Disc
B46
1568

Evaluation of Methanolic Extract of Wild Thyme (*Thymus serpyllum* L.)

By

Ateeq-ur-Rehman

Faculty of Biological Sciences
Department of Biochemistry
Quaid-i-Azam University
Islamabad

2006

Evaluation of Methanolic Extract of Wild Thyme (*Thymus serpyllum* L.)

A thesis submitted in the partial fulfillment of the requirements for the degree of

Master of Philosophy

In

BIOCHEMIOSTRY/MOLECULARY BIOLOGY

By

Ateeq-ur-Rehman

Faculty of Biological Sciences
Department of Biochemistry
Quaid-i-Azam University
Islamabad

2006

DECLARATION

This thesis submitted by Ateeq-ur-Rehman is accepted in its present form by the Department of Biological Sciences, Quaid-I-Azam University, Islamabad as fulfilling the thesis requirement for the degree of Master of Philosophy in Biology (Biochemistry /Molecular biology).

Internal Examiner: _____
(Dr.Bushra Mirza)

External Examiner: _____
(Prof. Dr. Shahbaz Warraich)

Chairman: _____
(Dr. S.A. Malik)

Dated: February 18, 2006

DEDICATED TO THE MEMORY OF
LITTLE SCHOLARS WHO SET OF
IN SEARCH OF FAIRY OF
KNOWLEDGE BUT LOST THE
BATTLE OF LIFE IN THE
CATASTROPHE OF
8TH OCT.2005

CONTENTS

ACKNOWLEDGEMENTS

I feel utmost obligate to offer my best thanks giving to ***ALMIGHTY ALLAH****, all the praise, the glory and honor is for Him. The Greatest Compassionate and Beneficent to all the human being, who blessed me with the health, talented and passionate teachers, good friends and expression of thought, confidence and determination needed for completion of my research work. Countless salutation upon the Holy Prophet* ***HAZRAT MUHAMMAD*** *(P.B.U.H), city of knowledge and blessing for entire creature who has guided his Ummah to seek the knowledge from Cradle to Grave, and enabled me to win honor of life.*

I am grateful to ***Prof. Dr. Samina Jalali****, Chairperson, Department of Biological Sciences, Quaid-i-Azam University, Islamabad for providing access to facilities that ensured successful completion of this work.*

I owe my deep gratitude to a kind personality, my supervisor ***Dr Bushra Mirza*** *Assistant Professor, Department of Biological Sciences, for her guidance, precious advice, thoughtful suggestions and generous supervision through out my research work. Her co-operation, kind interest in this research work, moral encouragement and efforts towards the inculcation of constant hard working will always serve me as beacon throughout the course of life.*

I must express gratitude to ***Dr Mir Ajab Khan****, and* ***Dr Rizwana Aleem Qureshi,*** *Associate Professors, Department of Biological Sciences, Quaid-i-Azam University, Islamabad, for their outstanding help in collection and identification of Thymus species. A special word of thanks to* ***Dr. Fayyaz A. Chaudhary,*** *Professor, Department of Biological Sciences, Quaid-i-Azam University, Islamabad, for his cooperation.*

Heart felt mention goes to amiable Lab fellows, ***Rehan Naeem, Abdul Mannan Abida Yasmeen, Wasim Ahmad, Maryam Jameel, Samia Inayatullah, Waheed Arshad, Munawar Ahmad, Muhammad Zaeem, Ihsan ul Haq, Muhammad Hasan, Humaira Naureen, Nazia Shaheen, Rukhsana Iram, Muhammad Ahsan, Ibrar Ahmed, Sheeraz Ahmed, Shoaib, Aisha Aftab, Asma, Naila Safdar*** *and my friends,* ***Rizwanullah, Asjad Ali, Muhammad Rafique, Rizwan Anwar, Faisal Ali, Aqeel Ahmed, Muhmmad Asif, Muhammad Saeed, Faheem Asim, Malik Ashfaq, Raja Ishtiaq, Mirza Jamshaid, Bashir*** *and* ***Isfahan.***

Words are inadequate to express my thanks to my friends ***Shabana Manzoor*** *and* ***Naeem Arif*** *for their moral support, love and care which helped me put up with all the frustrations encountered during my research work and also for their pleasant companionship, which rejoice me day and night.*

I would like to thank the back bone of my research, my family. Especially I owe great deal of intellectual debt to my father, for his support, encouragement and belief in me. I pay my homage and most heart felt gratitude from the core of my heart to my loving mother, brothers and sisters for their prayers and holy wishes. Words fail to express the intensity of gratitude to my parents who have devoted their lives and sacrificed their comforts for my studies and spiritual training. Their constant love affection and prayers made me able to pursue my goals and made them true.

Before bidding a farewell, I would also record my thanks giving to all those researchers and companies who send me their valuable research papers, articles and chemicals to carry out my research work.

ATEEQ UR REHMAN
CHAUDHRY

LIST OF ABBREVIATIONS

$BaCl_2$	Barium chloride
Cm	Centimeter
Conc.	Concentration
°C	Degree Centigrade
CFU	Colony forming unit
DMSO	Dimethyl Sulfoxide
gm	Gram
gm/L	Gram per liter
kb	Kilo base
KI	Potassium iodide
KOH	Potassium hydroxide
L	Liter
MIC	Minimum Inhibitory Concentration
mm	Millimeter
mL	Milliliter
M	Molar
mg	Milligram
mg/mL	Milligram per milliliter
mg/L	Milligram per liter
ppm	Part per million
USP	United States Pharmacopoeia
UV	Ultra Violet
µ	Micro
µL	Micro liter
µL/mL	Micro liter per milliliter
µg /mL	Microgram per milliliter
V	Volume

LIST OF FIGURES

DRSM LIBRARY Quaid-i-Azam University Reference Section

LIST OF TABLES

ABSTRACT

Thymus serpyllum L. is a traditional medicinal plant that is used for the treatment of inflammatory diseases, allergic rashes, skin irritations, dermatitis, asthma, bronchitis, colds and flu, wounds and other pulmonary diseases. The objective of the present study was to access the biological activity of *Thymus serpyllum* L. extract using selected bench top bioassays, including antifungal, cytotoxic, antitumor, antibacterial and two phytotoxicity assays i.e. radish seed and *Lemna minor* bioassay. Extract was prepared in methanol. Moderate antifungal activity (41-51%) was seen against *Fusarium moniliforme, Alternaria species, Candida albicans, Candida glabarata* and *Fusarium solani* while low activity (below 40%) was seen against *Mucor species* and *Aspergillus flavus*. Extract showed no linear growth inhibition but inhibited spore formation of *Aspergillus flavus green, Aspergillus nigar and Aspergillus fumigatus*. Plant extract showed highly significant (ED_{50} 466ppm<1000ppm) impact on % death of brine shrimp. *Agrobacterium tumefaciens* (*At*-10) induced tumors in potato disc tissue were inhibited ($P<0.05$) by the test extract however no antibacterial activity against *Agrobacterium tumefaciens* was observed. Phytotoixicity to *Lemna minor* L. ($P<0.05$) and radish seed germination and growth ($P<0.05$) was observed at higher concentration of the plant extract. Plant extract was also used against eight strains of bacteria. Three were gram positive, *Staphylococcus aureus, Bacillus subtilis and Micrococcus luteus* and five were gram negative, *Escherichia coli, Salmonella setubal, Pseudomonas picketii, Bordetella bronchiseptica,* and *Enterobacter aerogens*. Extract showed no antibacterial activity against all the eight strains of bacteria tested.

CHAPTER 1

INTRODUCTION

INTRODUCTION

Medicinal plants existed long before the first human appeared on earth. No one knows how long it took for humans to discover the curative power of medicinal plants. Higher and aromatic plants have traditionally been used in folk medicines as well as to extend the shelf life of foods, showing inhibition against bacteria, fungi and yeasts. Most of their properties are due to essential oils produced by their secondary metabolism (Adam *et al.*, 1998). Essential oils and extracts from several plant species are able to control microorganisms related to skin (Adam *et al.*, 1998) and food spoilage, including gram-negative and gram-positive bacteria. About 80 % individuals from developed countries use traditional medicine, which has compounds derived from medicinal plants. Therefore, such plants should be investigated to better understand their properties, safety and efficacy (Ellof, 1998).

Pakistan is one of the few places on earth with such a unique biodiversity, comprising of different climatic zones with a wide range of plant species. Approximately 6000 plant species with medicinal properties are found in Pakistan. There is a dire need on the part of manufacturers of allopathic and herbal medicines to carryout systematic research on medicinal plants to save foreign exchange spent on their imports (Shinwari and Malik, 1989).

Thymus serpyllum L. (Fig. 1.1, Fig.1.2) is commonly known as "Wild thyme". The name thyme, in its Greek form, was first given to the plant by the Greeks as a derivative of a word which meant 'to fumigate,' either because they used it as incense, for its balsamic odour, or because it was taken as a type of all sweet-smelling herbs.

Fig. 1.1: Dried plant of *Thymus serpyllum* L.

Fig. 1.2: Fresh plants of *Thymus serpyllum* L.

Thyme is the general name for many herbs of the *Thymus* species, all of which are small perennial plants native to Europe and Asia. The market demand for thyme is rather high, yearly estimates running at about 500 tons in USA and 1000 tons in Europe. Owing to a general popularity of the use of natural substances instead of synthetic compounds, an increase in that demand is predictable (Meriçli-Ilisulu and Tanker, 1986).

Thymol and carvacrol (Fig. 1.3) constitute the main phenolic compounds of thyme oil. The major non-phenolic compounds are linalool and p-cymene (Piccaglia and Marotti, 1991). It is reported that thymol has the higher medicinal activity, followed by carvacrol and geraniol, but linalool, terpineol and thujone exhibited the least effect (Broucke, 1983). There are several reports in the literature on the chemical composition of thyme oil. Most reports indicate thymol or carvacrol to be the major compounds in the oil (Adilson *et al*., 2004; Lattaoui and Tantaoui, 1994).

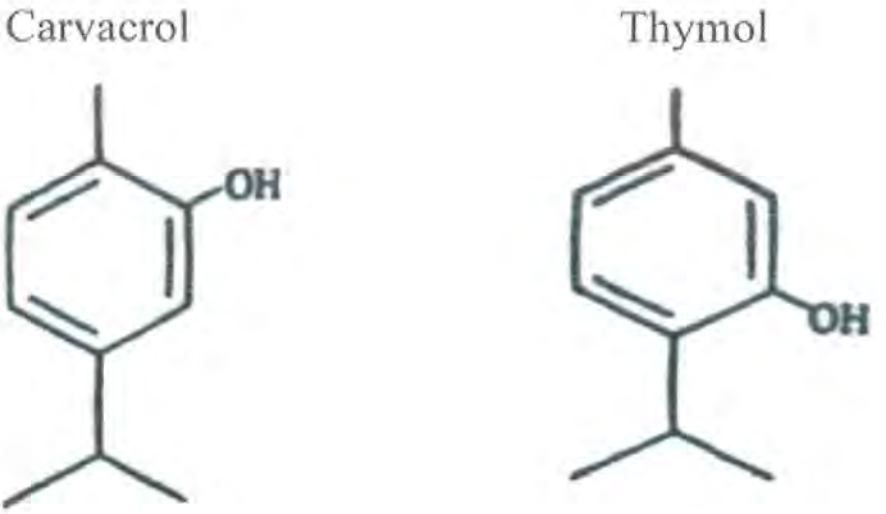


Fig.1.3: Chemical structure of carvacrol and thymol

1.1.1: Taxanomy

The genus *Thymus* belongs to family *Labiatae* (alternate name *Lamiaceae*), sub-family *Nepetoideae* and tribe *Mentheae*. It includes about 350 species worldwide and is widely distributed in temperate zones (Demissew, 1993). There is relatively little diversity in Pakistan and only one specie is recognized. The genus is easily distinguished in Pakistan by its creeping habit (Hedge, 1990).

1.1.2: Morphology

Thymus specie is a perennial dwarf shrub, strongly aromatic. Leaves undivided, entire linear-lanceolate to obovate, nerves prominent beneath, ciliate near base of lamina, sessile or petiolate. Verticillasters 2-many flowered, borne in axils of upper leaves, forming capitate inflorescence. Bracts very small. Flower often male sterile. Calyx strongly bilabiate, 10-13 nerved densely bearded in throat, tubular to campanulate; upper lip broad, 3-toothed, spreading or recurved; lower 2 teeth subulate, prominently ciliate and curved upwards. Corolla bilabiate; tube straight, exannulate; upper lip straight, emarginate; lower lip 3-lobed. Stamens 4, exerted; thecae 2-locular. Style 2-lobed. Nutlets ovoid or oblong (Hedge, 1990).

