51 kDa was observed without any contaminating protein both in SDS and native PAGE of coomassie stained gel. Thus it was found to be a monomeric protein of molecular mass 51 kDa. This 51 kDa purified protein from *N. odorum* leaves was named as *Nerium odorum* Protein-51 (NOP-51). The purity of the NOP-51, an antimicrobial protein was also checked by silver staining. Increasing concentration of purified protein was loaded in each well and the gel after electrophoresis was stained with silver nitrate. Silver staining does not showed any other contaminating protein even at higher concentrations. The purity of the purified protein (NOP-51) was also checked by RP-HPLC and MALDI TOF. A single sharp peak was obtained both in RP-HPLC and MALDI TOF, which further confirmed its purity. Molecular

mass of the NOP-51 was also determined by MADLI-TOF and was found to be 51.4 kDa. This was almost similar as revealed from SDS-PAGE, in which molecular weight of the protein was found to be around 51 kDa. Though the MALDI-TOF result seems to be more accurate usually, however, for simplicity mass 51 kDa has been used throughout the text.

Evaluation of antimicrobial activity of NOP-51

Evaluation of antibacterial activity

Antibacterial susceptibility of the crude protein and purified protein (NOP-51) was assayed against Gram positive (*S. aureus* and *B. substilis*) and Gram negative bacteria (*E. coli* and *P. putida).* Both crude protein and NOP-51 showed broad spectrum antibacterial activity as acted against all the tested bacteria. NOP-51 was found to be more effective as significant antibacterial activity was observed against most of the bacteria even at much lower concentration in comparison to crude protein which showed antibacterial activity at relatively higher concentrations. There found to be little differences in the susceptibility among different bacteria. Minimum inhibitory concentration (MIC) of NOP-51 against *S. aureus* and *E. coli* was 12µg/ml and 25µg/ml for *B. substilis* and *P. putida.* MBC of NOP-51 against *S. aureus, B. substilis, P. putida* and *E. coli* was found to be 25, 50, 50 and 50 µg/ml, respectively and it was very close to reference antibiotic gentamicin. IC_{50} of NOP-51 for bacterial growth was determined and it was found to be 15.2, 18.4, 19.3 and 16.0 µg/ml for *P. putida, S. aureus, B. substilis* and *E. coli,* respectively. The SEM and TEM micrographs of control and treated bacteria with sub lethal (10 & 30 µg/ml) and lethal concentration (50 µg/ml) for 30 min, clearly indicated that protein treatment cause deformity in cell wall and membrane. All tested bacteria showed wide range of significant abnormalities like surface roughing, shape deformity, cell clustering and damaged

cell debris in the protein treated cells while untreated cells were in normal shape and smooth surface.

Evaluation of antifungal activity

The *in vitro* antifungal activity of crude protein and NOP-51 against several filamentous fungi (*A. fumigatus, A. flavus, A. niger* and *F. oxysporum*) and *C. albicans* were tested using disk diffusion method. Similar to the antibacterial activity, both crude protein and NOP-51 showed significant antifungal activity against all the tested fungi as could be seen from inhibition zone formation. The NOP-51 was more effective as compared to crude protein, 25 µg/disk of NOP-51 and 40 µg/disk of crude protein was found to be most effective concentrations. The zone of inhibition at 30 µg/disk of fluconazole and 30 µg/disk of NOP-51 against these fungi were quite comparable and in some cases, NOP-51 found to be even better. The MIC of NOP-51 against *A. fumigatus, A. flavus, A. niger, F. oxysporum* and *C. albicans* were 25, 25, 25, 50 and 12 µg/ml, respectively, while in case of crude protein it was 50, 25, 50, 75 and 25 µg/ml, respectively. The MFC of NOP-51 against most of the fungi was found to be 50 µg/ml except for *C. albicans,* which had 25 µg/ml. The MFC of crude protein against these fungi were 50, 50, 75, 75 and 50 µg/ml, respectively. The MIC and MFC of NOP-51 against the above mentioned fungi were almost similar as that of fluconazole and in some cases it was found to be lower than fluconazole. The IC_{50} of NOP-51 against *A. fumigatus, A. flavus, A. niger, F. oxysporum* and *C. albicans* found to be 19.3, 15.1, 18.3, 20 and 18.3 µg/ml, respectively.

Both SEM and TEM micrographs of *A. fumigatus, A. flavus, A. niger, F. oxysporum* and *C. albicans,* control and cells treated with different concentration of NOP-51 for 30 min indicated that NOP-51 treatment cause drastic alteration and deformity in cell wall and membrane of all fungal hyphae as compared to control, whose hyphae were smooth, normal in

shape without any alteration in morphology. Even at lower concentration (10 μg/ml) treatment for 30 min caused significant changes in *A. fumigatus, A. flavus, A. niger, F. oxysporum.* However, drastic changes could be seen at higher concentration (30 and 50 μg/ml) and collapsing damaged hyphae could be easily seen in all fungi. Similar observation was also made in *C. albicans* where untreated cells were smooth and normal shapes while the protein treated cells surface became rough and irregular in shape. The cells with damaged cell wall forming aggregates could be seen at 50 μg/ml protein concentration TEM micrographs of control and protein treated *C. albicans* cells indicated that the walls of untreated cells were intact and regular in shape while those of treated cells were irregular in outline, without showing deposition of membranous material in the wall as observed in the control cultures. The treated cell interior looked completely necrotic; internal subcellular organelles were barely identified.

Antimicrobial activity of the NOP-51 was also tested by treating protein at different temperature up to 100°C for 20 min. The antibacterial activity was retained up to 75% and 50%, at 70°C and 80°C, respectively. However, it was drastically reduced at 100°C. The effect of one month storage of NOP-51 at room temperature on antimicrobial activity was also studied, 100% activity was observed on 4^{th} day, 50% and 5 % activity was retained on 20^{th} and 30^{th} day, respectively. The antimicrobial activity was stable in between pH 5 and 9, but remarkably reduced at pH 10.

Evaluation of immunomodulatory activity of NOP-51

Besides, antimicrobial activity the immunomodulatory activity of the NOP-51 was also evaluated both under *in vitro* and *in vivo* treatment conditions in Swiss albino mice. The effect of NOP-51 in *in vitro* and *in vivo* treatments on macrophages and splenocytes functions were studied. The levels and persistence of pro-inflammatory cytokines and NO play an important

role in determining the extent of inflammation. The effect of NOP-51 on the release of inflammatory mediators like NO and TNF-α in activated elicited mouse peritoneal macrophages and splenocytes proliferation was investigated.

Both *in vitro* and *in vivo* treatment of NOP-51 found to have effect on NO production. Treatment of macrophages with protein (25-150 μg/ml) alone did enhance nitrite production in unstimulated peritoneal macrophages to a low level but significant at higher concentration. There found to be substantial increase in nitrite production when cells were activated with LPS (5 μg/ml) for 24 h. However, the co-treatment of peritoneal macrophages with LPS (5 μg/ml) and different concentrations of protein (25-150 μg/ml) evoked a significant inhibition of nitrite release in a concentration dependant manner.

Similarly both *in vitro* and *in vivo* treatment of NOP-51 found to have effect on TNF-α release in peritoneal macrophages. The treatment of macrophages with protein (25-150 μg/ml) alone did not have much effect on TNF-α production in unstimulated peritoneal macrophages however at higher concentrations, little enhancement was observed as compared to control. On the other hand, stimulation of cells with LPS (5 μg/ml) induced elevated release of TNF-α but concentration dependent inhibition of TNF-α production was observed when peritoneal macrophages were co-treated with LPS (5 μg/ml) for 24 h and different concentrations of protein (25-150 μg/ml). Nearly 50% inhibition was observed at protein concentration of 100-150 μg/ml. The cells viability found to be greater than 95% at these protein concentrations as checked by Trypan blue dye exclusion method. Thus, the observed inhibition of TNF-α and NO production was not due to cell death.

The effect of purified protein (NOP-51) *in vitro* and *in vivo* treatment on splenocytes proliferation in presence or absence of mitogens Con-A and LPS were also evaluated. In both *in*

vitro and *in vivo* treatment protein alone demonstrated a strong stimulation of splenocytes proliferation as compared to untreated cells and a dose response relationship was observed. Con-A and LPS treatment of untreated cells caused the stimulation of splenocytes proliferation by activating T and B lymphocytes, respectively. However, in cells co-treated with Con-A and NOP-51 or LPS and NOP-51, there were augmentation of Con-A and LPS stimulatory effects in a dose dependant manner and it was highest at 150 µg/ml concentration. Thus NOP-51 has stimulatory effects on splenocytes proliferation alone and has synergistic effects on Con-A and LPS induced proliferations.

Since NOP-51 has stimulatory effect on splenocyte proliferation and augmented the Con-A activated proliferation. The IL-2 release by splenocytes in control and protein treated animals groups in the presence and absence of Con A was assayed. The i.p. administration of NOP-51 had a stimulatory effect on IL-2 release as compared to control (untreated animals group) but the overall stimulation was low. Con-A treatment of splenocytes of control group has pronounced effect on IL-2 release. Similar to splenocytes proliferation, the cells from treated group were co-treated with Con-A and have augmented the stimulation on IL-2 release by splenocytes in dose dependent manner. Thus is has synergistic effect on IL-2 production.

In vitro cytotoxicity

During experimentation in the 24 h treatment of peritoneal macrophage and splenocytes cultures with different concentration of protein (25-150 µg/ml), no cytotoxicity was observed at the highest concentration (150 µg/ml) by Trypan blue dye exclusion method. All protein concentration treatment did not cause any cell death with the percentages of viability of macrophages and splenocytes were more than 97 and 96%, respectively.

Effect on body weight, lymphoid organ weight

Different concentrations of purified protein (NOP-51) were administered in swiss male albino mice i.p. for 15 consecutive days, in a dose volume was 200 µl. Animals were divided into six groups (Group I: Control (50 mM Tris-HCl pH 8.0); Group II: 25 µg/ml; Group III: 50 µg/ml; Group IV: 100 µg/ml; Group V: 150 µg/ml). None of the protein doses showed any type of toxicity or mortality in the treated animals. No significant body weight differences were recorded in the various groups of animals. No effect was observed in the spleen, thymus and liver weight at any dose compared with control.

It is clear from the *in vitro* and *in vivo* study that NOP-51, a potent anti-inflammatory agents and it inhibited significantly the production of pro-inflammatory cytokines like TNF-α and NO by mitogen induced macrophages. Besides, it also had stimulatory effects on splenocytes (T & B lymphocytes) proliferation. Thus, it affects both the non-specific and specific immune system. However, the immune response is a complex and net effect depends on kind of subset of non-specific and specific cell activation and subsequent release of type of cytokines. In order to understand the precise mechanism, there is need for further investigation to identify subset of cell population activated for example Th1 or Th2 using cell surface specific monoclonal antibodies and the set of cytokines release.

Toxicological investigations

No Toxicity was confirmed after Lactate dehydrogenase cytotoxicity assay in cell culture supernatant of macrophages and splenocytes as compared to control. Total RBC, WBC, hemoglobin, blood urea, bilirubin, SGPT, SGOT and serum protein level did not show any appreciable change. There was no significant elevation was observed in the level of SGPT, SGOT, urea, bilirubin, and serum protein at any of the doses used in this study.

Identification of NOP-51 by N-terminus amino acid sequence and LC-MS/MS

The 12 N-terminal amino acid sequence of the purified protein NOP-51 was determined by Edman degradation and found to be Ala-Pro-Ala-Tyr-Asn-Tyr-Gly-Val-Ala-Val-Tyr-Glu. NCBI BLAST short sequence homology search of the N-terminal sequence of first 12 amino acids did not show significant similarity with any of the antimicrobial proteins; however, showed 33.3 % resemblance with antifungal protein from *Arabidopsis thaliana* and *Triticum aestivum*. Since NOP-51 has strong inhibition on LPS induced NO production by macrophages and also found to have antioxidant activity in preliminary study. The N-terminal sequence of first 12 amino acid was also compared with antioxidant protein present in NCBI database using BLAST. The search showed homology with various superoxide dismutase in first 12 amino acids but it was very low 33.3 % only. The N-terminus sequence homology search result was inconclusive as 12 amino acid residues from a 51 kDa protein is too small and 33% homology shown with mentioned protein is very low. The partial digest internal amino acid sequencing of 51 kDa protein was analyzed by LC-MS/MS analysis, twenty eight peptides of varying length were obtained studies after tryptic digestion. These peptides exhibited similarities to the sequences from Manganese superoxide dismutase (SOD) enzyme, Glutathione S-transferase, Vicilin, Heat shock protein 17, Caleosin, membrane protein MP27, Protein PV100 using mass spectrometery database (MSDB). Though the short sequence base search of a specific peptide showed high homology to the above mentioned proteins but overall homology was not that high. Therefore, there is need for further investigation using complete *de novo* sequence of protein in precise identification the NOP-51.

Conclusion

A potent antimicrobial and immunomodulatory protein from the leaves of *N. odorum* has been purified and named as NOP-51. This is the first reports of purification and identification of an active protein from *Nerium odorum*. The molecular mass of the protein was determined to be 51 kDa by SDS-PAGE and MALDI-TOF. Purified protein found to be monomeric as a single band of 51 kDa obtained on both native-PAGE and SDS-PAGE. The NOP-51 was found to have a potent broad spectrum antibacterial and antifungal activity as it acted on both Gram positive (*S. aureus and B. substilis)* and Gram negative bacteria (*P. putida and E. coli)* and fungi (*C. albicans, A. fumigatus, A. flavus, A. niger* and *F. oxysporum).* SEM and TEM indicated that NOP-51 has major effects on bacterial and fungal cell wall and membrane which lead to the pore formation and ultimately cell lysis. Purified protein (NOP-51) also exhibited strong immunomodulatory activity *in vitro* and *in vivo,* and found to affect both non-specific as well as specific immune system. It was found to be a strong anti-inflammatory agent. Besides, it has stimulatory effect of its own and also augmented the Con-A and LPS activation. Protein did not exhibit any toxicity in *in vitro* cell assay and in mice at the all tested concentrations. The 12 amino acids N-terminal sequence and the partial internal amino acid sequencing by LC-MS/MS, homology search with NCBI and MSDB databases using BLAST and MASCOT showed match with antimicrobial protein and superoxide dismutase, Glutathione S-transferase, Heat shock protein 17, Caleosin, membrane protein MP27 and Protein PV100. The over all homology of NOP-51 with each of these proteins was poor (33.3%) which clearly indicates that at the complete sequence level NOP-51 differ a lot and thus it seems to be new protein. However, the short sequence homology search of specific peptides showed significant match to vicilin (72%), an antimicrobial seed protein isolated from various plants; (100%) with superoxide dismutase;

and more than 90% homology to Gluthione S-tranferase present in various plants, clearly indicates that NOP-51 share domain level homology with these proteins. It is logical that the observed antimicrobial and immunomodulatory activity of NOP-51 in present study may be due to presence of these domains. Though the present data clearly indicates the basis of multifunctional activities of the NOP-51, but the exact nature of NOP-51 could not be confirmed with certainty from the present observation and this to further analyzed and confirm using complete *de novo* amino acids sequence data.

REFERENCES

Afolayan A.J. and Meyer J.J.M. 1997. The antimicrobial activity of 3,5,7-trihydroxyflavone isolated from the shoots of *Helichrysum aureonitens*. *J. Ethnopharmacol.* **57**: 177-181.

Agarbath B., Gunne H., Odeberg J., Kogner P., Boman H.G. and Gudmudsson G.H. 1995. FALL-39, a putative human petide antibiotic, is cycteine free and expressed in bone marrow and testes. *Proc. Natl. Acad. Sci. USA.* **92**: 195-199.

Agrawal G.K., Jwa N.S. and Rakwal R. 2000. A novel rice (*Oryza sativa L.*) acidic PR1 gene highly responsive to cut, phytohormones, and protein phosphatase inhibitors. *Biochem. Biophys. Res. Commun.* **274**: 157–165.

Ahmad I., Perkins W.R., Lupan D.M., Selsted M.E. and Janoff A.S. 1995. Liposomal entrapment of the neutrophil-derived peptide indolicidin endows it with *in vivo* antifungal activity. *Biochim. Biophys. Acta* **1237**: 109-114.

Ahmed A., Sesti F., Ilan N., Shih T.M., Sturley S.L. and Goldstein S.A. 1999. A molecular target for viral killer toxin: TOK1 potassium channels. *Cell* **99**: 283-291.

Akinpelu D.A. and Onakoya T.M. 2006. Antimicrobial activities of medicinal plants used in folklore remedies in south-western. *African J. of Biotech.* **5(11)**:1078-1081.

Almeida M.S., Cabral K.M., Zingali R.B. and Kurtenbach E. 2000. Characterization of two novel defense peptides from pea (*Pisum sativum*) seeds. *Arch. Biochem. Biophys.* **378**: 278-286.

Amaral J.A., Ekins A., Richards S.R. and Knowles R. 1998. Effect of selected monoterpenes on methane oxidation, denitrification, and aerobic metabolism by bacteria in pure culture. *Appl. Environ. Microbiol.* **64**: 520-525.

Arias F.J., Rojo M.A., Ferreras J.M., Iglesias R., Munoz R., Soriano F., Mendez E., Barbieri L. and Girbes T. 1994. Isolation and characterization of two new N-glycosidase type-1 ribosome-inactivating proteins, unrelated in amino-acid sequence, from *Petrocoptis* species. *Planta* **194**: 487-491.

Aureli P., Costantini A. and Zolea S. 1992. Antimicrobial activity of some plant essential oils against *Listeria monocytogenes*. *J. Food Prot.* **55**: 344-348.

Baeuerle P.A. and Henkel T. 1994. Function and activation of NF-kappa B in the immune system. *Ann. Rev. of Immunol.* **12**: 141-179.

Baker M.A., Maloy W.L., Zasloff M. and Jacob L.S. 1993. Anticancer efficacy of Magainin 2 and analogue peptides. *Cancer Res.* **53**: 3052-3057.

Bals R., Weiner D.J., Moscioni A.D., Meegalla R.L. and Wilson J.M. 1999. Augmentation of innate host defense by expression of a cathelicidin antimicrobial peptide. *Infect. Immun.* **67**: 6084-6089.

Barbieri L., Batelli M.G. and Stirpe F. 1993. Ribosome-inactivating proteins from plants. *Biochim. Biophys. Acta* **1154**: 237-282.

Barre J.T., Bowden B.F., Coll J.C., Jesus J., Fuente V.E., Janairo G.C. and Ragasa C.Y. 1997. A bioactive triterpene from *Lantana camara*. *Phytochemistry* **45**: 321-324.

Batista O. Duarte A., Nascimento J. and Simones M.F. 1994. Structure and antimicrobial activity of diterpenes from the roots of *Plectranthus hereroensis*. *J. Nat. Prod.* **57**: 858-861.

Bechinger B. 1999. The structure, dynamics and orientation of antimicrobial peptides in membranes by multidimensional solid-state NMR spectroscopy. *Biochim. Biophys. Acta* **1462**: 157-183.

Beffa R. and Meins F. Jr. 1996. Pathogenesis-related function of plant β-1,3-glucanases investigated by antisense transformation-a review. *Gene* **179**: 97-103.

Berkada B. 1978. Preliminary report on warfarin for the treatment of herpes simplex. *J. Irish Coll. Phys. Surg.* **22**(Suppl.): 56-63

Bindschedlera L.V., Whitelegge J.P., Millar D.J. and Bolwell G.P. 2006. A two component chitin-binding protein from French bean association of a proline-rich protein with a cysteine-rich polypeptide. *FEBS Lett.* **580:** 1541-1546.

Blanco-Labra A. and Iturbe-Chinas F.A. 1980. Purification and characterization of an α-amylase inhibitor from maise (*Zea maize*). *J. Food Biochem.* **5**: 1-17.

Bloch C. Jr., Patel S.U., Baud F., Zvelebil M.J., Carr M.D., Sadler P.J. and Thornton J. M. 1998. ^{1}H NMR structure of an antifungal γ-thionin protein SIa1: similarity to scorpion toxins. *Proteins* **32**: 334-349.

Boman H.G. 1995. Peptide antibiotics and their role in innate immunity. *Annu. Rev. Immunol.* **13**: 61-92.

Boman H.G., Agerberth B. and Boman A. 1993. Mechanisms of action on *Escherichia coli* of cecropin P1 and PR-39, two antibacterial peptides from pig intestine. *Infect. Immun.* **61**: 2978-2984.

Borgmeyer J.R., Smith C.E. and Huynh Q.K. 1992. Isolation and characterization of a 25 kDa antifungal protein from flax seeds. *Biochem. Biophys. Res.* Commun. **187**: 480-487.

Borris R.P. 1996. Natural products research: perspectives from a major pharmaceutical company. *J. Ethnopharmacol.* **51**: 29-38.

Bradford M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248-254.

Breukink E. and De Kruijff B. 1999. The lantibiotic nisin, a spezial case or not? *Biochim. Biophys. Acta* **1462**: 223-234.

Brodgen K.M. 2005. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria. *Nature* **3**: 238-250.

Broekaert W.F., Cammue B.P.A., De Bolle M.F.C., Thevissen K., De Samblanx G.W. and Osborn R.W. 1997. Antimicrobial peptides in plants. *Crit. Rev. Plant Sci.* **16**: 297-323.

Broekaert W.F., Terras F.R., Cammue B.P. and Osborn R.W. 1995. Plant defensins: novel antimicrobial peptides as components of host defense system. *Plant Physiol.* **108**: 1353-358.

Brotz H., Josten M., Wiedemann I., Schneider U., Gotz F. and Bierbaum G. 1998. Role of lipid-bound peptidoglycan precursors in the formation of pores by nisin, epidermin and other lantibiotics. *Mol. Microbiol.* **30**: 317-327.

Brown G.D. and Gordon S. 2003. Fungal b-glucans and mammalian immunity. *Immunity* **19**: 311-315.

Brown S.E., Howard A., Kasprzak A.B., Gordon K.H. and East P.D. 2008. The discovery and analysis of a diverged family of novel antifungal moricin-like peptides in the wax moth *Galleria mellonella*. *Insect Biochem. Mol. Biol.* **38(2)**: 201-212.

Butler M.S. and Buss A.D. 2006. Natural products - The future scaffolds for novel antibiotics? *Biochem. Pharmacol.* **71**: 919-929.

Cammue B.P.A., Thevissen K., Hendriks M., Eggermont K., Goderis I.J. and Proost P. 1995. A potent antimicrobial protein from onion seeds showing sequence homology to plant lipid transfer proteins. *Plant Physiol* **109**: 445-455.

Carlsson A., Engstrom P., Palva E.T and Bennich H. 1991 Attacin, an antibacterial protein from *Hyalophora cecropia*, inhibits synthesis of outer membrane proteins in *Escherichia coli* by interfering with *omp* gene transcription. *Infect. Immun.* **59:** 3040-3045.