Thymus serpyllum L. is much branched dwarf, creeping mat-forming herb. Basal branches procumbent with fascicles of leaves at the nodes and short ascending erect flowering shoots. Stems are quadrangular, pilose all round the stem or on the angles only with spreading or shortly retrorse eglandular hairs; flowering stems 2-6cm long. Leaves elliptic obovate to linear lanceolate, 1-11 x 2-5 mm with prominent or obsolete lateral veins, densely dotted with reddish sessile oil globules above and below, glabrous or with few scattered eglandular pilose hairs. Inflorescence ovoid-capitate. Bracts 0.5-1.5 mm. Calyx 3.5 - 4 mm tubular to campanulate, usually purplish with oil globules; lower teeth up to 2mm, subulate. Corolla 0.6mm long, pale lilac, pink purplish or violet, Stamens clearly exerted in hermaphrodite flower. Nutlets pale brown, ovoid, 0.1 x 75mm with a small V shaped attachment scar (Hedge, 1990).

1.1.3: Chemo types

The chemotypes of common thyme (*Thymus vulgaris* L.), which is quite close to wild thyme (*Thymus serpyllum* L.) with respect to the essential oil content, are widely known. Depending upon the source, six or even seven chemotypes are distinguished (Thompson *et al.*, 2003; Pothier *et al.*, 2001). Each chemotype is named after the main component of its essential oil, which is either a phenolic monoterpene, thymol (T) or carvacrol (C), or a non-phenolic monoterpene, linalool (L), thuyanol (U), a-terpineol (A) or geraniol (G). All six are genetically controlled by an epistatic series of five loci and are part of the same biosynthetic pathway (Vernet *et al.*, 1986). In *T. serpyllum*, the essential oil is sequestered within glandular trichomes at the surfaces of leaves, calices and young shoots. Phenolic chemotypes are common close to the Mediterranean Sea, whereas non-phenolic chemotypes predominate in inland sites, particularly above 400m elevation or in basins which experience a winter temperature inversion (Gouyon *et al.*, 1986; Thompson, 2002). Although phenolic chemotypes are generally more toxic than non-phenolic chemotypes to potential herbivores in controlled conditions, such deterrence varies dramatically across the spectrum of different antagonists (Linhart and Thompson, 1999).

1.1.4: Culturing and propagation.

Thymus serpyllum L. (wild thyme) can grow on any soil, but prefers light; sandy or gravel ground exposed to the sun and can tolerate partial shade. Propagate by seeds, cuttings, or division of roots. Care must be taken to weed. Requires farmyard manure in autumn or winter and nitrates in spring. Cut when in full flower in July and August. Bees love the blossom (www.fortunecity.com/roswell/chaney).

1.1.5: Distribution

There are more than 100 *Thymus* taxa distributed throughout Europe. It originates from the Mediterranean Coast and Asia Minor. It is now cultivated in numerous European countries and Russia, Canada, America and some North African countries (Taumara *et al.*,1993). It is cultivated all over the world and has naturalized in some areas including the northeastern US (www.floridata.com/ref/l/laur_nob.cfm).

Spain is the main producer of thyme oil although the oil is produced from *T. zygis* (Bauer *et al.*, 1997).

It is reported that only one specie is grown naturally in Pakistan (Hedge, 1990) but no significant work on this has been reported before. Northern area is rich in medicinal plants including *thymus* specie. There is a rather variable species widespread in Himalayas and reaching its western extremities in Pakistan and Afghanistan. Its two sub species are found in Hazara division (Stewart, 1972). This genus is also naturalized in Dir, Chitral, Swat, Balti, Gilgit, Ladakh, Zanskar, Kashmir, Northern Areas of Pakistan and in many others scattered places of Baluchistan (Hedge, 1990).

1.1.6: Medicinal constituents

Oil of thyme is the important product obtained by distillation of the fresh leaves and flowering tops of *T. serpyllum* L. Its chief constituents are from 20 to 25 per cent of the phenols thymol and carvacrol, rising in rare cases to 42 per cent. The phenols are the principal constituents of thyme oil, thymol being the most valuable for medicinal purposes but carvacrol, an isomeric phenol, preponderate in some oils has been investigated for its bactericidal effect (Ultee *et al.*, 1998). Moreover, due to the phenolic structures of the two principal constituents, the essential oil has shown significant evidence of antioxidant function. The components of the essential oil of wild thyme were determined in the 1960s, and include geraniol, terpineol, citronellol, borneol, linalol, nerolidol, citral, cineol (eucalypol), carvacrol, thymol, bornylacetat, geranylacetat, nerylacetat, linalylacetat, terpinylacetat, citronellal, camphen, -pinen, and limonen (Schratz *et al.*,1965). Nowadays, the main components are considered to be thymol, carvacrol, p-cymol, linalol, a-pinene and other terpenes (Wichtl, 1994). In general, the dried leaves contain 0.7-2.5% oil (Taumara *et al.*,1993). However, if the fresh material cannot be processed immediately, it is dried under controlled temperature conditions and stored at 4°C away from light until oil extraction (Fehrand *et al.*,1979).

1.1.7: Medicinal value

The oil of thyme and its different components are becoming increasingly popular as a naturally occurring antimicrobial and also as an antioxidant agent (Dursun *et al.*, 2003). Thyme showed broad antibacterial activity by inhibiting the growth of both gram-positive and gram-negative bacteria. However, gram positive bacteria *Clostridium botulinum* and *Clostridium perfringens* appeared to be more sensitive than the gram-negative organisms (Nevas *et al.*, 2004). The alcohol and ethanol extracts of thyme, thyme essential oil, thymol and carvacrol were found to have strong inhibition activity against *Bacillus subtilis*, *S. sonnei*, *E. coli* (Fan and Chen, 2001). The significance of aqueous extracts of thyme is that it inhibits the growth of *H. pylori*, reducing its growth (Tabak *et al.*, 1996). The essential oil of thyme, orits constituent thymol, decreased viable counts of *S. typhimurium* on nutrient agar (NA) (Juven *et al.*, 1994). Thymol showed antagonistic effect against *S. sonnei* in anaerobic conditions in vitro (Juven *et al.*, 1994). Carvacrol, a compound present in the essential oil fraction of oreganum and thyme showed adose-related inhibition of growth of the pathogen *Bacillus cereus* (Ultee *et al.*, 2000). The lowest 12 minimum inhibitory concentrations were 0.03% (v/v) thyme oil against *C. albicans* and *E. coli* (Hammer *et al.*, 1999). Thyme extracts exerted no microbicidal activity against *Porphyromous aeruginosa* (Thuille *et al.*, 2003). However, antibacterial growth-inhibitory effect of thyme on *Shigella sonnei* (*S. sonnei*) was noted. The addition of basil and thyme to spaghetti sauce prior to autoclaving and *S. sonnei* inoculation indicated that basil and thyme contributed to the reduction of *S. sonnei* after 16 days at 120°C, but not at 40°C temperatures. This study indicated that pH and NaCl concentrations affect the activity of thyme (Bagamboula *et al.*, 2003). Thyme essential oil exhibited bacteriostatic and bactericidal properties against the non-toxigenic strain of *E. coli* O157:H7 in a broad temperature range. It was found that lecithin diminished the antibacterial properties (Burt and Reinders, 2003). In an *in vitro* antibacterial study, thyme showed greatest inhibition against *A. hydrophila* compared to other psycrotrophic food-borne bacteria such as *Aeromonas hydrophila*, *Listeria monocytogenes* and *Yersinia enterocolitica*. Inhibition of growth was tested by using the paper disc agar diffusion method, while the MIC was determined by the broth micro dilution method (Fabio *et al.*, 2003). Thyme oil was tested for its

antibacterial activity against *Campylobacter jejuni* (*C. jejuni*), *E. coli*, *Listeria monocytogenes*, and *S. enterica* obtained from food and clinical sources and was found most effective against *E. coli*, *L. monocytogenes*, *S. enterica* and *C. jejuni* (Fridman *et al.*, 2002). Feeding thyme leaves to male Wister rats at 2% or 10% of standard diet for 6 weeks showed that thyme leaves were not toxic to rats (Haroun *et al.*, 2002). The powdered aerial parts of the thyme are used to treat cutaneous abcesses and for wound healing (Merzouki *et al.*, 2000).

1.1.8: Allergies

Study has assessed that exposure to thyme dust have potential health hazards to skin. Besides the potential allergenic and or irritant action of thyme, the possible adverse effect of epiphytic microorganisms present in thyme dust has also been observed. In past decades, it has been documented that bacteria and fungi may occur abundantly in airborne organic dusts and cause allergic and / or immunotoxic diseases of the respiratory tract, skin and conjunctiva (Dutkiewicz, 1997). In recent studies, large quantities of airborne bacteria, fungi and bacterial endotoxin were found during thyme threshing, within ranges of 10^4 - 10^5 colony forming units/m^3 (CFU/m^3), 10^3 - 10^4 CFU/m^3, and 10^1 - 10^3 CFU/m^3, respectively (Mackiewicz *et al.*, 1999).

Large amounts of thyme can cause upset stomach, cause headache, dizziness, convulsions (uncontrollable shaking) and coma. It can also cause stop breathing or heart to stop beating (Newall *et al.*,1996) Thyme oil is considered to be toxic because it is very concentrated (strong). The oil should not be taken by mouth (Newall *et al.*, 1996; Blumenthal *et al.*, 2000). Thyme should not be taken by people with intestinal (digestive) problems or heart problems (Fetrow and Avila, 1999). People with allergies to plants such as grass should not take thyme (Fetrow and Avila, 1999). Other possible side effects of thyme oil are irritation of the inside of the mouth and other membranes of the body (Newall *et al.*, 1996). Skin rashes have occurred if thyme is used on the skin (Fetrow and Avila, 1999).

1.1.9: Ecological variation in essential oil contents

Variation in essential oil contents of thyme and its components were investigated by Mensure and Sezen, (1998) during the different growing periods under both lowland and mountainous conditions. The highest drug yields were obtained from lowland conditions during the post flowering stage. Also, essential oil content and its components were significantly affected by both climatic and ecological conditions and various harvest dates. (Mensure and Sezen, 1998). The yield of plant material, the essential oil content and quantitative composition of *thymus* can be influenced by harvest time, ecological and climatical conditions (Cabo *et al.*, 1982; Putievsky *et al.*, 1977). It has been reported that a fairly tight correlation exists between the soil type and the chemotypic structure of the thyme population growing on it. Where the soil type varies, distinct differences among chemotypes can be found over a few meters (Gouyon *et al.*, 1988). Altitude seems to play a role in the case of oil rich and oil-intermediate aromatic plants, affecting their essential oil content. It does not seem to influence oil poor plants (Kokkini *et al.*, 1990). Drug yield, essential oil content and composition in thyme plants showed big variation from years to years because of perennial plants (Merino and Martin, 1990).

1.2: Biological screening

Screening programmes for biologically active natural products require the right bioassays. Detection of compounds with the desired activity in complex plant extracts depends on the reliability and sensitivity of the test systems used. Bioassays are also essential for monitoring the required effects throughout activity-guided fractionation: all fractions are tested and those continuing to exhibit activity are carried through further isolation and purification until the active mono substances are obtained. The search for promising plant extracts and subsequent activity guided isolation put specific requirements on the bioassays to be used. They must be simple, inexpensive and rapid in order to cope with the large number of samples, including extracts from the screening phase and all fractions obtained during the isolation procedure. They must also be sensitive enough to detect active principles which are generally present only in small concentrations in crude extracts. Their selectivity

should be such that the number of false positives is reasonably small (Hostettmann *et al.*, 1995). Following biological assay were used in this project to study the medicinal properties of *Thymus serpyllum* L.

1. Antifungal assay
2. Toxicity testing against the brine shrimp
3. Antitumor assay
4. Lemna bioassay for inhibitors and promoters of plant growth
5. Radish seed bioassay
6. Antibacterial assay

1.3: Antimicrobial assay

A large number of human, animal and plant diseases are caused by pathogenic microbes (fungi, bacteria and algae). Infections due to fungi and bacteria are major cause of death in higher organisms. There is no single, all embracing bioassay to evaluate the antimicrobial activity of a sample. Therefore, the evaluation process generally involves the use of a number of bioassay methods and careful comparison of all the data in order to arrive at an appropriate conclusion (Linton, 1983).

About 70,000 species of fungi have been described; however, some estimates of total numbers suggest that 1.5 million species may exist (Hawksworth, 1991; Hawksworth *et al.*, 1995). A large number of human, animal and plant diseases are caused by pathogenic fungi. Infections due to fungi and bacteria are major cause of death in higher organisms. Some of the pathogenic characteristics of fungi are as follows.

Aspergillus species are ubiquitous fungi (Rinaldi, 1983). Invasive aspergillosis which is often fatal is the second most common opportunistic mycosis, after candidiasis, affecting humans (Rinaldi, 1983). Of the more than 200 *Aspergillus* species known, *Aspergillus fumigatus* and *Aspergillus flavus* are frequently noted to cause infections in immuno-compromised patients. In certain high-risk populations, such as bone marrow transplant recipients (Fridkin *et al.*, 1996; Rhame, 1989), the

the attributable mortality rate approaches 85% to 95% (Fridkin and Jarvis, 1996; Pannuti, *et al.*, 1992). Outbreaks of *Aspergillus* infection have been reported in association with hospital construction (Buffington *et al.*, 1994; Iwen *et al.*, 1994; Loudon *et al.*,1994; Sarubbi *et al.*, 1982), prompting experts to recommend strategies for minimizing dust production in areas where high-risk patients may be exposed (Buffington *et al.*, 1994; Pannuti, 1993; Rhame, 1989; Sarubbi *et al.*, 1982). *Fusarium solani* (seed born pathogens) is commonly considered a contaminant, but disease has been reported in individuals after ingestion of food prepared from seeds had been over grown by species that produce toxins.

As well as being a common contaminant and a well-known plant pathogen, *Fusarium* spp. may cause various infections in humans. *Fusarium* is one of the emerging causes of opportunistic mycoses (Anaissie *et al.*, 1988; Anaissie *et al.*, 1989; De Hoog *et al.*, 2000). The most virulent *Fusarium* spp. is *Fusarium solani* (Mayayo *et al.*, 1999). Keratitis (Deshpande and Koppikar, 1999; Liesegang *et al.*, 1980; Moriarty *et al.*, 1993), endophthalmitis (Goldblum *et al.*, 2000), onychomycosis (Gupta and Summerbell, 1999), cutaneous infections (Romano *et al.*, 1998), particularly of burn wounds, mycetoma, sinusitis (Schell, 2000), pulmonary infections (Rolston, 2001), endocarditis, peritonitis, central venous catheter infections, septic arthritis, disseminated infections (Anaissie *et al.*, 1988; Anaissie *et al.*, 1989) and fungemia (Farina *et al.*, 1999; Guarro *et al.*, 2000; Kovacicova *et al.*, 2001) due to *Fusarium* spp. have been reported (De Hoog *et al.*, 2000).

Twenty years ago, *Candidia species was* commonly regarded as little more than culture contaminant; however, because of developed antimicrobial-resistance, in less than 2 decades this organism has become a major human pathogen (Michael and Pharm, 2001). Fungal yeasts used in this study were chosen primarily based on their importance as opportunistic human pathogens. *Candidia albicans* while naturally occurring in the intestinal flora, can cause oral thrush and systemic infections (Michael and Pharm, 2001).

There are numerous methods for detecting antifungal and antibacterial activities. The currently available methods are:

1) Agar diffusion method (Perez *et al.*, 1990).
2) Agar dilution method (Choudhary *et al.*, 1995).
3) Bioautographic method (Janaki *et al.*, 1998).

1.3.1: Agar diffusion method:

In this method, wells are made in seeded agar plates with the help of borer and the test sample is then introduced directly into these wells. After incubation the diameter of the clear zones around each well is measured and compared against zone of inhibition produced by solution of known concentration of standard antibiotics. Five or six samples may be tested simultaneously by the diffusion method.

1.3.2: Agar dilution method:

In agar dilution method, medium is inoculated with the test organisms and the samples to be tested are mixed with the inoculated medium. The material is inoculated and growth of the microorganisms is viewed and compared with a control culture, which does not contain the same sample. The experiment is repeated with various dilutions of the test sample in the culture medium and the highest dilution at which the sample just prevents the growth of microorganism (MIC) was determined.

1.3.3: Bioautographic procedure:

The bioautographic procedure for screening of antimicrobial activity involves localizing the antibacterial activity on a chromatogram. The antimicrobial agent is transferred from the TLC plate or paper chromatogram to an inoculated agar plate by diffusion and the zones of inhibition visualized.

1.4: Toxicity testing against the brine shrimp

Since most active plant principles are toxic at elevated dose, a possible approach to develop an effective general bioassay might be simply to screen for substances that are toxic to zoological systems. Once such substances have been

isolated, a battery of specific and more sophisticated bioassays could then be employed. Desiring a rapid, inexpensive, in-house bioassay for screening and fractionation monitoring of physiologically active plant extracts, a tiny crustacean, brine shrimp is used as the general bioassay tool as described by Meyer *et al.* (1982).

The brine shrimp lethality assay consists of exposing larvae to plant extracts in saline solution and lethality is evaluated after a day. It is an easy, low cast and safe assay and does not require any special equipment. These features make this a very helpful bench-top assay for the phytochemistry laboratory (McLaughlin *et al.*, 1991). This assay has a wide application in research towards the discovery of cytotoxic and other active principles present in plants (Mongelli, 1996). For example, a very positive correlation between the lethality to brine shrimp and antitumor activity has been established by researchers developing new anti-cancer drugs from the plants at the National Cancer Institute (NCI) in the USA (Anderson, 1991).

This correlation is considered so good that lethality to brine shrimp is recommended by these authors as an effective prescreen to existing cytotoxic and antitumor assays. More recently it has been shown that there is a very good correlation between median lethal concentrations (LC_{50}) of plant extract to brine shrimp larvae and the median lethal dose (LD_{50}) of these same extracts, administrated orally in mice (Parra, 2001). A number of studies have demonstrated the use of the brine shrimp assay to screen plants popularly used as pesticide (Fatope, 1993). Also successfully used to biomonitor the isolation of cytoxic, antineoplastic (Badaway and Kappe, 1997), antimalarial (Parez *et al.*, 1997), insecticidal (Oberlies, 1998) and anti-feedant (Labbe *et al.*, 1993) compounds from plant.

1.5: Antitumor Assay

In the 1960s and 1970s, the search for anticancer agents continued through the use of one or two cytotoxicity assay coupled with an in vivo murine lymphocytic model. As cancer chemotherapy and cell biology evolved, it was found that a number of tumor types were resistant to chemotherapy and agents could be evaluated for their potential to intercede in discrete biological event in tumor genesis or cell proliferation (Geoffrey *et al.*, 1994).

1.5.1: Description and Significance of *Agrobacterium tumefaciens*

At the turn of the century *Agrobacterium tumefaciens* was identified as the causal agent in crown gall disease in dicotyledonous plants. Since then, thorough research has been done on this bacterium's mechanism of tumor induction. In addition, *Argorbacterium* is used in numerous research projects as a means to introduce new genes into the genomes of a number of plants (Tinland and Hohn, 1995).

1.5.2: Genome Structure

Agrobacterium tumefaciens has an unusual chromosomal organization; it has a 2Mb linear and a 2.8Mb circular chromosome as well as a 206-479 kb Ti (tumor-inducing) plasmid. The genes that cause gall formation in plants are located for the most part on the Ti plasmid. Interestingly, if *Agrobacterium* is grown near its maximum temperature of about 30°C, then the plasmid is lost as well as the pathogenicity of the bacterium. The bacterium itself is still functional and can thrive in culture (Deacon, 2005). The genome of *Agrobacterium vitis* is currently being sequenced.

1.5.3: Cell Structure and Metabolism

Agrobacterium tumefaciens is a Gram-negative, non-sporeforming, rod-shaped bacterium. *Agrobacterium* strains use different carbohydrates and are classified into three main biovars. The differences among biovars are mainly determined by the genes on the circular chromosome. When a wound opens on the plant tissue, the motile cells of *A. tumefaciens* move into the tissue by chemotaxis as a response to the release of sugars and other components normally in the roots. While *A. tumefaciens* cells without Ti plasmids recognize and move towards plant wounds, the strains containing the Ti plasmids respond even more strongly because they recognize phenolic compounds such as acetosyringone that come out of the wound (Deacon, 2005).

1.5.4: Ecological behavior

Agrobacterium tumefaciens can generally be found on and around root surfaces known as the rhizosphere. There it seems to use nutrients that leak from the root tissue. It can infect the tissue at wound sites formed from transplanting seedlings, burrowing animals or bugs, etc. *Agrobacterium radiobacter* grows on various explosives such as nitroglycerine, they use this as their sole source of nitrogen. It removes two nitro groups from nitroglycerine by an NADH-dependent oxidoreductase, but can not use the carbon in nitroglycerine for growth because it cannot remove the third nitro group to release glycerol (White *et al.*, 2004).

1.5.5: Crown gall disease

Agrobacterium tumefaciens is most widely known for causing crown gall disease that affects many dicotyledonous (broad-leaved) plants; another strain called *biovar 3* causes crown gall disease in grapevines. The disease causes the formation of tumor-like swellings called galls that can generally be found on the crown of the plant just above the soil. Crown gall disease does not usually seriously harm older plants; however, it may reduce the value of a plant in a nursery. *Agrobacterium tumefaciens* causes crown gall disease by first transferring part of its DNA into an opening in the plant. The DNA attaches itself to the plant's DNA through histones. The DNA then integrates itself into the plant's genome.

When plant tissue is wounded it releases phenols, etc., which activate Ti-plasmid in *A. tumefaciens*. The Ti-plasmid cause plants cells to multiply rapidly without going through apoptosis and causes the formation of the tumors. These tumors (masses) may be spongy or hard, and may or may not have deleterious effect on plant. The tumors produced are histologicaly similar to those tumor found in humans and animals (Agirose, 1997). Because of how smoothly *Agrobacterium tumefaciens* is able to transfer DNA it has become a helpful tool for scientists to use.

1.5.6: Potato disc assay

In nature there are some plants having some metabolites that are capable to inhibit the production of these tumors. Potato disc assay is used to identify the tumor inhibiting potential of plant extract, in this assay potato plant is subjected to infection by *agrobacterium* and then different concentration of test sample (plant extract) are applied, which gives information that the test sample is tumor inhibitor or not. The key organism is *agrobacterium* that causes tumor in plants. McLaughlin, (1991) concludes that the crown gall tumor (potato disc) assay could be used as a fairly rapid, inexpensive and reliable prescreen for antitumor activity.

1.6: Phytotoxicity assay

Various laboratory screening techniques have been developed to measure and quantify allelopathy without the interference of resource competition (Leather and Einhellig, 1986; Navarez and Olofsdotter, 1996; Kawaguchi *et al.*, 1997). Large screenings of germplasm collections require reliable test species. Traditionally, easily grown, sensitive and reliable species such as *Lemna minor*, lettuce (*Lactuca sativa*) and radish (*Raphanus sativus*) seeds have been used as test plants in allelopathy studies (Putnam *et al.*, 1983; Einhellig *et al.*, 1985; Leather and Einhellig, 1985). Recently, chemical identification procedures have become more advanced, and biologically active substances with phytotoxic potential that can explain allelopathic behaviour; have been found (Duke *et al.*, 1998).

The term allelopathy was first introduced by Molisch in 1937 and refers to chemical interactions among plants, including those mediated by microorganisms. This definition, although broad, is reasonable as considerable research has recently indicated the involvement of microorganisms and lower plants in the production of phytochemically-active compounds (Inderjit and Weiner, 2001; Putnam, 1986).

Allelopathy is an important mechanism of plant interference, mediated by the addition of plant produced secondary products to the rhizosphere. Chemicals with allelopathic potential are present in nearly all plants and their respective tissues, including leaves, stems, roots, flowers, seeds, bark and buds. Under the appropriate environmental conditions, these phytotoxins may be released into the environment in sufficient quantities to affect the growth of neighboring plants (Weston, 1996).

Allelopathic interactions in soil environments depend greatly on the turn over rate of allelochemicals in the soil rhizosphere and their interaction with clay, organic matter and other factors which change the physico-chemical and biotic characteristics of the soil (Blum, 1995; Blum and Shafer, 1998). The major concern in using plant species such as radish and lemna is that they have no agronomic importance as weeds and the results have to be confirmed on weed species.

1.6.1: Radish seed bioassay

Radish (*Raphanus sativus* L.) seeds have been used in general toxicity studies because of their sensitivity to phytotoxic compounds (Einhelling and Rasmussen, 1978) and are a standard assay in allelopathic studies (Patterson, 1986). Radish seed was the most sensitive to allelochemicals (De Feo *et al.*, 2003). The radish seed bioassay consists of exposing seeds to plant extracts in petri plates and toxixity is evaluated after a five days. It is an easy, low cast and safe assay and does not require any special equipment. These features make this a very helpful bench-top assay for the phytochemistry laboratory. This assay has a wide application in research towards the discovery of active principles present in plants (Arzu and Camper, 2002).

1.6.2: *Lemna* bioassay for inhibitors and promoters of plant growth

Lemna minor (duckweed) is a miniature aquatic monocot (Rahman, 1991). It belongs to family *Lemnaceae* and also known as duckweed. *Lamna* means water plant and minor means lesser. *Lemna* plant consists of a central oral frond or mother frond with two attached daughter fronds and filamentous root. The fronds consist of one to several conspicuous air spaces and one to several veins. Fronds are flattened, generally symmetrical with smooth upper surface. It has only one root. Fronds

Fronds absorbs nutrients though the whole plant. Duckweed consists of largely metabolically active cells and has very little structure fiber. The tissue contains twice as much protein, fats, nitrogen and phosphorous as compared to other vascular plants. Smaller duckweed is distinguished from larger duckweed by its single root and much smaller size.