Casteels P., Ampe C., Jacobs F. and Tempst P. 1993. Functional and chemical characterization of hymenoptaecin, an antibacterial polypeptide that is infection-inducible in the honeybee (*Apis mellifera*). *J. Biol. Chem.* **268**: 7044-7054.

Chabot S., Bel-Rhlid R., Chenevert R. and Piche Y. 1992. Hyphal growth promotion *in vitro* of the VA mycorrhizal fungus, *Gigaspora margarita* Becker and Hall, by the activity of structurally specific flavonoid compounds under CO_2-enriched conditions. *New Phytol.* **122**: 461-467.

Chaly Y.V., Paleolog E.M., Kolesnikova T.S., Tikhonov I.I., Petratchenko E.V. and Voitenok N.N. 2000. Neutrophil alpha-defensin human neutrophil peptide modulates cytokine production in human monocytes and adhesion molecule expression in endothelial cells. *Eur Cytokine Netw.* **11**: 257-266.

Chambers H.F. 2001. The changing epidemiology of *Staphylococcus aureus*? *Emerg. Infect.* Dis. **7**: 178-182.

Charp P.A., Rice W.G., Raynor R.L., Reimund E., Kinkade Jr.J.M., Ganz T., Selsted M.E., Lehrer R.I. and Kuo J.F.. 1988 Inhibition of protein kinase C by defensins, antibiotic peptides from human neutrophils. *Biochem. Pharmacol.* **37**: 951-956.

Chen Z.Y., Brown R.L., Lax A.R., Guo B.Z., Cleveland T.E., and Russin J.S. 1998. Resistance to *Aspergillus flavus* in corn kernels is associated with a 14-kDa protein. *Phytopathology* **88**: 276-281.

Cheong N.E., Choi Y.O., Kim W.Y., Bae I.S., Cho M.J., Hwang, I. Kim J.W. and Lee S.Y. 1997. Purification and characterization of an antifungal PR-5 protein from pumpkin leaves. *Mol. Cells* **7**: 214-219

Chernin L.S., De la Fuente L., Sobolev V., Haran S., Vorgias C.E., Oppenheim A.B. and Chet I. 1997. Molecular cloning, structural analysis, and expression in *Escherichia coli* of a chitinase gene from *Enterobacter agglomerans*. *Appl. Environ. Microbiol.* **63**: 834-839.

Chernin L.S., De la Fuente L., Sobolev V., Haran S., Vorgias C.E., Oppenheim A.B., and Chet I. 1997. Molecular cloning, structural analysis, and expression in *Escherichia coli* of a chitinase gene from *Enterobacter agglomerans*. *Appl. Environ. Microbiol.* **63**: 834-839.

Chesnokova L.S., Slepenkov S.V. and Witt S.N. 2004. The insect antimicrobial peptide, L-pyrrhocoricin, binds to and stimulates the ATPase activity of both wild-type and lidless DnaK. *FEBS Lett.* **565**: 65-69.

Chilosi G., Caruso C., Caporale C., Leonardi L., Bertini L. and Buzi A. 2000. Antifungal activity of a Bowman–Birk type trypsin inhibitor from wheat kernel. *J Phytopathol* **148**: 477-481.

Chin Y.W., Balunas M.J., Chai H.B. and Kinghorn A.D. 2006. Drug discovery from natural sources. *The AAPS Journal* **8(2)**: E239-E53.

Chopra I., Hodgson J., Metcalf B. and Poste G. 1997. The search for antimicrobial agents effective against bacteria resistant to multiple antibiotics. *Antimicrob. Agents and Chemother.* **41(3)**: 497-503.

Chopra R.N., Nayar S.L. and Chopra I.C., 1956. Glossary of Indian Medicinal Plants, New Delhi, Council of Scientific and Industrial Research, 475 p.

Chryssanthou E. and Sjölin J. 2004. Post-antifungal effect of amphotericin B and voriconazole against *Aspergillus fumigatus* analysed by an automated method based on fungal CO_2 production: dependence on exposure time and drug concentration. *J.* Antimicrob.Chemotherap. **54(5)**: 940-943.

Chu K.T. and Ng T.B. 2003. Isolation of a large thaumatin-like antifungal protein from seeds of the Kweilin chestnut Castanopsis chinensis. *Biochem Biophys Res Commun.* **301**: 364-730.

Chu K.T., Xia L. and Ng T.B. 2005. Pleurostrin, an antifungal peptide from the oyster mushroom. Peptides 26(11): 2098-2103.

Classen B., Thude S., Blaschek W., Wack M. and Bodinet C. 2006. Immunomodulatory effects of arabinogalactan-proteins from *Baptisia* and *Echinacea*. *Phytomedicine* **13(9-10)**: 688-694.

Clausen M., Kraute R., Schachermayr G., Potrykus I. and Sautter C.. 1999. Antifungal activity of a virally encoded gene in transgenic wheat. *Nat. Biotechnol.* **18**: 446-449.

Cole A.M., Liao H.I., Ganz T. and Yang O.O. 2003. Antibacterial activity of peptides derived from envelope glycoproteins of HIV-1. *FEBS Letters* **535 (1-3)**: 195-199.

Com E., Bourgeon F., Evrard B., Ganz T., Colleu D., Jegou B. and Pineau C. 2003 Expression of antimicrobial defensins in the male reproductive tract of rats, mice, and humans. *Biol. of Rep.* **68**: 95-104.

Cordell GA 1981. Introduction to the alkaloids: Biogenetic approach. John Willey and Sons. New York. 1055 p.

Costerton J.W., Stewart P.S. and Greenberg E.P. 1999. Bacterial biofilms: a common cause of persistent infections. *Science* **284**: 1318-1322.

Cotter P.D., Hill C. and Ross R.P. 2005. Bacteriocins: developing innate immunity for food. *Nat. Rev. Microbiol.* **3**: 777-782.

Cowan M.M. 1999. Plant products as antimicrobial agents. *Clin. Microbiol. Rev.* **12(4)**: 564-582.

Dagan A., Efron L., Gaidukov L., Mor A. and Ginsburg H. 2002. *In vitro* antiplasmodia effects of dermasceptin S derivatives. *Antimicrob. Agents Chemother.* **46**: 1059-1066.

Dahanukar S.A., Kulkarni R.A and Rege N.N. 2000. Pharmacology of medicinal plants and natural products. *Indian J. of Pharmacol.* **32**: S81-S118.

Dahot U.M. 1999. Antibacterial and antifungal activity of small protein of *Indigofera oblongifolia* leaves. *J. Ethnopharmacol.* **64**: 277-282.

Dathe M. 2002. General aspects of peptide selectivity towards lipid bilayers and cell membranes studied by variation of the structural parameters of amphipathic helical model peptides. *Biochim. Biophys. Acta* **1558**: 171-186.

Dathe M. and Wieprecht T. 1999. Structural features of helical antimicrobial peptides: their potential to modulate activity on model membranes and biological cells. *Biochim. Biophys. Acta* **1462**: 71-87.

Davidson B.S. 1995. New dimensions in natural products research: cultured marine microorganisms. *Curr. Opin. in Biotech.* **6(3)**: 284-291.

De Clercq E. 2004. Antiviral drugs in current clinical use. *J. Clin. Virol.* **30**: 115-133.

Devadas S.K. and Raina R. 2002. Preexisting systemic acquired resistance suppresses hypersensitive response-associated cell death in *Arabidopsis hrl1* mutant. *Plant Physiol.* **128**: 1234-1244.

Duke J. A. 1985. Handbook of medicinal herbs. CRC Press, Inc., Boca Raton, Fla. 696 p.

Edelson P.J. and Cohn Z.A.1976. Purification and cultivation of monocytes and macrophageiIn *in vitro* Methods in cell mediated and tumor immunity, Bloom, B.A. and Davis, J.R. (eds). Academic Press, San Diego, **15**: 333-339.

Elsbach P. 2003. What is the real role of antimicrobial polypeptides that can mediate several other inflammatory responses? *J. Clin. Invest.* **111**: 1643-1645.

Espinel-Ingroff A. 2003. Evaluation of Broth Microdilution Testing Parameters and Agar Diffusion Etest Procedure for Testing Susceptibilities of *Aspergillus* spp. to Caspofungin Acetate (MK-0991) *J. Clin. Microbiol.* **41(1)**: 403-409.

Faber C., Stallmann H.P., Lyaruu D.M., Joosten U., von Eiff C., van Nieuw Amerongen A. and Wuisman P.I. 2005. Comparable efficacies of the antimicrobial peptide human lactoferrin 1–11 and gentamicin in a chronic methicillin-resistant *Staphylococcus aureus* osteomyelitis model. *Antimicrob. Agents Chemother.* **49**: 2438-2444.

Fabricant D.S. and Farnsworth N.R. 2001. The Value of plants used in traditional medicine for drug discovery. *Environmental Health Perspectives* **109(1)**: 69-75.

Fernandez M.A., Garcia M.D and. Saenz M.T. 1996. Antibacterial activity of the phenolic acids fraction of *Scrophularia frutescens* and *Scrophularia sambucifolia*. *J. Ethnopharmacol.* **53**: 11-14.

Fessenden R.J. and Fessenden J. S. 1982. Organic chemistry, 2nd ed. Willard Grant Press, Boston, Mass. 1069 p.

Fisher M. and Yang L.X. 2002. Anticancer effects and mechanisms of polysaccharide-K (PSK): implications of cancer immunotherapy. *Anticancer Research* **22(3)**: 1737-1754.

Florach D.E. and Stiekema W.J. 1994. Thionins: properties, possible biological roles and mechanisms of action. *Plant Mol. Biol.* **26**: 25-37.

Fostel J.M. and Lartey P.A. 2000. Emerging novel antifungal agents. *DDT* **5(1)**: 25-32.

Francis J.S., Doherty M.C. and Lopatin U. 2005 Severe community-onset pneumonia in healthy adults caused by methicillin-resistant *Staphylococcus aureus* carrying the Panton-Valentine leukocidin genes. *Clin. Infect. Dis.* **40**: 100-107.

Freeman B.D. and Natanson C. 2000. Anti-inflammatory therapies in sepsis and septic shock. *Expert Opin. on Invest. Drugs.* **9**: 1651-1663.

Freiburghaus F., Kaminsky R., Nkunya M.H.H. and Brun R. 1996. Evaluation of African medicinal plants for their *in vitro* trypanocidal activity. *J. Ethnopharmacol.* **55**: 1-11.

Fritig B., Heitz T. and Legrand M. 1998. Antimicrobial proteins in induced plant defense. *Curr. Opin. in Immunol.* **10(1)**: 16-22

Fujioka T. and Kashiwada Y. 1994. Anti-AIDS agents. 11. Betulinic acid and platanic acid as anti-HIV principles from *Syzigium claviflorum*, and the anti-HIV activity of structurally related triterpenoids. *J. Nat. Prod.* **57**: 243-247.

Futaki S. 2001. Arginine-rich peptides. An abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery. *J. Biol. Chem.* **276**: 5836-5840.

Galkowska H.and Olszewski W.L. 2003. Antimicrobial peptides - their role in immunity and therapeutic potential. *Cen. Eur. J. Immunol.* **28(3)**: 138-142.

Ganz T. 2002. The role of hepcidin in iron sequestration during infections and in the pathogenesis of anemia of chronic disease. *Israeli Med. Assoc. J.* **4(11)**: 1043-1045.

Gazit E., Boman A., Boman H.G. and Shai Y. 1995. Interaction of the mammalian antibacterial peptide cecropin P1 with phospholipid vesicles. *Biochemistry* **34**: 11479-11488.

Geissman T.A. 1963. Flavonoid compounds, tannins, lignins and related compounds, 265 p. *In:* M. Florkin and E. H. Stotz (ed.), Pyrrole pigments, isoprenoid compounds and phenolic plant constituents, vol. 9. Elsevier, New York.

Ghebremichaela K.A., Gunaratnab K.R., Henrikssonc H., .Brumerc H. and Dalhammar G. 2005. A simple purification and activity assay of the coagulant protein from *Moringa oleifera* seed. *Water Res.* **39**: 2338-2344.

Ghosh D. and Maiti T.K. 2007. Imunomodulatory and anti-tumor activities of native and heat denatured Abrus agglutinin *Immunobiology.* **212(7)**: 589-599.

Ghoshal S., Prasad K.B.N. and Lakshmi V. 1996. Antiamoebic activity of *Piper longum* fruits against *Entamoeba histolytica in vitro* and *in vivo*. *J. Ethnopharmacol.* **50**: 167-170.

Girbes T., Citores L., Iglesias R., Ferreras J.M., Munoz R., Rojo M.A., Arias F.J., Garcia J.R., Mendez E. and Calonge M. 1993. Ebulin 1, a nontoxic novel type 2 ribosome-inactivating protein from *Sambucus ebulus* L. leaves. *J. Biol. Chem.* **268**: 18195-18199.

Gordon Y.J., Ramanowaski E.G. and McDermott A.M. 2005. A review of antimicrobial peptides and their therapeutic potential as anti-infective drugs. *Curr. Eye Res. 30*: 505-515.

Gough M., Hancock R.E. and Kelly N.M. 1996. Antiendotoxin activity of cationic peptide antimicrobial agents. *Infect. Immun.* **64**: 4922-4927.

Graham J.S., Burkhart W., and Xiong J. and Gillikin JW. 1992. Complete amino acid sequence of soybean leaf P21-similarity to the thaumatin-like polypeptides. *Plant Physiol.* **98**: 163-165.

Green L.C., Wagner D.A., Gloglowski J., Skipper P.L., Wishnok J.S. and Tannenbaum S.R. 2004. Analysis of nitrate, nitrite and [15N] nitrate in biological fluids. and quantification of cytokines. *J. Immunol. Methods* **284**: 99-106.

Groisman E.A., Kayser J. and Soncini F.C. 1997. Regulation of polymyxin resistance and adaptation to low-Mg^{2+} environments. *J. Bacteriol.* **179**: 7040-7045.

Guerbette F., Grosbois M., Jolliot-Croquin A., Kader J.C. and Zachowski A. 1999. Lipid-transfer proteins from plants: structure and binding properties. *Mol. Cell. Biochem.* **192**: 157-161.

Guleria S. and Kumar A. 2006. Antifungal activity of some Himalayan medicinal plants using direct bioautography. *J. Cell & Mol. Biol.* **5**: 95-98.

Guo B.Z., Cleveland T.E., Brown R.L., Widstrom N.W., Lynch R.E. and Russin J.S. 1999. Distribution of antifungal proteins in maize kernel tissues using immunochemistry. *J. Food Prot.* **62**: 295-299

Ha C., Weng C., Wang L., Lian T. and Wu M. 2006. Immunomodulatory effect of *Glossogyne tenuifolia* in murine peritoneal macrophages and splenocytes *J. of Ethnopharmacol.* **107**: 116-125.

Hancock R.E., Chapple D.S. 1999 Peptide antibiotics. *Antimicrob Agents Chemother.*; **43**: 1317-23.

Hao J.J., Geng C., Xie W., Gong Z., Liu W.Y. and Wang E. 1999. Isolation and characterization of viridin, a new 65 kDa antifungal protein from the mould *Trichoderma viride*. *Biol. Chem.* **380**: 1243-1245.

Hao J.J., Ye J.Q., Yang Q., Gong Z.Z., Liu W.Y. and Wang E. 2000. A silent antifungal protein (AFP)-like gene lacking two introns in the mould *Trichoderma viride*. *Biochim. Biophys. Acta* **1475**: 119-124.

He K., Ludtke S.J., Huang H.W. and Worcester D.L. 1995. Antimicrobial peptide pores in membranes detected by neutron in-plane scattering. *Biochemistry* **34**: 15614-15618.

Heinrich M. 2003. Ethnobotany and Natural Products: The Search for New Molecules, New Treatments of Old Diseases or a Better Understanding of Indigenous Cultures? *Curr. Topics in Med. Chem.* **3**: 29-42.

Hejgaard J., Jacobsen S. and Svendsen I. 1991. Two antifungal thaumatin- like proteins from barley grain. *FEBS Lett.* **291**: 127-131.

Herold B.C., Immergluck L.C. and Maranan M.C. 1998. Community-acquired methicillin-resistant *Staphylococcus aureus* in children with no identified predisposing risk. *JAMA* **279**: 593-598.

Hiraga K., Hayashi S., Kitazawa M. and Oda K. 1999. Isolation and some properties of a novel killer toxin-like protein produced by *Streptomyces* sp. F-287. Biosci. *Biotechnol. Biochem.* **63**: 1037-1044.

Hobta A., Lisovskiy I., Mikhalap S., Kolybo D., Romanyuk S., Soldatkina M., Markeyeva N., Garmanchouk L., Sidorenko S.P. and Pogrebnoy P.V. 2001. Epidermoid carcinoma-derived antimicrobial peptide (ECAP) inhibits phosphorylation by protein kinases in vitro. *Cell Biochem. Funct.* **19**: 291-298.

Hoult J.R.S., and Paya. M. 1996. Pharmacological and biochemical actions of simple coumarins: natural products with therapeutic potential. *Gen. Pharmacol.* **27**: 713-722.

Hsieh K.Y., Hsu C.I., Lin J.K., Tsai C.C. and Lin R.H. 2003. Oral administration of an edible-mushroom-derived protein inhibits the development of food-allergic reactions in mice. *Clin. Exp. Allergy* **33**: 1595-1602.

Hsu H.C., Hsu C.I., Lin R.H., Kao C.L. and Lin J.Y. 1997. Fip-*vvo*, a new fungal immunomodulatory protein isolated from *Volvariella volvacea*. *Biochem. J.* **323**: 557-565.

Hsu S.Y., Hua K.F., Lin C.C., Lin C.H., Hsu J. and Wong, C.H. 2004. Extract of Reishi polysaccharides induces cytokine expression via TRL4-modulated protein kinase signaling pathways. *J. Immunol.* **173**: 5989-5999.

Hu X. and Reddy A.S. 1997. Cloning and expression of a PR5-like protein from *Arabidopsis*: inhibition of fungal growth by bacterially expressed protein. *Plant Mol. Biol.* **34**: 949-959.

Huang H.J., Ross C.R. and Blecha F. 1997. Chemoattractant properties of PR-39, a neutrophil antibacterial peptide. *J Leukoc Biol.* **61**: 624-629.

Huang X., Xie W.J. and Gong Z.Z. 2000 Characterization and antifungal activity of a chitin binding protein from Ginkgo biloba. *FEBS Lett* **478**: 123-126.

Hunter M.D. and Hull L.A. 1993. Variation in concentrations of phloridzin and phloretin in apple foliage. *Phytochemistry* **34**: 1251-1254.

Hurst A. 1981. Nisin. In: Advances in Applied Microbiology, Academic Press, London **27**: 85-123.

Hussain M.A. and Gorsi M.S. 2004. Antimicrobial activity of *Nerium oleander* Linn. *Asian J. of Plant Sci.* **3 (2)**: 177-180.

Huynh Q.K., Bergmeyer J.R. and Zobel J.F. 1992. Isolation and characterization of a 22-kDa protein with antifungal properties from maize seeds. *Biochem Biophys Res Commun.* **182**: 1–5.

Huynh Q.K., Borgmeyer J.R., Smith C.E., Bell L.D. and Shah D.M. 1996. Isolation and characterization of a 30 kDa protein with antifungal activity from leaves of *Engelmannia pinnatifida*. *Biochem. J.* **15**: 723-727.

Hwu L., Huang C.C., Chen D.T. and Lin A. 2000. The action mode of the ribosome-inactivating protein a-sarcin. *J. Biomed. Sci.* **7**: 420-428.

Ibrahim E.H., Sherman G., Ward S., Fraser V.J. and Kollef M.H. 2000. The influence of inadequate antimicrobial treatment of bloodstream infections on patient outcomes in the ICU setting. *Chest* **118**: 146-155.

Iglesias R., Arias F.J., Rojo M.A., Escarmis C., Ferreras J.M. and Girbes T. 1993. Molecular action of the type 1 ribosome-inactivating protein saporin 5 on *Vicia sativa* ribosomes. *FEBS Lett.* **325**: 291-294.

Ignacio S.N.R., Ferreira J.L.P., Almeida M.B. and Claire F. Kubelka C.F. 2001. Nitric oxide production by murine peritoneal macrophages *in vitro* and *in vivo* treated with *Phyllanthus tenellus* extracts. *J. Ethnopharmacol.* **74**: 181-187.

Iijima R., Kurata S. and Natori S. 1993. Purification, characterization, and cDNA cloning of an antifungal protein from the hemolymph of *Sarcophaga peregrina* (flesh fly) *larvae. J. Biol. Chem.* **268**: 12055-12061.

Izgu F., Altinbay D. and Sagiroglu A.K. 1999. Isolation and characterization of the K6 type yeast killer protein. *Microbios* **99**: 161-172.

Jacob L. and Zasloff M. 1994. Potential therapeutic application of magainins and other antimicrobial agents of animal origin. *Ciba Found. Symp.* **186**: 197-223.

Jagadeeswaran G., Raina S., Acharya B.R. Maqbool S.B. Mosher S.L., Appel H.M., Schultz J.C. Klessig D.F. and Raina R. 2007. *Arabidopsis GH3*-like defense gene 1 is required for accumulation of salicylic acid, activation of defense responses and resistance to *Pseudomonas syringae*. *Plant J.* **51**: 234-246.

Janovská D., Kkubíková K. and Kokoška L. 2003. Screening for antimicrobial activity of some medicinal plants species of traditional chinese medicine. *Czech J. Food Sci.* **21**: 107-110.

Jaynes J. M., Burton C.A., Barr S.B., Jeffers G.W., Julian G.R., White K.L., Enright F.M., Klei T.R. and Laine R.A. 1988. *In vitro* cytocidal effect of novel lytic peptides on *Plasmodium falciparum*. *FASEB J.* **2**: 2878-2883.

Joshi B.N., Sainani M.N., Bastawade K.B., Gupta V.S. and Ranjekar P.K. 1998. Cysteine protease inhibitor from pearl millet: a new class of antifungal protein. *Biochem. Biophys. Res. Commun.* **246**: 382-387.

Kaiserer L., Oberparleiter C., Weiler-Görz R., Burgstaller W., Leiter E. and Marx F. 2003. Characterization of the *Penicillium chrysogenum* antifungal protein PAF. *Arch. of Microb.* **180(3)**: 204-210.

Muller C.A., Markovic-Lipkovski J., Klatt T., Gamper J., Schwarz G., Beck H., Deeg M., Kalbacher H., Widmann S., Wessels J.T., Becker V., Muller G.A. and Flad T. 2002 Human alpha-defensins HNPs-1, -2, and -3 in renal cell carcinoma: influences on tumour cell proliferation. *Am. J. Pathol.* **160**: 1311-1324.

Kalfa V.C. 2001. Congeners of SMAP29 kill ovine pathogens and induce ultrastructural damage in bacterial cells. *Antimicrob. Agents Chemother.* **45**: 3256-3261.

Kanai A and Natori S. 1990. Analysis of a gene cluster for sarcotoxin II, a group of antibacterial proteins of *Sarcophaga peregrina*. *Mol Cell Biol.* **10(12)**: 6114-6122.