It is widely spread through temperate region of the North America, Eurasia, Australia and New Zealand. It spreads rapidly across stable bodies of water rich in nutrients, growing best in water with high levels of nitrogen and phosphate. It can tolerate a wide pH range, but survives at pH 4.5-7.5 and grows in full sunlight as well as dense shade. In the ecosystem, it acts as an essential link in the food chain, useful as a water crop as it can acclimatize itself to almost all growing conditions, with some thriving in manure-rich water. Duckweed reproduces at twice the rate of other vascular plants under ideal conditions, the fronds can double in a few days (www.resk.org/earl /bwac/nature /aquatic/lemna.htm-24k).

The *Lemna minor* is used in various complains like; throat inflammation, twisting pain in nose and disturbance of bowels to set in after a dose of lemna (Edward, 2002). The *lemna* bioassay is quick measurement of phytotoxicity of the materials. It is a useful primary screening for weedicide research. In this bioassay the growth stimulating effects of test samples are also determined. It has been observed that natural antitumor compounds can inhibit the *Lemna* growth, it was also discovered that some substances stimulate frond proliferation, and the assay might be useful to detect new plant growth stimulants (Lewis *et al.*, 1995).

1.9: Objectives

A main objective was to evaluate the medicinal values of *Thymus serpyllum* L. For this purposes aim was to evaluate antifungal, cytotoxic, antitumor, phytotoxic, antibacterial activities of *Thymus serpyllum* L.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

The present research work was carried out in the Molecular Biology Laboratory, Department of Biological Sciences, Quaid-i-Azam University, Islamabad. A brief account of materials as well as procedures used in it is described below.

2.1. Plant material

Plant material was collected from upper Mahu dand lake, Kalam valley District Abbotabad, Pakistan on 11th Aug. 2004. The plants were identified as *Thymus serpyllum* L. by Dr. Mir Ajab Khan (Taxonomist), Department of Biological Sciences, Quaid-i-Azam University Islamabad. He followed the Flora of Pakistan for determination of name of *Thymus* specie.

2.2. Extraction

Fresh aerial plant material was taken, rinsed with distilled water and kept under shade till drying. Aerial parts of the plants were separated and weighed.

2.2.1. Extraction from aerial part

Extraction from the aerial parts was carried out by simple maceration process. In total 500g of the aerial parts were taken and ground in 3.0 liters of methanol using kitchen blender. The poorly homogenized mixture was kept for 4 weeks at room temperature (25°C ± 2°C) in extraction bottle. After 4 weeks, maximum amount of methanol was separated from the mixture. Filtrate was filtered twice, first using ordinary filter paper and then Whatman-41 filter paper. Methanol was completely evaporated at room temperature. After evaporation 3.94gm methanolic extract of aerial parts was obtained.

2.3. Antifungal assay

The agar tube dilution method was used for antifungal activity of plant extract as reported by Choudhary *et al.* (1995).

2.3.1. Microorganisms used

Ten strains of fungus were used which were *Mucor species* (0300), *Aspergillus nigar* (0198), *Aspergillus flavus* (0064), *Aspergillus flavus g.*, *Aspergillus fumigatus* (66), *Fusarium moniliforme* (0056), *Alternaria species* (0297), *Fusarium solani*, *Candida albicans* and *Candida glabarata*. The fungus was maintained on Sabouraud dextrose agar medium at 4°C.

2.3.2. Preparation of samples

The sample prepared for antifungal assay was of methanolic extract of aerial part. A total of 72mg of the extract was dissolved in 3mL of DMSO to get 24mg/mL concentration. 66.6µL of this stock solution was used for each test tube containing 4mL media. Solutions of terbinafine and clutrimazole, 12mg/mL in DMSO, were prepared for positive control. Pure DMSO was used as negative control.

2.3.3. Media for fungus

Sabouraud dextrose agar (Merck) was used to grow fungus for inoculums preparation. Its composition was:

a) Peptone complex = 10 gm / L

b) Glucose = 40 gm / L

c) Agar =15 gm / L

2.3.4. Preparation of media for fungus

Sabouraud dextrose agar (Merck) was prepared by dissolving 6.5gm/100mL in distilled water; pH was adjusted at 5.6. The contents were dissolved and dispensed as 4mL volume into screw capped tubes and were autoclaved at 121°C for 20 minutes.

2.3.5. Loading of sample:

Tubes were allowed to cool to 50 °C and non-solidified SDA is loaded with 66.6 µL of compound pipette from the stock solution. This will give the final concentration of 400 and 200 µg /mL of the crude extract and pure compound respectively in media. Tubes were then allowed to solidify in slanting position at room temperature. Tubes were prepared in triplicate for each fungus species

2.3.6. Inoculation of fungus, incubation and measurement of growth inhibition

Each tube was inoculated with 4mm diameter piece of inoculums, removed from a seven days old culture of fungus. Other media supplemented with DMSO and reference antifungal drugs were used as negative and positive control respectively. The tubes were incubated at 27-29 °C for 7-10 days. Cultures were examined twice weekly during the incubation. Growth in the media was determined by measuring linear growth (mm) and growth inhibition was calculated with reference to negative control.

$$\text{Percentage inhibition of fungal growth} = 100 - \frac{\text{Linear growth in test (mm)}}{\text{Linear growth in control (mm)}} \times 100$$

2.4. Toxicity testing against the brine shrimp

The method used for brine shrimp lethality bioassay was as reported by Mclaughlin *et al.* (1990).

2.4.1. Sample preparation

Sample was prepared by dissolving 60mg of methanolic plant extract in 4mL of methanol and the volume was raised up to 6mL (10mg/mL or 10,000ppm) stock solution. From the stock solution further dilutions (1000ppm, 100ppm) were made as given in Table 2.1.

Table No. 2.1: Dilutions prepared for brine shrimp toxicity assay

Concentration (ppm)	**Stock solution (mL)**	**Solvent (mL)**	**Final volume (mL)**
10,000	2.00	0.00	2.00
1000	0.2	1.8	2.00
100	0.02	1.98	2.00

2.4.2. Preparation of artificial seawater

Artificial seawater was prepared by dissolving 28gm commercial sea salt (Harvest Co. H. K.) in 1 liter distilled water with continuous stirring. It was aerated for two hours by vigorous shaking on magnetic stirrer.

2.4.3. Hatching shrimps

Brine shrimp (*Artemia salina*) eggs (Sera, Heidelberg, Germany) were hatched in shallow rectangular dish (22x32 cm) filled with prepared seawater. A plastic divider with 2mm holes was clamped in the dish to make two unequal compartments. The eggs (about 25mg) were sprinkled in the larger compartment which was darkened (covered with aluminum foil), while the smaller compartment was illuminated. After 24 hours of starting hatching, phototropic nauplii (brine shrimp larvae) were collected by pipette from the lightened side having been separated by the divider from their shells.

2.4.4. Bioassay

Vials were used for this bioassay. 0.5mL of the each solution (10,000ppm, 1000ppm, and 100ppm) was taken in the vials and methanol was vacuum evaporated. Residue was re-solublized in 2mL of seawater. Ten shrimps were transferred to each vial using pasteur pipette, and the volume was raised up to 5mL. It made the concentration of each vial 1000ppm, 100ppm, 10ppm, respectively. Three replicates were prepared for each concentration. Negative control was prepared by taking 0.5mL of solvent (methanol) in vial and methanol was vacuum evaporated. The nauplii can be counted macroscopically in the stem of pipette against a light background. The vials were maintained under illumination at room temperature 25°C to 28°C. Survivors were counted with the aid of 3x magnifying glass after 24 hours.

2.4.5. ED_{50} Determination:

The resulting data were analyzed by probit analysis (Finny, 1971) for the determination of ED_{50} value for the extract.

2.5. Antitumor Assay

The potato disc method was used for antitumor activity of plant extract as reported by Ferrigini *et al.* (1982).

2.5.1. Preparation of bacterial culture

Luria broth (Miller's LB broth) medium was prepared by dissolving 25gm/L. in distilled water; pH was adjusted at 7.0 and was autoclaved in 100mL flask. A total of 5µL of 50mg/mL rifampicin from the stock solution was added. In the final volume concentration of rifampicin was 10µg/mL. Single colony from culture plate of *agrobacterium tumefaciens* (At10) was inoculated and culture was grown for 48 hours at 28°C in shaking incubator.

2.5.2. Sample preparation

Sample was prepared by dissolving 60mg of methanolic plant extract in 4mL of DMSO and the volume was raised up to 6mL (10mg/mL or 10,000ppm) to make stock solution. From the stock solution further dilutions (1000ppm, 100ppm) were made as given in Table 2.2.

Table No. 2.2: Dilutions prepared for antitumor assay

Concentration (ppm)	**Stock solution (mL)**	**Solvent (mL)**	**Final volume (mL)**
10,000	2.00	0.00	2.00
1000	0.2	1.8	2.00
100	0.02	1.98	2.00

2.5.3. Preparation of inoculum

To prepare final concentration of 10ppm, 100ppm and 1000ppm, 5mL of inoculum was prepared from initial stocks by adding 0.5mL of each of the stock solution in three autoclaved test tubes. Add 2.5mL of autoclaved distilled water and 2mL of bacterial culture.

2.5.4. Preparation of control solutions

In this experiment we used three controls:

1. Negative control

Negative control was prepared by taking 0.5mL of DMSO in test tube and adding 2mL of bacterial culture and 2.5mL of autoclaved distilled water in it.

2. Positive control

Positive control was prepared by taking 0.5mL of DMSO in test tube and adding 4.5mL of autoclaved distilled water in it.

3. Blank

Blank was prepared by taking 5mL of autoclaved distilled water in test tube.

All of the solutions were prepared in Laminar flow hood by considering all precautionary measures to avoid contamination.

2.5.5. Preparation of agar plates

Plane agar medium was prepared by dissolving 15gm/L of plane agar in distilled water and was autoclaved. For one plant extract 6 petri plates were used. Three plates for three concentrations (1000ppm, 100ppm, 10ppm) and three plates for three control. 25mL, 1.5% autoclaved agar solution was poured in each petri plate and allowed to solidify.

2.5.6. Preparation of potato discs

Surface sterilization of red skinned potato was done in 0.1% mercuric chloride solution in 1L beaker for 2-3 minute. Red skinned potato was then rinsed thrice with autoclaved, distilled water and dried. Cylinders of potato were made with the help of sterilized borer. These cylinders were washed in autoclaved distilled water in another petri plate. Both ends of these cylinders were cut about 1cm with the help of sterilized blade and were discarded. The 5mm thick discs of these potato cylinders were made in petri plates. Discs were washed with autoclaved distilled water and placed on solidified agar plates (10 discs per plate). 50µL of inoculum was added on surface of each disc of respective concentration as well as controls. The inoculum was allowed to diffuse for 10-20 minutes. Petri plates were wrapped with parafilm to make the

plates air tight. Placed these petri plates in an incubator at 28°C for 21 days. The experiment was repeated thrice.

2.5.7. Staining procedure

Lugol's solution was prepared in distilled water (10% KI, 5% Iodine). The discs were covered with lugol's solution for staining purpose and were allowed for 5 minutes to diffuse. The discs were examined under dissecting microscope with side illumination of light. Detained portion of discs are actually tumors. Number of tumors per disc was counted. Percentage inhibition for each concentration was determined by using the following formula. Twenty percent tumor inhibition was considered significant.

$$\text{Percentage of inhibition} = 100 - \frac{\text{No of tumor with sample}}{\text{No of tumor with control}} \times 100$$

2.7. Radish seed Bioassay

The method for radish seed bioassay was followed as reported by Arzu *et al.* (2002)

2.7.1. Preparation of samples

For radish (*Raphanus sativus* L.) seed germination, two different parameters were determined, namely root length and percent of seed germination. Two different concentrations were used for each parameter. 500mg of the extract was dissolved in 50mL of solvent (methanol) to get 10,000mg/L concentration. This stock solution was used for further two dilutions with methanol as shown in Table 2.3. Pure methanol was used as negative control.

Table No. 2.3: Dilutions prepared for radish seed bioassay

S. No.	Concentration (mg/L)	Stock Solution (mL)	Methanol (mL)	Final volume (mL)
1	10,000	30.0	00.00	30.0
2	75,00	22.5	07.50	30.0
3	1,000	03.0	27.00	30.0

2.7.2. Surface sterilization of radish seeds

Surface sterilization of seeds was done in 0.1% mercuric chloride solution in 1L beaker for 2-3 minutes. Seeds were then rinsed thrice with autoclaved distilled water and dried with sterilized blotting paper.

2.7.3. Bioassay

In the first experiment for the effect of test extract on radish seedling root length, two different concentrations (10,000mg/L and 10,00mg/L) were used. 5ml of each concentration was added to sterilize 10cm petri plates containing a sterilized filter paper (whatman#1). Methanol was vacuum evaporated. Then 5ml autoclaved distilled water was added to each plate. Three replicates were prepared for each concentration. For control 5mL of methanol was added to sterilized 10cm petri plates containing a sterilized filter paper (whatman#1). Methanol was vacuum evaporated. Then 5mL autoclaved distilled water was added to each plate. Three replicates were prepared for control.