Kang C.I., Kim S.H. and Park W.B. 2005 Bloodstream infections caused by antibiotic-resistant gram-negative bacilli: risk factors for mortality and impact of inappropriate initial antimicrobial therapy on outcome. *Antimicrob. Agents Chemother.* **49**: 760-766.

Kang S.C., Park S. and Lee D.G. 1999. Purification and characterization of a novel chitinase from the entomopathogenic fungus, *Metarhizium anisopliae*. *J. Invert. Pathol.* **73**: 276-281.

Karlowsky J.A., Draghi D.C., Jones M.E., Thornsberry C., Friedland I.R. and Sahm D.F. 2003. Surveillance for antimicrobial susceptibility among clinical isolates of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* from hospitalized patients in the United States, 1998 to 2001. *Antimicrob. Agents Chemother.* **47**: 1681-1688.

Kawata M., Nakajima T., Yamamoto T., Mori K., Oikawa T., Fukomoto F. and Kuroda S. 2003. Genetic engineering for disease resistance in rice (*Oryza sativa* L.) using antimicrobial peptides. *JARQ* **37(2)**: 71-76.

Kazmi M.H., Malik A., Hameed S., Akhtar N. and Ali S.N. 1994. An anthraquinone derivative from *Cassia italica*. *Phytochemistry* **36**: 761-763.

Kilbourn R., Jubran G. Gross A., Griffith S.S., Levi O.W., Adams R. and Lodato R.F. 1990. Reversal of endotoxin mediated shock by NG-methyl-L-arginine, an inhibitor of nitric oxide synthesis. *Biochem. Biophys. Res. Comm.* **172**: 1132-1138.

Kim Y.J. and Hwang B.K. 1997. Isolation of a basic 34 kDa β-1,3-glucanase with inhibitory activity against *Phytophthora capsici* from pepper stems. *Physiol. Mol. Plant Pathol.* **50**: 103-115.

Kino K., Yamashita A., Yamaoka K., Watanabe J., Tanaka S., Ko K., Shimizu, K. and Tsunoo H., 1989. Isolation and characterization of a new immunomodulatory protein, ling Zhi-8 (LZ-8), from *Ganoderma lucidium*. *J. Biol. Chem.* **264**: 472-478.

Kirsch K., Kensinger M., Hanafusa H. and August A. 2002. A p130Cas tyrosine phosphorylated substrate domain decoy disrupts v-crk signaling. *BMC Cell Biol.* **3**: 18-21.

Kirtikar K.R., Basu B.D. and Sing M.P. 1975. Indian medicinal plants, International book distributors, India: Dehradun, vol II., 1584 p.

Koehn F.E. and Carter G.T. 2005. The evolving role of natural products in drug discovery. *Nat. Rev. Drug Dis.* **4**: 206-220.

Koiwa H., Kato H., Nakatsu T., Oda J., Yamada Y. and Sato F. 1997. Purification and characterization of tobacco pathogenesis-related protein PR-5d, an antifungal thaumatin-like protein. *Plant Cell Physiol.* **38**: 783-791.

Komiyama T., Shirai T., Ohta T., Urakami H., Furuichi Y., Ohta Y. and Tsukada Y.. 1998. Action properties of HYI killer toxin from *Williopsis saturnus* var. *saturnus* and antibiotics, aculeacin A and papulacandin B. *Biol. Pharm. Bull.* **21**: 1013-1019.

Kristensen A.K., Brunsted J., Nielsen J.W., Mikkelsen J.D., Roepstorff P. and Nielsen K.K. 1999. Processing, disulfide pattern, and biological activity of a sugar beet defensin, AX2, expressed in *Pichia pastoris*. *Protein Exp. Pur.* **16**: 377-387.

Kubo I., Muroi H and Himejima M. 1992. Antibacterial activity of totarol and its potentiation. *J. Nat. Prod.* **55:**1436-1440.

Kurome T. 1998. Aureobasidins as new inhibitors of P-glycoprotein in multidrug resistant tumor cells. *J. Antibiot.* **51**: 353-358.

Ladokhin A.S. and White S.H. 2001. 'Detergent-like' permeabilization of anionic lipid vesicles by melittin. *Biochim. Biophys. Acta* **1514**: 253-260.

Laemmli U.K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.

Lam S.S., Wang H. and Ng T.B. 1998. Purification and characterization of novel ribosome inactivating proteins, α- and β-pisavins, from seeds of the garden pea *Pisum sativum*. *Biochem. Biophys. Res. Commun.* **253**: 135-142.

Lam Y.W., Wang H.X. and Ng T.B. 2000. A robust cysteine-deficient chitinase-like antifungal protein from inner shoots of the edible chive *Allium tuberosum*. *Biochem Biophys Res Commun* **279**: 74-80.

Lamberty M., Ades S., Uttenweiler-Joseph S., Brookhart G., Bushey D., Hoffmann J.A. and Bulet P. 1999. Insect immunity. Isolation from the lepidopteran *Heliothis virescens* of a novel insect defensin with potent antifungal activity. *J. Biol. Chem.* **274**: 9320-9326.

Leah R., Tommerup H., Svendsen I. and Mundy J. 1991. Biochemical and molecular characterization of three barley seed proteins with antifungal properties. *J. Biol. Chem.* **266**: 1564-1573.

Leah R., Tommerup H., Svendsen I. and Mundy J. 1991. Biochemical and molecular characterization of three barley seed proteins with antifungal properties. *J Biol Chem* **246**: 1564-1573.

Lee D. G., Kim P.I., Park Y., Park S., Woo E. and Hahm K. 2002. Antifungal mechanism of SMAP-29 (1-18) isolated from sheep myeloid mRNA against *Trichosporon beigelii. Biochem. Biophys. Res Comm.* **295**: 591-596.

Lee J.R., Park S.C., Kim J.Y., Lee S.S., Park Y., Cheong G.W., Hahm K.S. and Lee S.Y. 2007. Molecular and functional characterization of a cyclophilin with antifungal activity from Chinese cabbage. *Biochem. and Biophy. Res. Comm.* **353**: 672-678.

Lee K.H. 2005. Recent advances in the discovery and development of plant-derived chemotherapeutic agents. *Int. J. App. Sci. & Eng.* **3(3)**: 151-155.

Lee K.M., Kim D.H., Lee Y.H., Choi B.S., Chung J.H. and Lee B.L. 1999. Antifungal activities of recombinant antifungal protein by conjugation with polyethylene glycol. *Mol Cells* **9**: 410-416.

Lee S.Y., Moon J.H., Kurata S., Natori S. and Lee B.L. 1995. Purification and cDNA cloning of an antifungal protein from the hemolymph of *Holotrichia diomphalia* larvae. *Biol. Pharm. Bull.* **18**: 1049-1052.

Lee Y.T., Kim K.H., Suh J.Y., Chung J.H., Lee B.L., Lee Y. and Choi B.S. 1999. Structural characteristics of tenecin 3, an insect antifungal protein. *Biochem. Mol. Biol. Int.* **47:** 369-376.

Lehrer R.I. 1989. Interaction of human defensins with *Escherichia coli.* Mechanism of bactericidal activity. *J. Clin. Invest.* **84**: 553-561.

Lehrer R.I., Lichtenstein A.K. and Ganz T. 1993. Defensins: antimicrobial and cytotoxic peptides of mammalian cells. *Annu. Rev. Immunol.* **11**: 105-128.

Leung K.C., Meng Z.Q. and Ho W.K. 1997. Antigenic determination fragments of α-momorcharin. *Biochim. Biophys. Acta* **1336**: 419-424.

Lewis K. and Ausubel F.M. 2006. Prospects for plant-derived antibacterials. *Nature Biotech.* **24(12)**: 1504-1507.

Lichtenstein A., Ganz T., Selsted M.E. and Lehrer R.I. 1986. *In vitro* tumour cell cytolysis mediated by peptide defensin of human and rabbit granulocytes. *Blood.* **68**: 1407-1410.

Linda S. Kim, N.D., Robert F. and Peter M. 2002. Immunological Activity of Larch arabinogalactan and Echinacea: A preliminary, randomized, double-blind, placebo-controlled trial. *Altern. Med. Rev.* **7(2)**: 138-149.

Liu L., Roberts A.A. and Ganz T. 2003. By IL-1 Signaling, monocyte-derived cells dramatically enhance the epidermal antimicrobial response to lipopolysaccharide. *J. of Immunol.* **170(1)**: 575-580.

Liu Y., Luo J., Xu C., Ren F., Peng C., Wu G. and Zhao J.. 2000. Purification, characterization, and molecular cloning of the gene of a seed specific antimicrobial protein from pokeweed. *Plant Physiol.* **122**: 1015-1024.

Liu Y.W. Shang H.F., Wang C.K., Hsu F.L. and Hou W.C. 2007. Immunomodulatory activity of dioscorin, the storage protein of yam (*Dioscorea alata* cv. Tainong No. 1) tuber. *Food and Chemical Toxicol.* **45**: 2312-2318.

Lixin H and Ng T.B. 2004. Actinchinin, a novel antifungal protein from the gold kiwi fruit. *Peptides* **25**: 1093-1098.

Lorito M., Broadway R.M., Hayes C.K., Woo S.L., Noviello C. and Williams D.L. 1994. Proteinase inhibitors from plants as a novel class of fungicides. *Mol Plant Microbe Interact* **7**: 525-527.

Luzhetskyy A., Pelzer S. and Bechthold A. 2007. The future of natural products as a source of new antibiotics. *Curr. Opin. in Inves. Drugs* **8(8)**: 608-613.

Ly I.A. and Mishell R.I. 1974. Separation of mouse spleen cells by passage through columns of sephadex G-10. *J Immunol Methods* **5**: 239-247.

Mainous III A. and Pomeroy C. 2001. Management of antimicrobials in infectious diseases. Humana Press, 349 p.

Mangoni M.L., Papo N., Barra D., Simmaco M., Bozzi A., Giulio A.D. and Rinaldi A.C. 2004. Effects of the antimicrobial peptide temporin L on cell morphology, membrane permeability and viability of Escherichia coli. *Biochem J.* **380**: 859-865.

Manna S.K., Sah N.K., Newman R.A., Cisneros A. and Aggarwal B.B. 2000. Oleandrin Suppresses Activation of Nuclear Transcription Factor-ηB, Activator Protein-1, and c-Jun NH_2-Terminal Kinase. *Cancer Res.* **60**: 3838-3847.

Marshall S.H. and Arenas G. 2003. Antimicrobial peptides: A natural alternative to chemical antibiotics and a potential for applied biotechnology. *Elec. J. Biotech.* **6(2)**: 271-284.

Matsuzaki K., Murase O., Fujii N. and Miyajima K. 1996. An antimicrobial peptide, magainin 2, induced rapid flip-flop of phospholipids coupled with pore formation and peptide translocation. *Biochemistry* **35**: 11361-11368.

Matsuzaki K., Sugishita K., Fujii N. and Miyajima K. 1995. Molecular basis for membrane selectivity of an antimicrobial peptide, magainin 2. *Biochemistry* **34**: 3423-3429.

Matsuzaki K., Yoneyama S. and Miyajima K. 1997. Pore formation and translocation of melittin. *Biophys. J.* **73**: 831-838.

McDevitt J.T., Schneider D.M., Katiyar S.K. and Edlind T.D. 1996. Berberine: a candidate for the treatment of diarrhea in AIDS patients. 175 p. *In:* Program and Abstracts of the 36th Interscience Conference on antimicrobial agents and chemotherapy. American Society for Microbiology, Washington, D.C.

McGowan Jr. J.E. 2001 Economic impact of antimicrobial resistance. *Emerg. Infect. Dis.* **7**: 286-292.

Meer J.W.M. 2003. Immunomodulation by antimicrobial drugs. *The J. Med.* **61(7)**: 233.

Melchers L.S., Apotheker-de Groot M., van der Knaap J.A., Ponstein A.S., Sela-Buurlage M.B., Bol J.F., Cornelissen B.J., van den Elzen P.J and Linthorst H.J. 1994. A new class of tobacco chitinases homologous to bacterial exo-chitinases displays antifungal activity. *Plant J.* **5**: 469-480.

Mendoza L., Wilkens M. and Urzua A. 1997. Antimicrobial study of the resinous exudates and of diterpenoids and flavonoids isolated from some Chilean *Pseudognaphalium* (Asteraceae). *J. Ethnopharmacol.* **58**: 85-88.

Mentag R., Luckevich M., Morency M.J. and Seguin A. 2003. Bacterial disease resistance of transgenic hybrid poplar expressing the synthetic antimicrobial peptide D4E1. *Tree Physiol.* **23**: 405-11.

Meyer J.J.M., Afolayan A.J., Taylor M.B. and Erasmus D. 1997. Antiviral activity of galangin from the aerial parts of *Helichrysum aureonitens*. *J. Ethnopharmacol.* **56**: 165-169.

Miyasaka N. and Hirata Y. 1997. Nitric oxide and inflammatory arthritis. *Life Sci.* **61**: 2073-2081.

Miyauchi H., Kaino A., Shinoda I., Fukuwatari Y. and Hayasawa H. 1997. Immunomodulatory effect of bovine lactoferrin pepsin hydrolysate on murine splenocytes and Peyer's patch cells. *J. Dairy Sci.* **80**: 2330-2339.

Montville T.J. and Chen Y. 1998. Mechanistic action of pediocin and nisin: recent progress and unresolved questions. *Appl. Microbiol. Biotech.* **50(5)**: 511-519.

Morassutti C., De Amicis F., Skerlavaj B., Zanetti M. and Marchetti S. 2002. Production of a recombinant antimicrobial peptide in transgenic plants using a modified VMA intein expression system. *FEBS Lett.* **519**: 141-146.

Morsy N., Konoki K., Houdai T., Matsumori N., Oishi T., Murata M. and Aimoto S. 2008. Roles of integral protein in membrane permeabilization by amphidinols *Biochimica et Biophysica Acta.* (in press).

Mossman T. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assay. *J. Immunol. Methods* **65**: 55-63.

Mueller B.M., Rosskopf F., Paper D.H., Kraus J., and Franz G. 1991. Polysaccharides from *Nerium oleander:* structure and biological activity. *Pharmazie.* **46**: 657-663.

Ng T.B. 2004. Peptides and proteins from fungi. *Peptides* **25**: 1055-1073.

Ng T.B. and Ngai P.H.K. 2004. The trypsin-inhibitory, immunostimulatory and antiproliferative activities of a napin-like polypeptide from Chinese cabbage seeds. *J. Peptide Sci.* **10**: 103-108.

Ng T.B. and Wang H.X. 2000. Panaxagin, a new protein from Chinese ginseng possesses antifungal, antiviral, translation inhibiting and ribosome-inactivating activities. *Life Sci* **68**: 739-749.

Ng T.B., Lam Y.W. and Woo N.Y.S. 2003. The immunostimulatory activity and stability of grass carp (*Ctenopharyngodon idellus*) roe lectin. Verter. *Immuno. Immunopath.*. **94**: 105-112.

Ngai P.H. and Ng T.B. 2004. Coccinin, an antifungal peptide with antiproliferative and HIV-1 reverse transcriptase inhibitory activities from large scarlet runner beans. *Peptides* **25**: 2063-2068.

Nibbering P.H., Danesi R., van't Wout J.W., van Dissel J.T., Senesi S and Lupetti A. 2002. Antimicrobial peptides: therapeutic potential for the treatment of *Candida* infections. *Expert Opin Investig Drugs* **11**: 309-318.

Niderman T., Genetet I., Buryere T., Gees R., Stintzi A., Legrand M., Fritig B. and Mosinger E. 1995. Pathogenesis-related PR-1 proteins are antifungal. Isolation and characterization of three 14-kilodalton proteins of tomato and of a basic PR-1 of

tobacco with inhibitory activity against *Phytophthora infestans*. *Plant Physiol.* **108**: 17-27.

Nielsen K.K., Nielsen J.E., Madrid S.M. and Mikkelsen J.D. 1997. Characterization of a new antifungal chitin-binding peptide from sugar beet leaves. *Plant Physiol.* **113**: 83-91.

Nielsen K.K., Nielsen J.E., Madrid S.M. and Mikkelsen J.D. 1996. New antifungal proteins from sugar beet (*Beta vulgaris* L.) showing homology to non-specific lipid transfer proteins. *Plant Mol. Biol.* **31:** 539-552.

Nizet V., Ohtake T., Lauth X., Trowbridge J., Rudisil J. and Dorschner R.A. 2001. Innate antimicrobial peptide protects the skin from invasive bacterial infection. *Nature* **414**: 454-457.

Nozawa R.T. and Yokoto Z. 1982. Microbicidal activity of bone marrow cells. Augmentation of candida killing activity of the cells by culturing with fibroblast conditioned medium. *Microbiol. Immun.* **26**: 153-161.

O'Kennedy R. and Thornes R.D. 1997. Coumarins: biology, applications and mode of action. John Wiley & Sons, Inc., New York. 358 p.

Ogata C.M., Gordon P.F., de Vos A.M. and Kim S.H. 1992. Crystal structure of a sweet tasting protein Thaumatin I, at 1.65 A° resolution. *J. Mol. Biol.* **228**: 893-908.

Oh J., Cajal Y., Skowronska E.M., Belkin S., Chen J., Van Dyk T.K., Sasse R.M. and Jain M.K. 2000. Cationic peptide antimicrobials induce selective transcription of micF and osmY in *Escherichia coli*. *Biochim. Biophys. Acta*, **1463**: 43-54.

Omulokoli E., Khan B. and Chhabra S.C. 1997. Antiplasmodial activity of four Kenyan medicinal plants. *J. Ethnopharmacol.* **56**: 133-137.

Ostoa-Saloma P., Carrero J.C., Petrossian P., Herion P., Landa A. and Laclette J.P. 2000. Cloning, characterization and functional expression of a cyclophilin of *Entamoeba histolytica*. *Mol. Biochem. Parasitol.* **107**: 219-225.

Otvos Jr L., Insug O., Rogers M.E., Consolvo P.J., Condie B.A., Lovas S., Bulet P. and Blaszczyk-Thurin M. 2000 Interaction between heat shock proteins and antimicrobial peptides. *Biochemistry* **39**: 14150-14159.

Otvos Jr. L. 2002. The short proline-rich antibacterial peptide family. *Cell. Mol. Life Sci.* **59**: 1138-1150.

Otvos Jr. L. Snyder C., Condie B., Bulet P. and Wade J.D. 2005. Chimeric antimicrobial peptides exhibit multiple modes of action. *Int. J. Pep. Res. Therap.* **11(1)**: 29-42.

Ou H.T., Shieh C.J., Chen J.Y.J. and Chang H.M. 2005. The antiproliferative and differentiating effects of human leukemic U937 cells are mediated by cytokines from activated mononuclear cells by dietary mushrooms. *J. Agric. Food Chem.* **53**: 300-305.

Park C.B., Kim H.S. and Kim S.C. 1998. Mechanism of action of the antimicrobial peptide buforin II: buforin II kills microorganisms by penetrating the cell membrane and inhibiting cellular functions. *Biochem. Biophys. Res. Commun.* **244**: 253-257.

Park C.B., Yi K.S., Matsuzaki K., Kim M.S. and Kim S.C. 2000. Structure activity analysis of buforin II, a histone H2A derived antimicrobial peptide: the proline hinge is responsible for the cell-penetrating ability of buforin II. *Proc. Natl Acad. Sci. USA* **97:** 8245-8250.

Park K.S., Cheong J.J., Lee S.J., Suh M.C. and Choi D. 2000. A novel proteinase inhibitor gene transiently induced by tobacco mosaic virus infection. *Biochim. Biophys. Acta* **1492**: 509-512.

Park Y., Park N.P., Park S., Shin S.O., Kim J., Kan S., Kim M., Jeong C. and Hahm K. 2006. Synergism of Leu-Lys rich antimicrobial peptides and chloramphenicol against bacterial cells. *Biochemi. Biophys. Acta* **1764**: 24-32.

Parkinson J.F., Mitrovic B. and merril J.E. 1997. The role of nitric oxide in multiple sclerosis. *J. of Mol. Med.* **75**: 174-186.

Pogrebnoy P.V., Hobta A.I., Soldatkina M.A., Garmanchouk L.V., Markeeva N.V. and Eremenko T. 1998. A protein kinase inhibitor from A431 subline overexpressing TGF alpha possesses antimicrobial activity. *New Microbiol.* **21**: 269-273.

Ponstein A.S., Bres-Vloemans S.A., Sela-Buurlage M.B., van den Elzen P.J., Meichers L.S. and Cornelissen B.J. 1994. A novel pathogen-and wound-inducible tobacco (*Nicotiana tabacum*) protein with antifungal activity. *Plant Physiol.* **104**: 109-118.

Poole K. and Srikumar R. 2001. Multidrug efflux in *Pseudomonas aeruginosa*: components, mechanisms, and clinical significance. *Curr. Top. Med. Chem.* **1:** 59-71.

Powers J.P. and Hancock R.E. 2003. The relationship between peptide structure and antibacterial activity. *Peptides* **24**: 1681-1691.

Pressey R. 1997. Two isoforms of NP24: a thaumatin-like protein in tomato fruit. *Phytochemical.* **44**: 1241-1245.

Pugh N., Ross S.A., ElSohly H.N., ElSohly M.A. and Pasco D.S. 2001. Isolation of three high molecular weight polysaccharide preparations with potent immunostimulatory

activity from *Spirulina platensis*, Aphanizomenon flos-aquae, and Chlorella pyrenoidosa. *Planta Med.* **67**: 737-742.

Rajaskaran K., Cary J., Jaynes J. and Clevland T. 2005. Disease resistance conferred by the expression of a gene encoding a synthetic peptide in transgenic cotton (*Gossypium hirsutum* L.) plants. *Plant Biotechnol. J.* **3(6)**: 545-554.

Rana B.K., Singh U.P and. Taneja. V. 1997. Antifungal activity and kinetics of inhibition by essential oil isolated from leaves of *Aegle marmelos*. *J. Ethnopharmacol.* **57**: 29-34.

Reddy K.V.R., Yedery R.D. and Aranah C. 2004. Antimicrobial peptides: premises and promises. *Int. J. Antimicrob. Agents* **24**: 536-547.

Rezzonico E., Flury N., Meins Jr. F. and Beffa R. 1998. Transcriptional down-regulation by abscisic acid of pathogenesis-related β-1,3-glucanase genes in tobacco cell cultures. *Plant Physiol.* **117**: 585-592.

Ribeiro S.F.F., Agizzio A.P.,. Machado O.L.T, Neves-Ferreira AG.C., Oliveira M.A., Fernandes K.V.S. Carvalho A.O., Jonas Perales J. and Gomes V.M. 2007. A new peptide of melon seeds which shows sequence homology with vicilin: Partial characterization and antifungal activity. *Scientia Horticulturae* **111**: 399-405.