Sterilized twenty radish seeds were placed in each plate. Petri plates were incubated in dim light at 25°C. Root length was measured with the help of scale after 1, 3 and 5 days. The experiment was repeated thrice.

In the second experiment for the effect of test extract on radish seedling germination, two different concentrations (75,00mg/L and 10,00mg/L) were used. 5mL of each concentration was added to sterilized 10cm petri plates containing a sterilized filter paper (whatman#1). Methanol was vacuum evaporated. Then 5mL

autoclaved distilled water was added to each plate. Three replicates were prepared for each concentration. Sterilized 100 radish seeds were placed in each plate. Petri plates were incubated in the dark at room temperature, 25±2°C. Germination was recorded every day from the 1st to the 5th day. Germinated seeds were removed from the plates and counted. Experiment was repeated thrice. The results were statistically analyzed using ANOVA and Duncan's Multiple Range Test (SAS, 1996).

2.6. *Lemna* Bioassay for inhibitors and promoters of plant growth

The method used for *lemna* bioassay for inhibitors and promoters of plant growth was as reported by Rahman *et al.* (2003)

2.6.1. Media for *Lemna minor*

Inorganic medium was used to grow *Lemna minor* L. for inoculum preparation. Its composition is shown in Table 2.4.

Table No. 2.4: Composition of E-medium

Constituents	**Chemical names**	**mg/L**
Potassium dihydrogen phosphate	KH_2PO_4	6800
Potassium nitrate	KNO_3	15150
Calcium nitrate	$Ca(NO_3)_2.4H_2O$	11800
Magnesium sulfate	$MgSO_4.7H_2O$	4920
Boric acid	H_3BO_3	28.6
Manganous chloride	$MnCl_2.4H_2O$	36.2
Ferric chloride	$FeCl_3.4H_2O$	54.0
Zinc sulfate	$ZnSO_4.5H_2O$	2.2

Copper sulfate	$CuSO_4.5H_2O$	0.8
Sodium molybdate	$Na_2MoO_4.2H_2O$	1.2
Ethylene diamine tetra acetic acid	EDTA	112

E-medium was prepared by mixing various constituents in 1L distilled water and pH was adjusted at 5.5 to 7.0 by adding KOH pellets. Then working E-medium was prepared by mixing 100 mL of stock solution and 900 mL of tap water

2.6.2. Preservation and cultivation of *Lemna* plants

Cultivation of *Lemnaceae* generally includes cleaning of the new clones in water and cultivation under optimal conditions for one or two days. In this way a good number of healthy fronds can be obtained.

2.6.3. Preparation of samples

A total of 30mg of the extract was dissolved in 1.5mL of methanol to get 20mg/mL concentration. For the preparation of drug stock solution, 30mg of compound (paraquate) was dissolved in 1.5mL of methanol to get 20mg/mL concentration.

2.6.4. Preparation of control solutions

In this experiment we use two controls

1. Positive control

Paraquate (standard drug) serving as a positive control. Three flasks were used for positive control. Each flask was inoculated with 10, 100 and 1000µL of the solution from the standard drug stock solution and methanol was vacuum evaporated.

2. Negative control

Negative control was prepared by taking 10, 100 and 1000µL of solvent (methanol) in three flasks and methanol was vacuum evaporated.

2.6.5. Bioassay

Three flasks were used for this bioassay. Each flask was inoculated with 10, 100 and 1000µL of solution from the stock solution for 10, 100 and 1000µg/mL and methanol was vacuum evaporated. Residue was re-solublized in 40mL working E-medium. It made the concentration of each flask up to 1000ppm, 100ppm, 10ppm, respectively. Three replicates were prepared for each concentration. Ten plants of *lemna minor*, each containing a rosette of two fronds, were placed to each flask. A volume of 40mL working E-medium and ten plants of *lemna minor*, each containing a rosette of two fronds, were also placed in flasks containing solvent and reference standard drug serving as negative and positive control, respectively. The flasks were placed in growth chamber for 7 days. Plants were examined daily during incubation period, and number of fronds per flask was counted. The %age inhibition for each concentration was determined by using the following formula.

$$\text{Percentage of inhibition} = 100 - \frac{\text{No of fronds in test}}{\text{No of fronds in control}} \times 100$$

Growth inhibition above 70% was considered significant.

2.8. Antibacterial assay

The agar well diffusion method was used to screen for antibiotic activity (Perez *et al.* 1990).

2.8.1. Preparation of samples

A total 250mg of the extract was dissolved in 10mL of DMSO to get 25mg/mL concentration. This stock solution was used for further dilutions with DMSO as shown in Table 2.5. Solution of Roxithromycin and Cefixime-USP, 2mg/mL in DMSO, were prepared for positive control. Pure DMSO was used as negative control.

Table No. 2.5: Dilutions prepared for antibacterial assay

S. No.	Concentration (mg/mL)	Stock Solution (mL)	DMSO (mL)	Final volume (mL)
1	25.00	1.00	0.00	1.0
2	20.00	0.800	0.20	1.0
3	15.00	0.60	0.40	1.0
4	10.00	0.40	0.60	1.0
5	07.00	0.28	0.72	1.0
6	05.00	0.20	0.80	1.0
7	02.00	0.08	0.92	1.0
8	01.00	0.04	0.96	1..0

2.8.2. Media for bacteria

1. Nutrient Broth medium (Merck) was used to grow bacteria for inoculums preparation. Its composition was:

    a) Peptone from meat = 5 gm / L

    b) Meat extract = 3 gm / L

2. Nutrient agar medium (Merck) was used to perform antibacterial assay. Its composition was:

    a) Peptone from meat = 5 gm / L

    b) Meat extract = 3 gm / L

    c) Agar-agar = 12gm / L

2.8.3. Preparation of media for bacteria

Nutrient broth medium was prepared by dissolving 8.0gm/L nutrient broth in distilled water; pH was adjusted at 7.0 and was autoclaved. Nutrient agar medium was prepared by dissolving 20gm/L of nutrient agar in distilled water; pH was adjusted to 7.0 and was autoclaved.

2.8.4. McFarland 0.5 $BaSO_4$ turbidity standard

The standard was prepared by adding 0.5mL, 0.048M $BaCl_2$ to 99.5mL, 0.36N H_2SO_4. Barium sulfate turbidity standard (4 to 6mL) was taken in screw capped test tube and was used to compare the turbidity (Koneman, 1988).

2.8.5. Microorganisms used

Eight strains of bacteria were used, Three were gram positive, which were *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis* (ATCC 6633) *and Micrococcus luteus* (ATCC 10240) and five were gram negative, which were *Escherichia coli* (ATCC 15224), *Salmonella setubal* (ATCC 19196), *Pseudomonas picketii* (ATCC 49129), *Bordetella bronchiseptica* (ATCC 4617), and *Enterobacter aerogens* (ATCC 13048). The organisms were maintained on nutrient agar medium at 4°C.

2.8.6. Preparation of inocula

Centrifuged pallets of bacteria from twenty-four hours old culture in nutrient broth (SIGMA) of selected bacterial strain was mixed with physiological saline and turbidity was corrected by adding sterile physiological saline until a McFarland 0.5 $BaSO_4$ turbidity standard [10^8 colony forming unit (CFU) per mL]. This inoculum was used for seeding the nutrient agar.

2.8.7. Preparation of seeded agar plates

Nutrient agar medium was prepared by suspending nutrient agar (MERCK) 20gm in 1 liter distilled water; pH was adjusted at 7.0 and was autoclaved. It was allowed to cool up to 45°C. Then it was seeded with 10mL of prepared inocula to have 10^6 CFU per mL. Petri plates (14 cm) were prepared by pouring 75 mL of seeded nutrient agar and allowed to solidify. Eleven wells per plate were made with sterile cork borer (8mm).

2.8.7. Pouring of test solutions, incubation and measurement of zone of inhibition

Using Micropipette, 100µl. of test solutions was poured in respective well. Eight concentrations of extract, two solutions for positive control (Roxithromycin and Cefixime-USP, one for each) and one for negative control (DMSO) was applied to each petri plate. These plates were incubated at 37°C. After 24 hours and 48 hours of incubation, the diameter of the clear zones showing no bacterial growth around each well was measured. Triplicate plates were prepared for each strain. Mean clear zone of these plates was calculated with standard deviation. Antibacterial activity of all dilutions of extract was determined against eight bacterial strains.

CHAPTER 3

RESULTS

RESULTS

3.1 Antifungal Assay

Methanolic extract of *Thymus serpyllum* L was tested against ten fungal strains. Linear growth inhibition or any morphological changes were observed. Methanolic plant extract had an impact on % inhibition of test fungi (Table 3.1). Moderate activity was seen against *Fusarium moniliforme, Alternaria species, Candida albicans, Candida glabarata* and *Fusarium solani* while low activity was seen against *Mucor species* and *Aspergillus flavus* (Fig. 3.1). Methanolic extract of *Thymus serpyllum* L. showed no linear growth inhibition but inhibited spore formation of *Aspergillus flavus g. Aspergillus nigar and Aspergillus fumigatus* (Fig.3.2). Clutrimazole (standard drug) showed 100 percent inhibition against *Candida albican* and *Candida glabarata*. Terbinafine (standard drug) showed 100 percent inhibition against *Fusarium moniliforme, Alternaria specie, Fusarium solani, Mucor specie, Aspergillus flavus, Aspergillus flavus g., Aspergillus nigar and Aspergillus fumigatus*

$$\text{Percentage inhibition of fungal growth} = 100 - \frac{\text{Linear growth in test (mm)}}{\text{Linear growth in control (mm)}} \times 100$$

Criteria for significance:

Below 40 % Inhibition = Low Activity
40-60 % Inhibition = Moderate Activity
60-70 % Inhibition = Good Activity
70 % and above = Significant Activity

Table 3.1: In vitro antifungal bioassay

S.No.	Name of the Fungus	Linear Mean Growth in Neg.control (mm)	Linear Mean Growth with extract (mm)	Mean inhibition % ± S.E	Standard Drug	Morphological changes
1	*Fusarium moniliforme*	90.0±0.60	45.5±0.57	51 ± 0.57	Turbinafine	-
2	*Alternaria species*	48.0±1.15	26.6±0.60	45 ± 1.15	Turbinafine	-
3	*Candida albicans*	100±0.00	49.0±0.57	51 ± 1.15	Clutrimazole	-
4	*Candida glabarata.*	100±0.00	53.0±0.60	47 ± 0.88	Clutrimazole	-
5	*Fusarium solani*	85.0±0.60	50.0±0.60	41 ± 0.57	Turbinafine	-
6	*Mucor species*	100±0.00	90.0±0.60	10 ± 0.57	Turbinafine	-
7	*Aspergillus flavus*	100±0.00	73.3±0.60	27 ± 1.73	Turbinafine	-
8	*Aspergillus flavus g.*	100±0.00	100±0.00	0	Turbinafine	Spore formation inhibition
9	*Aspergillus nigar*	100±0.00	100±0.00	0	Turbinafine	
10	*Aspergillus fumigatus*	100±0.00	100±0.00	0	Turbinafine	

*The data represents the mean values of three replicates

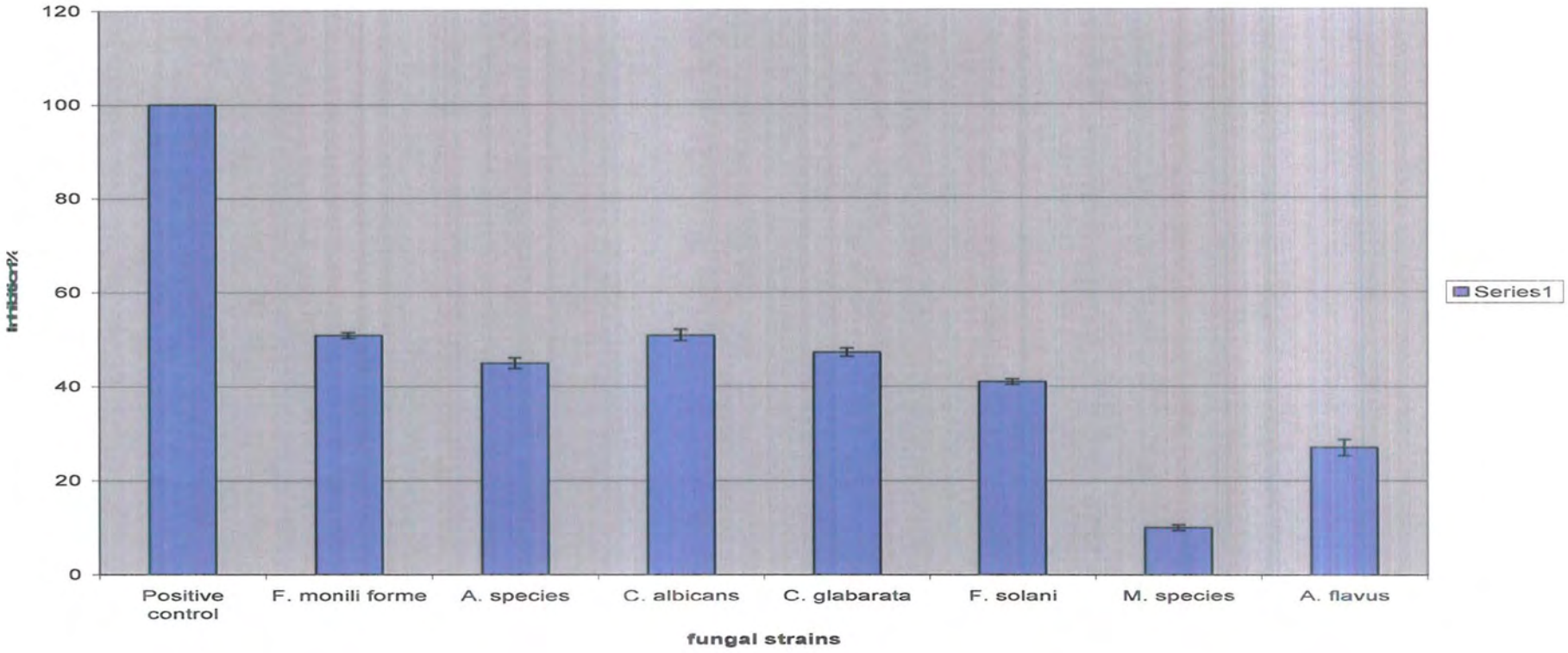


Fig. 3.1: Effect of different concentrations of the plant extract on % inhibition of different fungus strains

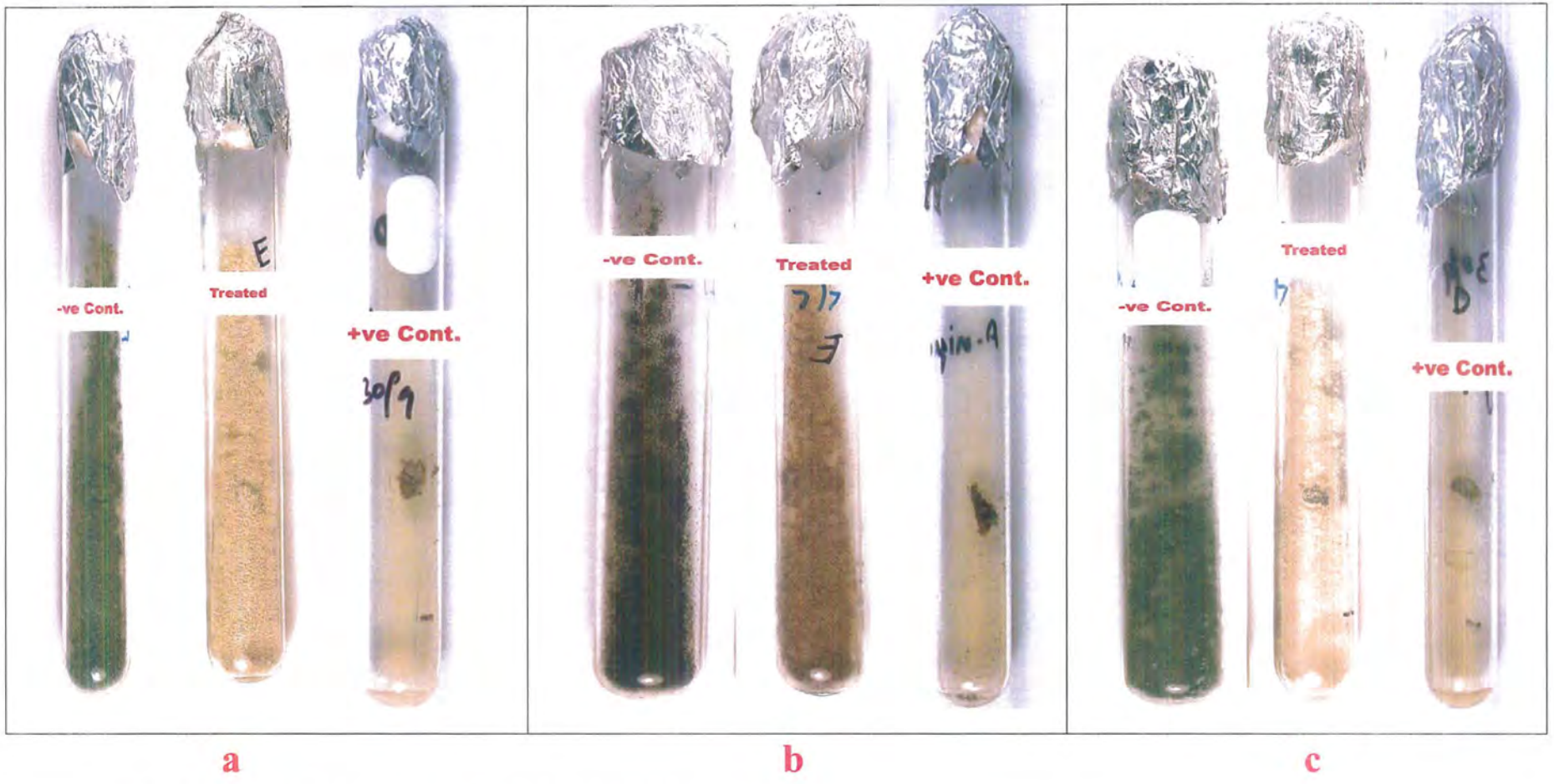


Fig. 3.2: Study of spore formation inhibition of fungus species

(a) Spore formation inhibition of *Aspergillus flavus green* at 24 mg/ml plant extract concentration

(b) Spore formation inhibition of *Aspergillus nigar* at 24 mg/mL plant extract concentration

(c) Spore formation inhibition of *Aspergillus fumigatus* at 24 mg/mL plant extract concentration

3.2. Brine shrimp assay

Methanolic extract of *Thymus serpyllum* L. were tested against brine shrimp for brine shrimp lethality assay. Methanolic plant extract had a highly significant (ED<1000ppm) impact on % death (Table 3.2). Results of the brine shrimp lethality assay are shown in table 3.2. The extract has ED_{50} values 466 ppm and 19.3% death at concentration of 10 ppm, 27% death at concentration of 100 ppm and 50% death at 1000 ppm (Fig 3.3).

Table 3.2: Percent Death and ED_{50} of brine shrimp assay.

S. No.	Conc. (ppm)	No. of shrimps	Mean no. of survivors± S.E	% death	ED50
1	Control	10	8.66±3.84	14	466.35
2	10	10	7.00±0.00	19.26	
3	100	10	6.33±3.84	26.95	
4	1000	10	4.33±3.84	50.02	

The data represents the mean values of three replicates.

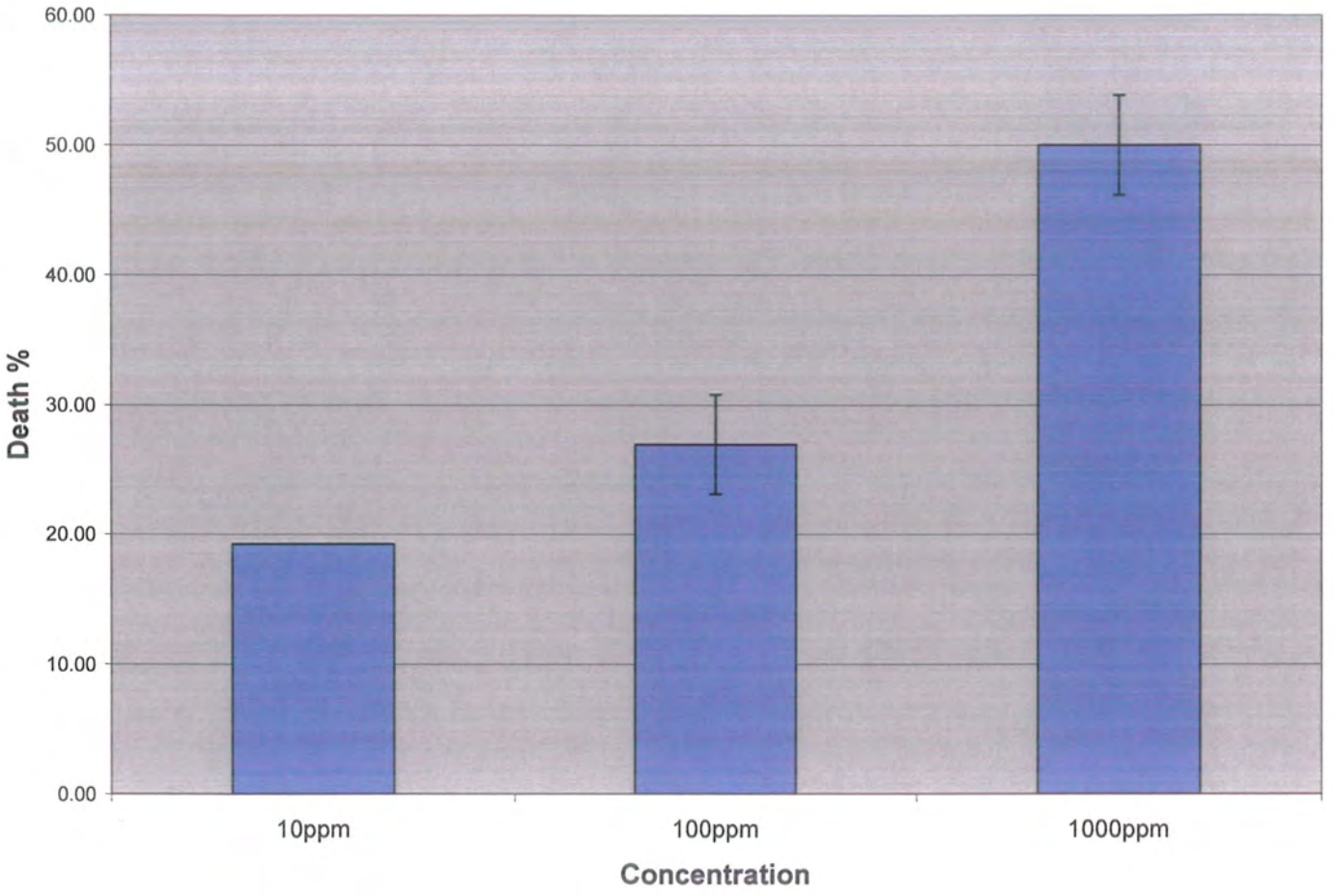


Fig. 3.3: Effect of different the concentrations of the plant extract on % death of brine shrimp

BNSM LIBRARY
Quaid-i-Azam University
Reference Section

3.3: Antitumor Assay

An *Agrobacterium tumefaciens* (At10) strain was used for antitumor assay. Tumor formation was observed after 12th and 21 day's incubation period. Large numbers of tumors were observed; showing differences in different plant extract concentrations and time of incubation. Major observations are described below:

3.3.1: Factors affecting tumor formation

This study was conducted in factorial experiment (10 X4 X 2) to see the effect of different concentrations and incubation time on tumor formation.

3.3.2: Effect of different plant extract concentrations

Methanolic extract of *Thymus serpyllum* L. had a highly significant ($P<0.05$) impact on tumor inhibition (Table 3.3). Mean values of tumor formation were higher in control as compared to extract treated. Discs untreated with plant extract showed maximum number of tumors formation while extract at 10ppm, 100ppm and 1000ppm showed about 2.7, 1.7 and 1.1 numbers of tumors formation respectively. Tumors formation differed highly significantly (LSD value 0.8819) at control, 10ppm and 100ppm but not at 100ppm and 1000ppm (Table 3.4 and Fig. 3.4).

3.3.3: Effect of incubation period

Incubation period had a highly significant ($P<0.05$) impact on tumors formation (Table 3.3). Tumors formation differed significantly (LSD value 0.8819) in 12th and 21st day of incubation period (Table3.4). The overall mean values of tumors were about 2 in 12th day and about 3 in 21st day of incubation period (Table 3.4 and Fig. 3.5).

3.3.4: Interaction effect between concentrations and incubation period

The interaction effect between the two factors i.e. concentrations and incubation period was highly significant ($P<0.05$) as shown in table 3.3. Potato discs untreated with extract yielded the highest number of tumors at 21st day while the number decreased significantly to 3.8 in the same discs untreated with extract at 12th day of incubation period (Table 3.4 and Fig. 3.5).

Discs treated with extract at 10ppm showed an average number of 2.3 tumors at 12th day of incubation periods while the number increased significantly to 3.1 in the same discs treated at 21st day of incubation period (Table 3.4). Discs treated with plant extract at 100ppm showed about 1.4 number of tumor formation at 12th day incubation period while number of tumor did not differ significantly at 21st day incubation period (Table 3.4). In the same way discs treated with plant extract at 1000ppm showed about 0.5 number of tumor formation at 12th day incubation period while number of tumors did not differ significantly at 21st day incubation period (Table 3.4).