Richard J.P. 2003. Cell-penetrating peptides. A re-evaluation of the mechanism of cellular uptake. *J. Biol. Chem.* **278**: 585-590.

Rimbach G., park Y.C., Guo Q., Moini H., Quereshi N., Saliou C., Takayama K., Virgili R. and Packer L. 2000. Nitric oxide synthesis and TNF-alpha secretion in RAW 264.7 macrophage: mode of action of a fermented papaya preparation. *Life Sci.* **67**: 679-694.

Roberts W. and Selitrennikoff C.P. 1990. Zeamatin, an antifungal protein from maize with membrane-permeabilizing activity. *J. Gen. Microbiol.* **136**: 1771-1778.

Ryu J.H., Ahn H., Kim J.Y. and kim Y.k. 2003. Inhibitory activity of plant extracts on nitric oxide synthase in LPS-activated macrophages. *Phytotherap. Res.* **17**: 485-489.

Sahl H.G., Pag U., Bonness S., Wagner S., Antcheva N. and A. Tossi. 2004. Mammalian Defensins: Structures and Mechanism of Antibiotic Activity. *J. Leukoc Biol.* **77 (4)**: 466-474.

Sakanaka S., Kim M., Taniguchi M. and Yamamoto T. 1989. Antibacterial substances in Japanese green tea extract against *Streptococcus mutans*, a cariogenic bacterium. *Agric. Biol. Chem.* **53**: 2307-2311.

Sakanaka S., Shimura N., Aizawa M., Kim M. and Yamamoto T. 1992. Preventive effect of green tea polyphenols against dental caries in conventional rats. *Biosci. Biotechnol. Biochem.* **56**: 592-594.

Sanglard D. and Odds F.C. 2002. Resistance of Candida species to antifungal agents: molecular mechanisms and clinical consequences. *Infect. Disease* **2**: 73-85.

Sanglard D., Kuchler K., Ischer F., Pagani J.L., Monod M. and Bille J. 1997. Mechanisms of resistance to azole antifungal agents in *Candida albicans* isolates from AIDS patients involve specific multidrug transporters. *Antimicrob Agents Chemother* 1995 **39**: 2378-2386.

Scalbert A. 1991. Antimicrobial properties of tannins. *Phytochemistry* **30**: 3875-3883.

Segura A., Moreno M. and Garcia-Olmedo F. 1993. Purification and antipathogenic activity of lipid transfer proteins (LTPs) from the leaves of *Arabidopsis* and spinach. *FEBS Lett.* **332**: 243-246.

Segura A., Moreno M., Madueno F., Molina A. and Garcia-Olmedo F. 1999. Snakin-1, a peptide from potato that is active against plant pathogens. *Mol. Plant Microbe Interact.* **12**: 16-23.

Selitrennikoff C.P. 2001. Antifungal proteins. *App. and Env. Microb.* **67(7)**: 2883-2894.

Sharma N., Gruszewski H.A., Park S.W., Holm D.G., Jorge M. and Vivanco J.M. 2004. Purification of an isoform of patatin with antimicrobial activity against *Phytophthora infestans*. *Plant Physiol. and Biochem.* **42**: 647-655.

Sheu F., Chien P.J., Chien A.L., Chen Y.F. and Chin K.L. 2004. Isolation and characterization of an immunomodulatory protein (APP) from the Jew's ear mushroom Auricularia polytricha. *Food Chemistry* **87**: 593-600.

Shi J. 1996. Antibacterial activity of a synthetic peptide (PR-26) derived from PR-39, a proline–arginine-rich neutrophil antimicrobial peptide. *Antimicrob. Agents Chemother.* **40**: 115-121.

Silphaduang U., Hincke M.T., Nys Y. and Mine Y. 2006. Antimicrobial proteins in chicken reproductive system. *Biochem. and Biophy. Res. Comm.* **340**: 648-655.

Silvestro L., Weiser J.N., and Axelsen P.H. 2000. Antibacterial and Antimembrane Activities of Cecropin A in *Escherichia coli*. *Antimicrob. Agents and Chemother.* **44(3)**: 602-607.

Singh N.K., Bracker C.A., Hasegawa P.M., Handa A.K., Buckel S., Hermodson M.A., Pfankoch E., Regnier F.E. and Bressan R.A. 1987. Characterization of osmotin. *Plant Physiol.* **85**: 529-536.

Singh P.K. Parsek M.R., Greenberg E.P. and Welsh M.J. 2002. A component of innate immunity prevents bacterial biofilm development. *Nature* **417(6888)**: 552-555.

Skerlavaj B. 1999. SMAP-29: a potent antibacterial and antifungal peptide from sheep leukocytes. *FEBS Lett.* **463(1-2)**: 58-62

Skerlavaj B., Romeo D. and Gennaro R. 1990. Rapid membrane permeabilization and inhibition of vital functions of gram-negative bacteria by bactenecins. *Infect. Immun.* **58**: 3724-3730.

Stenkvist B. 1999. Is digitalis a therapy for breast carcinoma? *Oncol. Rep.* **6**: 493-496.

Stuart L.M. and Ezekowitz R.A.B. 2005. Phagocytosis: elegant complexity. *Immunity* **22**: 539-550.

Subbalakshmi C. and Sitaram N. 1998. Mechanism of antimicrobial action of indolicidin. *FEMS Microbiol. Lett.* **160**: 91-96.

Sun H.D., Qiu S.X., Lin L.Z., Wang Z.Y., Lin Z.W., Pengsuparp T., Pezzuto J.M., Fong H.H., Cordell G.A. and. Farnsworth N.R. 1996. Nigranoic acid, a triterpenoid from *Schisandra sphaerandra* that inhibits HIV-1 reverse transcriptase. *J. Nat. Prod.* **59**: 525-527.

Sung W.S. and Lee D.G. 2008. Pleurocidin-derived antifungal peptides with selective membrane disruption effect. *Biochem. and Biophys. Res. Comm.* (in press).

Suresh B.S., Dhanaraj S.A., Elango K. and Chinnaswamy K. 1997. Anticandidal activity of *Santolina chamaecyparissus* volatile oil. *J. Ethnopharmacol.* **55**:151-159.

Suslow T.V., Thomas B.R. and Bradford K.J. 2002. Biotechnology provides new tools for planting. Oakland: University of California Division of Agriculture and Natural Resources, Publication 8043.

Suzuki I., Tanaka H., Kinoshita A., Oikawa S., Osawa M. and Yadomae T. 1990. Effect of orally administered β-glucan on macrophage function in mice. *Int. J. Immunopharmacol.* **12(6)**: 675-684.

Szabo C. and Thiemermann C. 1994. Invited opinion: role of nitric oxide in hemorrhagic, traumatic, and anaphylacticshock and thermal injusy. *Shock* **2**: 145-155.

Tan B.K.H. and Vanitha J. 2004. Immunomodulatory and Antimicrobial Effects of Some Traditional Chinese Medicinal Herbs: A Review. *Curr. Med. Chem.* **11** 1423-1430.

Tanaka K., Fujimoto Y., Suzuki M., Suzuki Y., Ohtake T., Saito H. and Kohgo Y. 2001. PI3-kinase p85alpha is a target molecule of proline-rich antimicrobial peptide to suppress proliferation of ras-transformed cells. *Jpn. J. Cancer Res.* **92**: 959-967.

Tassin S., Broekaert W., Marion D., Acland D., Ptak M., Vovelle F. and Sodano P. 1998. Solution structure of *Ace*-AMP1, a potent antimicrobial protein extracted from onion seeds. Structural analogies with plant nonspecific lipid transfer proteins. *Biochemistry* **37**: 3623-3637.

Taylor R.S.L., Edel F., Manandhar N.P. and Towers G.H.N. 1996. Antimicrobial activities of southern Nepalese medicinal plants. *J. Ethnopharmacol.* **50**: 97-102.

Tenover F.C. 2006. Mechanisms of antimicrobial resistance in bacteria. *The American J. of Med.* **119**: S3-S10.

Territo M.C., Ganz T., Selsted M.E. and Lehrer R. 1989. Monocyte-chemotactic activity of defensins from human neutrophils. *J. Clin. Invest.* **84**: 2017-2020.

Theis T. and Stahl U. 2004. Antifungal proteins: target, mechanism and prospective applications. *Cell Mol. Life. Sci.* **61**: 437-453.

Theis T., Marx F., Salvenmoser W., Stahl U. and Meyer V. 2005. New insights into the target site and mode of action of the antifungal protein of *Aspergillus giganteus*. *Res. in Microbiol.* **156**: 47-56.

Thomson W.A.R. 1978. Medicines from the Earth. McGraw-Hill Book Co., Maidenhead, United Kingdom. 669 p.

Toke O. 2005. Antimicrobial Peptides: New candidates in the fight against bacterial infections. *Pept. Sci.* **80**: 717-735.

Tossi A., Sandri L. and Giangaspero A. 2000. Amphipathic, α-helical antimicrobial peptides. *Biopolymers* **55**: 4-30.

Toutouzas P.C., Tousoulis D. and davies G.J. 1998. Nitric oxide synthesis in artherosclerosis. *Eur. Heart J.* **19**: 1504-1511.

Tracey K.J. and Cerami A. 1994. Tumor necrosis factor: a pleotropic cytokine and therapeutic target. *Ann. Rev. of med.* **45**: 1504-1511.

Trotti A., Garden A., Warde P., Symonds P., Langer C., Redman R., Pajak T.F., Fleming T.R., Henke M., Bourhis J., Rosenthal D.I., Junor E., Cmelak A., Sheehan F., Pulliam J., Devitt-Risse P., Fuchs H., Chambers M., O'Sullivan B. and Ang K.K. 2004. A multinational, randomized phase III trial of iseganan HCl oral solution for reducing the severity of oral mucositis in patients receiving radiotherapy for head-and-neck malignancy. *Int. J. Radiat. Oncol. Biol. Phys.* **58**: 674-681.

Tzi B.N. and Wang H. 2001. Panaxagin, a new protein from Chinese ginseng possesses anti-fungal, anti-viral, translation-inhibiting and ribonuclease activities *Life Sciences*. **68**: 739-749.

Utsugi T., Schroit A.J., Connor J., Bucana C.D. and Fidler I.J. 1991. Elevated expression of phosphatidylserine in the outer membrane leaflet of human tumour cells and recognition by activated human blood monocytes. *Cancer Res.* **51**: 3062-3066.

Van Damme E.J., Charels D., Roy S., Tierens K., Barre A., Martins J. C., Rouge P., Van Leuven F., Does M. and Peumans W. J. 1999. A gene encoding a hevein-like protein from elderberry fruits is homologous to PR-4 and class V chitinase genes. *Plant Physiol.* **119**: 1547-1556.

Van Damme E.J.M., Willems P., Torrekens S., Van Leuven F. and Peumans W.J. 1993. Garlic (Allium sativum) chitinases: characterization and molecular cloning. *Physiol Planta* **87**: 177-186.

Van Den Bergh K.P., Rouge P., Proost P., Coosemans J., Krouglova T. and Engelborghs Y. 2004. Synergistic antifungal activity of two chitin-binding proteins from spindle tree (Euonymus europaeus L.). *Planta* **219(2)**: 221-232.

van Dijk A., Veldhuizen E.J.A., Kalkhove S.I.C., Tjeerdsma-van Bokhoven J.L.M., Romijn R.A., Haagsman H.P. 2007. The {beta}-defensin gallinacin-6 is expressed in the chicken digestive tract and has antimicrobial activity against food-borne pathogens. *Antimicrob. Agents Chemother.* **51**: 912-922.

Van Loon L.C. 1985. Pathogenesis-related proteins. *Plant Mol. Biol.* **116**: 111-116.