Methanolic extract were also tested for antibacterial activity against *Agrobacterium tumefaciens* (AT 10). Eight concentrations (max. 15mg/ml and min. 1mg/ml) of extract were tested against this bacterium. Our results showed no antibacterial activity against this strain of *Agrobacterium*.

Table 3.3: Analysis of variance for factors affecting tumor formation

Source of variation	df	Sum of Squares	Mean Square	F Value	P-value
Concentrations	3	139.338	46.446	23.7339	0.0000
Incubation time	1	19.013	19.013	9.7154	0.0026
Concentrations ×Incubation time	3	2.637	0.879	0.4493	
Error	72	140.900	1.957		
Total	**79**	**301.888**			

Coefficient of Variation: 55.13%

Table 3.4 Effect of the extract concentration, incubation time and interaction of incubation time and concentration on mean value of tumors ($\alpha = 0.05$).

Concentration(ppm)	Mean value of tumor ± S.E	Ranked order
Control	4.60±0.93	A
10	2.70±0.58	B
100	1.70±0.96	C
1000	1.10±0.89	C
LSD value	**6.235**	
Time(days)		
12 days	2.05±1.80	B
21 days	3.02±1.56	A
LSD value	**6.235**	
Interaction between concentration(ppm) and time of incubation (days)		
control ×12days	3.8±0.63	AB
control ×21days	5.4±0.30	B
10 ppm ×12days	2.3±0.37	BC
10 ppm ×21 days	3.1±0.21	CD
100 ppm ×12 days	1.4±0.34	CD
100 ppm ×21days	2.1±0.62	DE
1000 ppm ×12 days	0.7±0.46	DE
1000 ppm ×21days	1.5±0.43	E
LSD value	**1.247**	

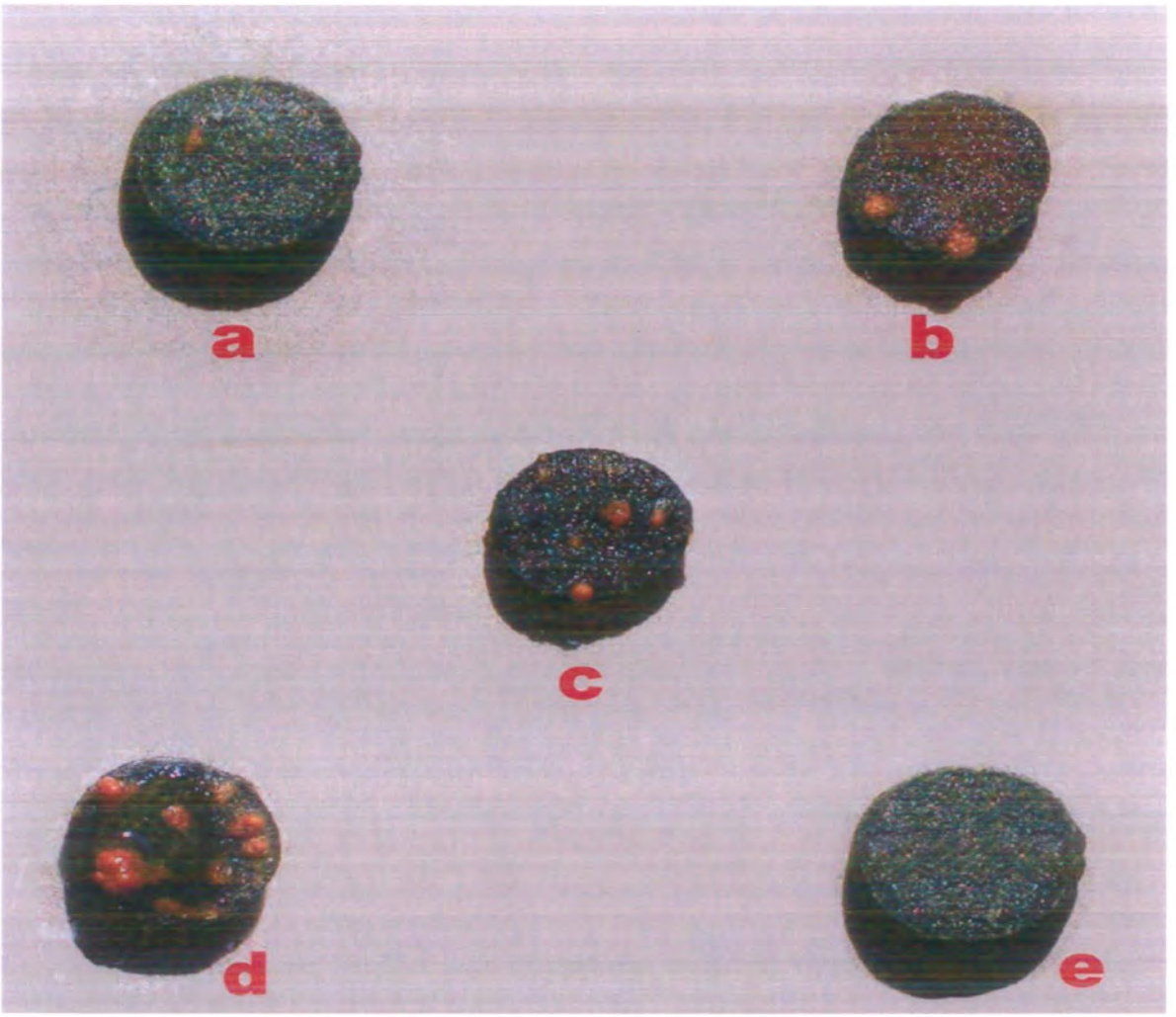


Fig. 3.4: Antitumor assay: Tumors observed on 21st day of incubation with

a) 1000 ppm concentration of the plant extract.

b) 100 ppm concentration of the plant extract.

c) 10 ppm concentration of the plant extract.

d) –ve control

e) Blank

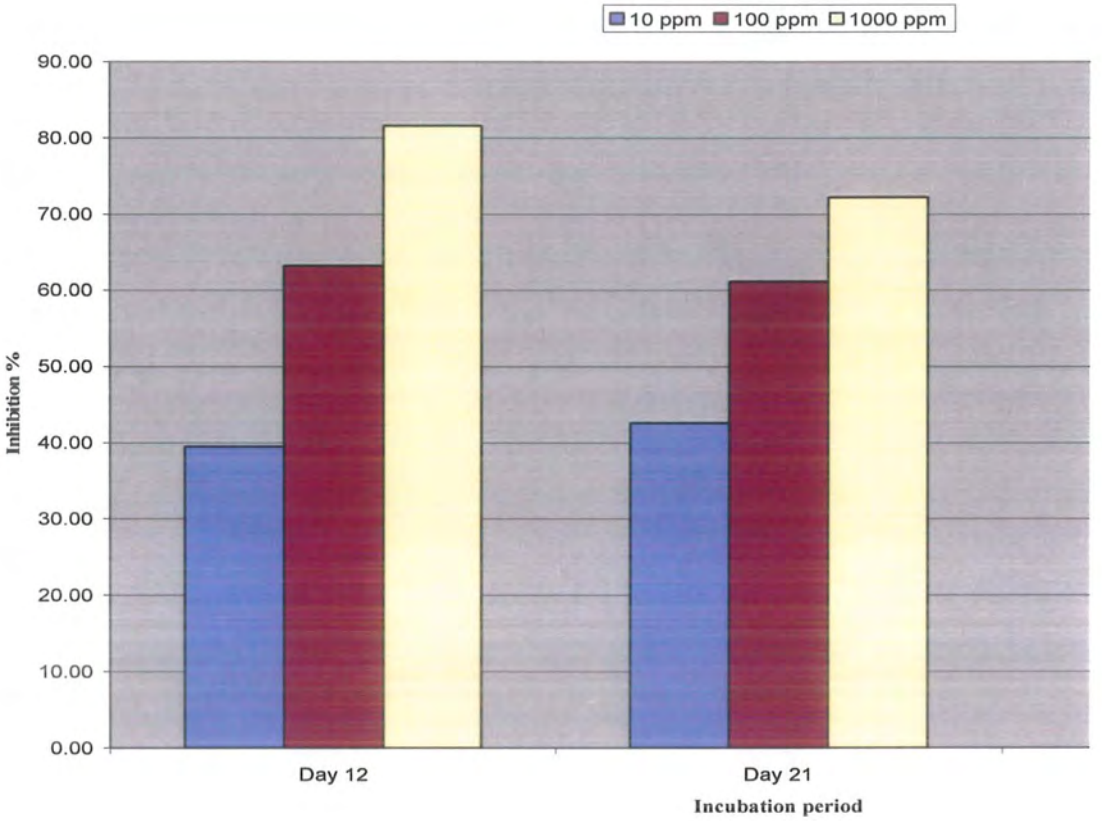


Fig. 3.5: Effect of different concentrations of the plant extract on % inhibition of tumor formation

3.4: Radish seed bioassay

For radish (*Raphanus sativus L.*) seed germination, two different parameters were determined, namely root length and percent of seed germination. Methanolic extract of *Thymus serpyllum* L was tested against radish seed. Seedling root length and percent germination inhibition were observed.

3.4.1: Radish seedling root length

Radish seeds root length was measured after one, three and five day's period of dark treatment. Etiolated seedlings having long radical of different lengths were measured; showing differences in time of germination of seedlings with their cotyledons attached. Major measurements are described below:

3.4.2: Factors affecting radish seedling root length

This study was conducted in factorial experiment (3 X 3 X 3) to see the effect of different plant extract concentrations and incubation time on radish seed root length.

3.4.3: Effect of different plant extract concentrations

Methanolic extract of *Thymus serpyllum* L. had a highly significant ($P<0.05$) impact on radish seeds root length (Table 3.5). Mean value of root length was higher in control as compared to treated seeds. Seeds untreated with extract showed 29.3mm root length while those treated with 1000mg/L concentration showed 18.22mm and at 10,000mg/L, 9.12mm root length (Table 3.6. and Fig 3.6).

3.4.4: Effect of incubation period

Incubation period had a highly significant ($P<0.05$) impact on radish seedling root length (Table 3.5). Seedling root length differed highly significantly (LSD value 5.359) at first, third and fifth day (Table 3.6). The overall mean values of seedling root length were 1.52mm , 17.35mm and 37.78mm at first, third and fifth day respectively (Table 3.6 and Fig. 3.7).

3.4.5: Interaction effect between different plant extract concentrations and incubation period

The interaction effect between the two factors i.e. different plant extract concentrations and incubation period was highly significant ($P<0.05$) as shown in table 3.5 Radish seeds untreated with extract yielded the lowest root length above 2.8mm at first day incubation period while the root length increased significantly in the same seeds at third day incubation period (25mm) and at fifth day incubation period (59mm) (Table 3.6 and Fig. 3.7).

Seeds treated with plant extract at 1000mg/L showed 1.3mm, 18.58mm and 34.73mm root length at first, third and fifth day incubation periods, respectively (Table 3.6). Seeds treated with plant extract at 10,000mg/L showed 0.3mm and 7.8mm root length at first and third day incubation periods, respectively did not differ significantly while the root length increased significantly by 19.2mm at fifth day incubation period (Table 3.6).

Table 3.5: Analysis of variance for factors affecting radish seedling root length

Source of variation	df	Sum of Squares	Mean Square	F Value	P-value
Concentrations	2	1840.094	920.047	31.4214	0.0000
Incubation time	2	5948.723	2974.362	101.5802	0.0000
Concentrations ×Incubation time	4	1124.086	281.021	9.5974	0.0002
Error	18	527.057	29.281		
Total	**26**	**9439.960**			

Coefficient of Variation: 28.64%

Table 3.6: Effect of the extract concentration, incubation period and interaction of incubation period and concentration on mean value of radish seedling root length (α = 0.05).