Van Wetering S., Mannesse-Lazeroms S.P., Dijkman J.H. and Hiemstra P.S. 1997. Effect of neutrophil serine proteinases and defensins on lung epithelial cells: modulation of cytotoxicity and IL-8 production. *J Leukoc Biol.* **62**: 217-226.

Vandenbergh P.A. 1993. Lactic acid bacteria, their metabolic products and interference with microbial growth. *FEMS Microbiol. Reviews* **12(1-3)**: 221-237.

Varadhachary A., Wolf J.S., Petrak K., O'Malley Jr. B.W., Spadaro M., Curcio C., Forni G. and Pericle F. 2004. Oral lactoferrin inhibits growth of established tumors and potentiates conventional chemotherapy. *Int. J. Cancer* **111**: 398-403.

Vivanco J.M., Savary B.J. and Flores H.E. 1999. Characterization of two novel type I ribosome-inactivating proteins from the storage roots of the andean crop *Mirabilis expansa*. *Plant Physiol.* **119**: 1447-1456.

Vizioli J. and Salzet M. 2003. Antimicrobial peptides: new weapons to control parasitic infections? *Trends in Parasitology* **19**: 475-457.

Vu L., and Huyhn Q.K. 1994. Isolation and characterization of a 27-kDa antifungal protein from the fruits of Diospyros texana. *Biochem Biophys Res Commun.* **202**: 666-72.

Wadia J.S., Stan R.V. and Dowdy, S.F. 2004. Transducible TATHA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. *Nature Med.* **10**: 310-315.

Wang C.N., Shino Y.J., Kuo Y.H., Chen C.C. and Lin Y.L. 2000. inducible nitric oxide synthase inhibitors from *Saposhnikovia divarticata* and *Panax quinquefolium. Planta* **66**: 644-647.

Wang H. and Ng T.B. 2002. Isolation of an antifungal thaumatin-like protein from kiwi fruit. *Phytochemistry* **61**: 1-7.

Wang H.X. and Ng T.B. 2000. Ginkbilobin, a novel antifungal protein from *Ginkgo biloba* seeds with sequence similarity to embryo-abundant protein. *Biochem Biophys Res Commun* **279**: 407-411.

Wang H.X. and Ng T.B. 2003. Alocasin, an anti-fungal protein from rhizomes of the giant taro Alocasia macrorrhiza. *Protein Exp. and Purif.* **28**: 9-14.

Wang X.Y., Wu J.H., Ng T.B., Ye X.Y. and Rao P.F. 2004. A non-specific lipid transfer protein with antifungal and antibacterial activities from the mung bean. *Peptides* **25**: 1235–1242.

Wasser S.P. 2002. Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. *Appl. Microbiol. Biotechnol.* **60**: 258-274.

Watanabe T., Kanai R., Kawase T., Tanabe T., Mitsutomi M., Sakuda S. and Miyashita K. 1999. Family 19 chitinases of *Streptomyces* species: characterization and distribution. *Microbiology* **145**: 3353-3363.

Welling M.M., Paulusma-Annema A., Balter H.S., Pauwels E.K. and Nibbering P.H. 2004. Technetium-99m labelled antimicrobial peptides discriminate between bacterial infections and sterile inflammations. *Eur. J. Nucl. Med.* **24**: 292-301.

Wild R. 1994. The complete book of natural and medicinal cures. Rodale Press, Inc., Emmaus, Pa. 784 p.

Wildman H.K.A., Lee D.K. and Ramamoorthy A. 2003. Mechanism of lipid bilayer disruption by the human antimicrobial peptide, LL-37. *Biochemistry* **42**: 6545-6558.

Wong J.H. and Ng T.B. 2003. Gymnin, a potent defensin-like antifungal peptides from the Yunnan bean (Gymnocladus chinensis Baill). *Peptides* **24**: 963-968.

Wong J.H. and Ng T.B. 2005. Sesquin, a potent defensin-like antimicrobial peptide from ground beans with inhibitory activities toward tumor cells and HIV-1 reverse transcriptase. *Peptides* **26**:1120-1126.

Wösten M.M. and Groisman E.A. 1999. Molecular characterization of the PmrA regulon. *J. Biol. Chem.* **274**: 27185-27190.

Wray W., Boulikas T., Wray V.P., Hancock R. 1981. Silver staining of proteins in polyacrylamide gels. *Anal Biochem.* **118(1)**:197-203.

Wu E., Maier M., Benz R. and Hancock R.E.W. 1999. Mechanism of interaction of different classes of cationic antimicrobial peptides with planar bilayers and with the cytoplasmic membrane of *Escherichia coli*. *Biochem.* **38**: 7235-7242.

Wurms K. and Greenwood D. 1999. Sharrock K, Long P. Thaumatin like protein in kiwi fruit. *J Sci Food Agric* **79**: 1448-1452.

Xia L.X. and Ng T.B. . Isolation of alliumin, a novel protein with antimicrobial and antiproliferative activities from multiple cloved garlic bulbs. *Peptides* **26**: 177-83.

Xiang Y., Huang R.H., Liu X.Z., Zhang Y. and Wanga D.C. 2004. Crystal structure of a novel antifungal protein distinct with five disulfide bridges from *Eucommia ulmoides* Oliver at an atomic resolution. *J. Str. Biol.* **148**: 86-97.

Xu H.X., Zeng F.Q., Wan M. and Sim K.Y. 1996. Anti-HIV triterpene acids from *Geum japonicum*. *J. Nat. Prod.* **59**: 643-645.

Yamaguchi S. 2001 Orientation and dynamics of an antimicrobial peptide in the lipid bilayer by solid-state NMR spectroscopy. *Biophs. J.* **81**: 2203-2214.

Yang L., Harroun T.A., Weiss T.M., Ding L. and Huang H. W. 2001. Barrel-stave model or toroidal model? A case study on melittin pores. *Biophys. J.* **81**: 1475-1485.

Yang L., Harroun T.A., Weiss T.M., Ding L. and Huang H.W. 2001. Barrel-stave model or toroidal model? A case study on melittin pores. *Biophys. J.* **81**: 1475-1485.

Ye X.Y. and Ng T.B. 2000. Hypogin, a novel antifungal peptide from peanuts with sequence similarity to peanut allergen. *J. Peptide Res.* **57**: 330-336.

Ye X.Y. and Ng T.B. 2000. Mungin, a novel cyclophilin-like antifungal protein from the mung bean. *Biochem. Biophys. Res. Commun.* **273**: 1111-1115.

Ye X.Y., Ng T.B. 2003. Isolation of vulgin, a new antifungal polypeptide with mitogenic activity from the pinto bean. *J. Pep. Sci.* **9(2)**: 114-119.

Ye X.Y., Wang H.X. and Ng T.B. 1999. First chromatographic isolation of an antifungal thaumatin-like protein from French bean legumes and demonstration of its antifungal activity. *Biochem. Biophys. Res. Commun.* **263**: 130-134.

Ye X.Y., Wang H.X. and Ng T.B. 2000. Dolichin, a new chitinase-like antifungal protein isolated from field beans (*Dolichos lablab*). *Biochem. Biophys. Res. Commun.* **269**: 155-159.

Ye X.Y., Wang H.X. and Ng T.B. 2000. Sativin, a novel antifungal miraculin-like protein isolated from the legumes of the sugar snap *Pisum sativum* var. macrocarpon. *Life Sci.* **67**: 775-781.

Zasloff M. 1987. Magainins, a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. *Proc. Natl Acad. Sci. USA* **84**: 5449-5453.

Zasloff M. 2002. Antimicrobial peptides of multicellular organisms. *Nature* **415**: 389-395.

Zhang G.P., Shi Y.L., Wang W.P. and Liu W.Y. 1999. Cation channel formed at lipid bilayer by Cinnamomin, a new type II ribosome-inactivating protein. *Toxicon* **37**: 1313-1322.

Zhang L. and Falla T.J. 2006. Antimicrobial peptides: therapeutic potential *Expert Opin. Pharmacotherp.* **7(6)**: 653-663.

Zhang Y. and Lewis K. 1997. Fabatins: new antimicrobial plant peptides. *FEMS Microbiol. Lett.* **149**: 59-64.

Zhao H., Mattila J.P., Holopainen J.M. and Kinnunen P.K. 2001. Comparison of the membrane association of two antimicrobial peptides, magainin 2 and indolicidin. *Biophys. J.* **81**: 2979-2991.

Zimeck M., Mazurier J., Machnicki M., Wieczorek Z., Montreuil J. and Spik G., 1991. Immunostimulatory activity of lactotransferrin and maturation of CD4 CD8 murine thymocytes. *Immunol. Lett.* **30**: 119-124.

RESEARCH PUBLICATIONS

A. Papers in Journals.

1. **Parveen S.** and Prasad R. (2007). A 53 kDa immunomodulatory protein from the leaves of *Nerium odorum. Allergy,* 62(s83): 459-460.

2. **Parveen S.**, Gupta A.D. and Prasad R. (2007). Arabinogalactan protein from *Arachis hypogea*: role as carrier in drug-formulations. *International Journal of Pharmaceutics*, 333(1-2): 79-86.

3. Parveen S. Prasad R (2005) Antimicrobial potential of arabinogalactan proteins (AGPs) like molecules from *Nerium odorum* leaves. *Glycoconjugate Journal*, 22(4): 265-266.

4. Parveen S. and Prasad R. A novel antibacterial protein isolated from the leaves of *Nerium odorum*. (communicated)

B. Book Chapter.

Prasad R, **Parveen S**, Gupta AD. Arabinogalactan and Arabinogalactan-Proteins complexes (AGPs) a Natural Plant derived macromolecules of Therapeutic importance. *In Recent Advances in Plant Biotechnology and Its Applications* Ed by Ashwani Kumar and Sudhir K. Sopory, Publisher I.K. International, New Delhi, Chapter 30.

C. Papers and Presentations in Conferences.

1. **Parveen S.** and Prasad R. A 53 kDa immunomodulatory protein from the leaves of *Nerium odorum.* XXVI Congress of the European Academy of Allergology and Clinical Immunology, **Göteborg, Sweden**, 9-13 June 2007

2. **Parveen S**. and Prasad R. Antimicrobial protein from the seeds of *Trichosanthes cucumerina.* "20th IUBMB International Congress of Biochemistry and Molecular Biology and 11th FAOBMB Congress, (IUBMB 2006) **Kyoto, Japan**". June 18th - 23rd 2006.

3. **Parveen S.** and Prasad R. Antimicrobial potential of arabinogalactan proteins (AGPs) like molecules from *Nerium odorum* leaves. XVIII International symposium on Glycoconjugates (Glyco XVIII 2005) **Florence, Italy**. September 4th - 9th 2005.

4. **Parveen S.** and Prasad R. Antimicrobial Potential of *Thymus vulgaris*. 7th National Symposium on Biochemical and Biotechnology (BIOHORIZON) 2005, IIT Delhi, New Delhi. March 11th - 12th 2005

5. Prasad R. and **Parveen S.** Gupta A.D. A novel glycoconjugate from *Arachis hypogea*: Its isolation, purification and pharmaceutical potentials. International conference on Recent Advances in Biomedical and Therapeutics Sciences 2004, organized by Bundelkhand University, Jhansi U.P. February 13th - 15th 2004,

6. **Parveen S.** and Gupta A.D. and Prasad R. Isolation and purification of arabinogalactan Proteins (AGPs) like molecules from *Catharanthus roseus* and its antifungal potential. Proceedings of National symposium on Biochemical Sciences Health and Environment Aspects 2003, organized by Dayalbagh Educational Institute, Dayalbagh Agra, October 13th - 15th 2003.