Concentration(mg/L)	Mean value of root length (mm) ± SE	Ranked order
Control	29.32 ±5.80	A
1000	18.23 ±4.38	B
10000	9.128 ±2.94	C
LSD value	**5.359**	
Incubation period (days)		
1	1.528 ±0.73	A
3	17.36 ±3.66	B
5	37.79 ±8.85	C
LSD value	**5.359**	
Interaction between concentration(mg/L) and incubation period (days)		
Control ×1day	2.85 ±0.38	D
Control ×3 days	25.67 ±1.43	BC
Control ×5 days	59.43 ±4.09	A
1000 ×1 day	1.367 ±0.24	D
1000 ×3 days	18.58 ±1.18	C
1000 ×5 days	34.73 ±2.96	B
10000 ×1 day	0.367 ±0.09	D
10000×3 days	7.817 ±1.05	D
10000 ×5days	19.20 ±1.80	C
LSD value	**9.282**	

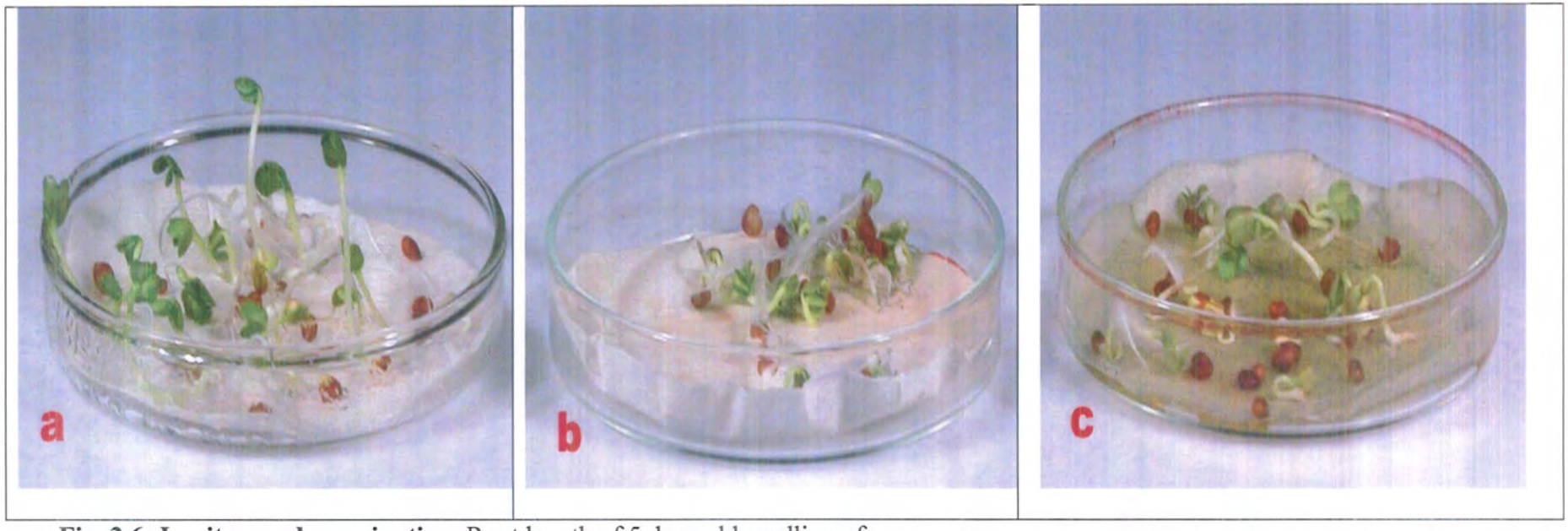


Fig. 3.6: In vitro seed germination: Root length of 5 days old seedling of

a) Control

b) Treated with1000 mg/L concentration of the plant extract

c) Treated with10, 000 mg/L concentration of the plant extract.

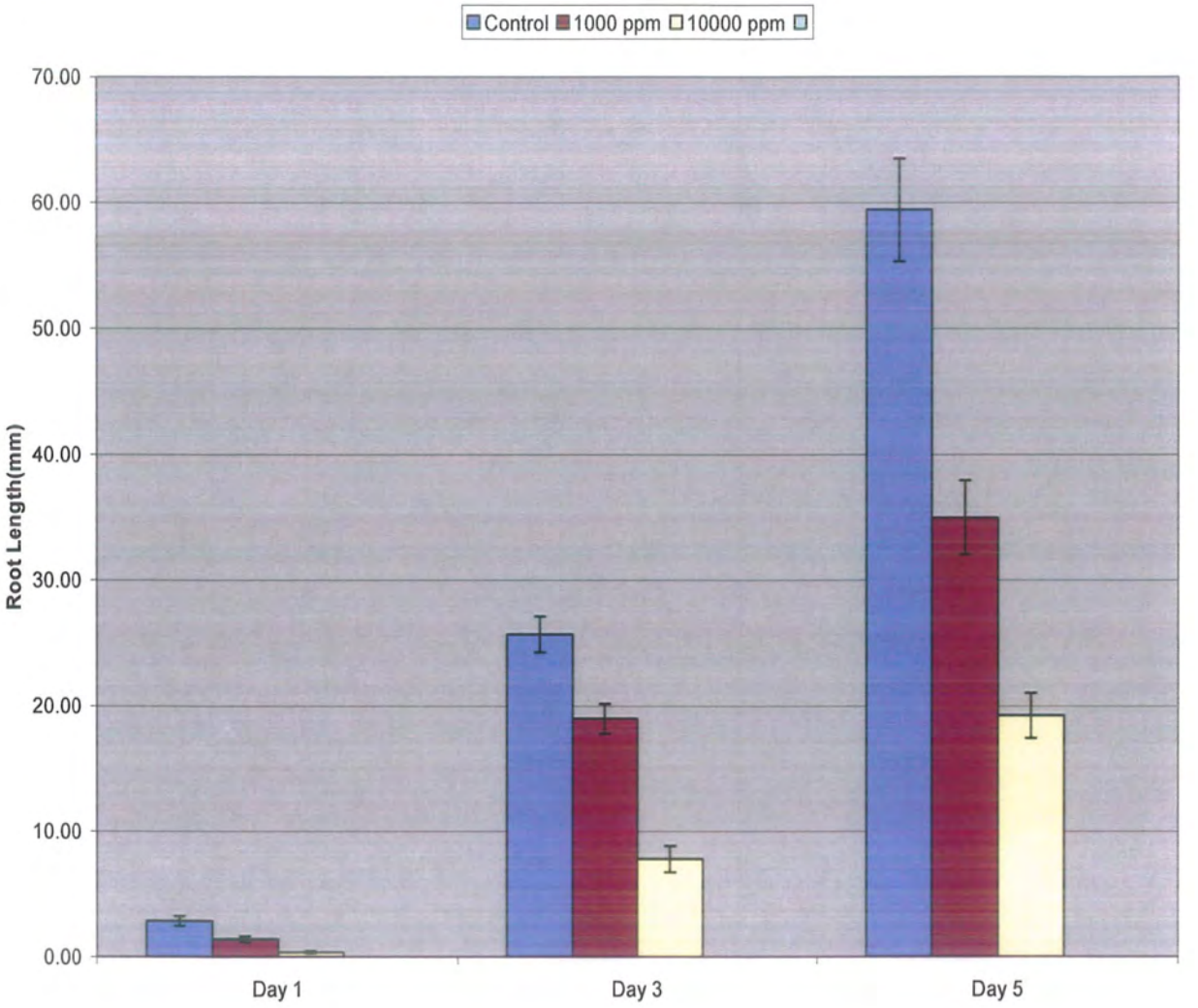


Fig. 3.7: Effect of different concentrations of the plant extract and incubation period interaction on radish seedling root length.

3.4.5: Radish seed germination

Seed germination was observed after one, three and five day's period of dark treatment. Total number of germinating seeds were counted showing differences in time of germination. Major observations are described below:

3.4.6: Factors affecting radish seed germination

This study was conducted in factorial experiment (3 X 3 X 3) to see the effect of different concentrations and incubation period on radish seed germination.

3.4.7: Effect of different plant extract concentrations

Methanolic extract of *Thymus serpyllum* L. had a highly significant ($P<0.05$) impact on radish seeds germination (Table 3.7). Mean values of percentage germination were higher in control as compared to extract treated seeds. Untreated seeds showed 84% germination while those treated with 1000mg/L and 7500mg/L showed 77% and 67% germination respectively (Table 3.8 and Fig. 3.8).

3.4.8: Effect of incubation period

Incubation period had a highly significant ($P<0.05$) impact on radish seeds germination (Table 3.7). Seed germination differed highly significantly (LSD value 6.235) at first and third day but not at third and fifth day (Table3.8). The overall mean values of percentage germination were 45%, 90% and 94% on first, third and fifth day (Table 3.8 and Fig. 3.10).

3.4.9: Interaction effect between concentrations and incubation period

The interaction effect between the two factors i.e. concentrations and incubation period was highly significant ($P<0.05$) as shown in table 3.7. Untreated seeds yielded the lowest percent germination at first day of incubation periods while the percentage increased significantly in the same seeds at third and fifth day of incubation period (Table 3.8 and Fig. 3.9).

Seeds treated with extract at 1000mg/L showed 50% at first day incubation period while the percentage increased significantly by 89-93 % germination in the same seeds treated with extract at 1000mg/L at third and fifth day incubation periods (Table 3.8). Seeds treated with extract at 7500mg/L showed 25% germination at first day incubation period at third and fifth day incubation periods while the percentage increased significantly by 86-89 % germination in the same seeds treated with extract at 7500mg/L (Table 3.8).

Table 3.7: Analysis of variance for factors affecting on radish seed germination

Source of variation	df	Sum of Squares	Mean Square	F Value	P-value
Concentrations	2	1362.889	681.444	17.1953	.0001
Incubation time	2	13130.889	6565.444	165.6701	0.0000
Concentrations ×Incubation time	4	736.889	184.222	4.6486	0.0094
Error	18	713.333	39.630		
Total	**26**	**15944.000**			

Coefficient of Variation: 8.25%

Table 3.8: Effect of the extract concentrations, incubation period and interaction of incubation period and concentration on mean value of radish seed germination (α = 0.05).

Concentration(mg/L)	Mean value of seed germination ± S.E.	Ranked order
Control	84.22 ±1.22	A
1000	77.78 ±1.66	B
7500	67.00 ±2.95	C
LSD value	**6.235**	
Incubation period (days)		
1	45.22 ±4.00	B
3	90.00 ±1.29	A
5	93.78 ±1.24	A
LSD value	**6.235**	
Interaction between concentration(mg/L) and incubation period (days)		
control ×1day	60.00 ±0.93	B
control ×3 days	94.00 ±0.22	A
control ×5 days	98.67 ±0.07	A
1000 ×1 day	50.00 ±1.44	B
1000 ×3 days	89.67 ±0.07	A
1000 ×5 days	93.67 ±0.15	A
7500 ×1 day	25.67 ±0.93	C
7500×3 days	86.33 ±1.00	A
7500 ×5	89.00 ±1.02	A
LSD value	**10.80**	

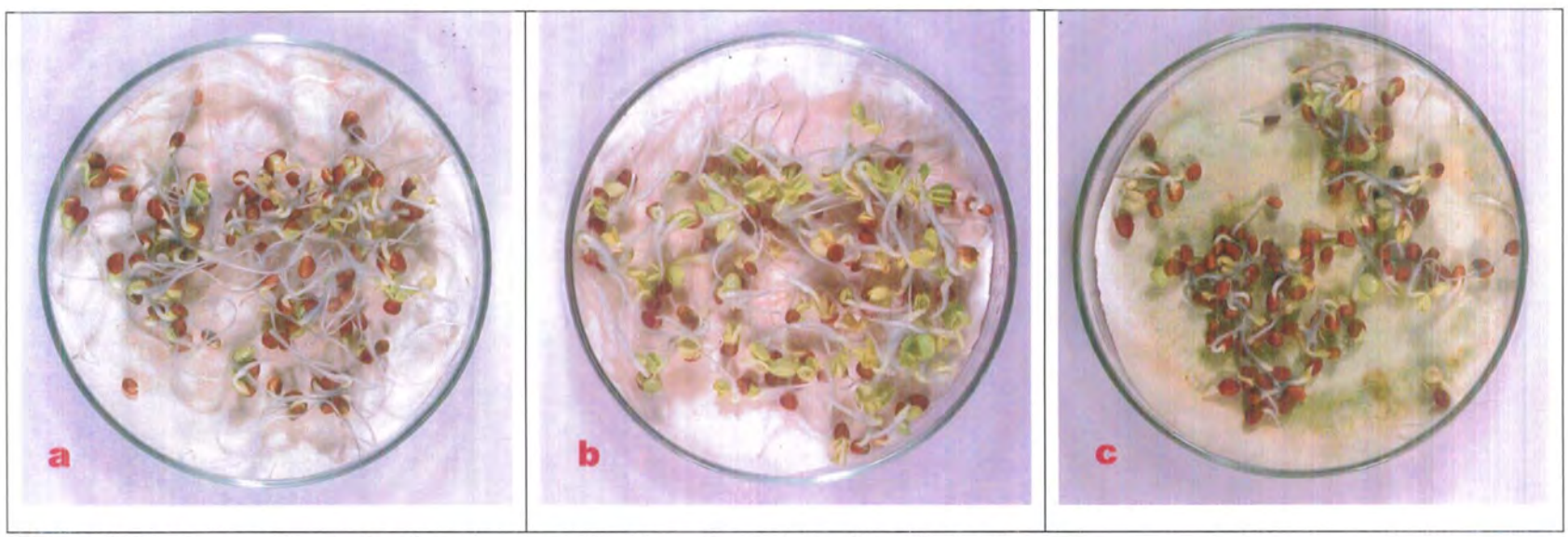


Fig. 3.8: In vitro seed germination: Seed germination after 5 days of incubation in

a) Control

b) Treated with1000 mg/L concentration of the plant extract.

c) Treated with10, 000 mg/L concentration of the plant extract.

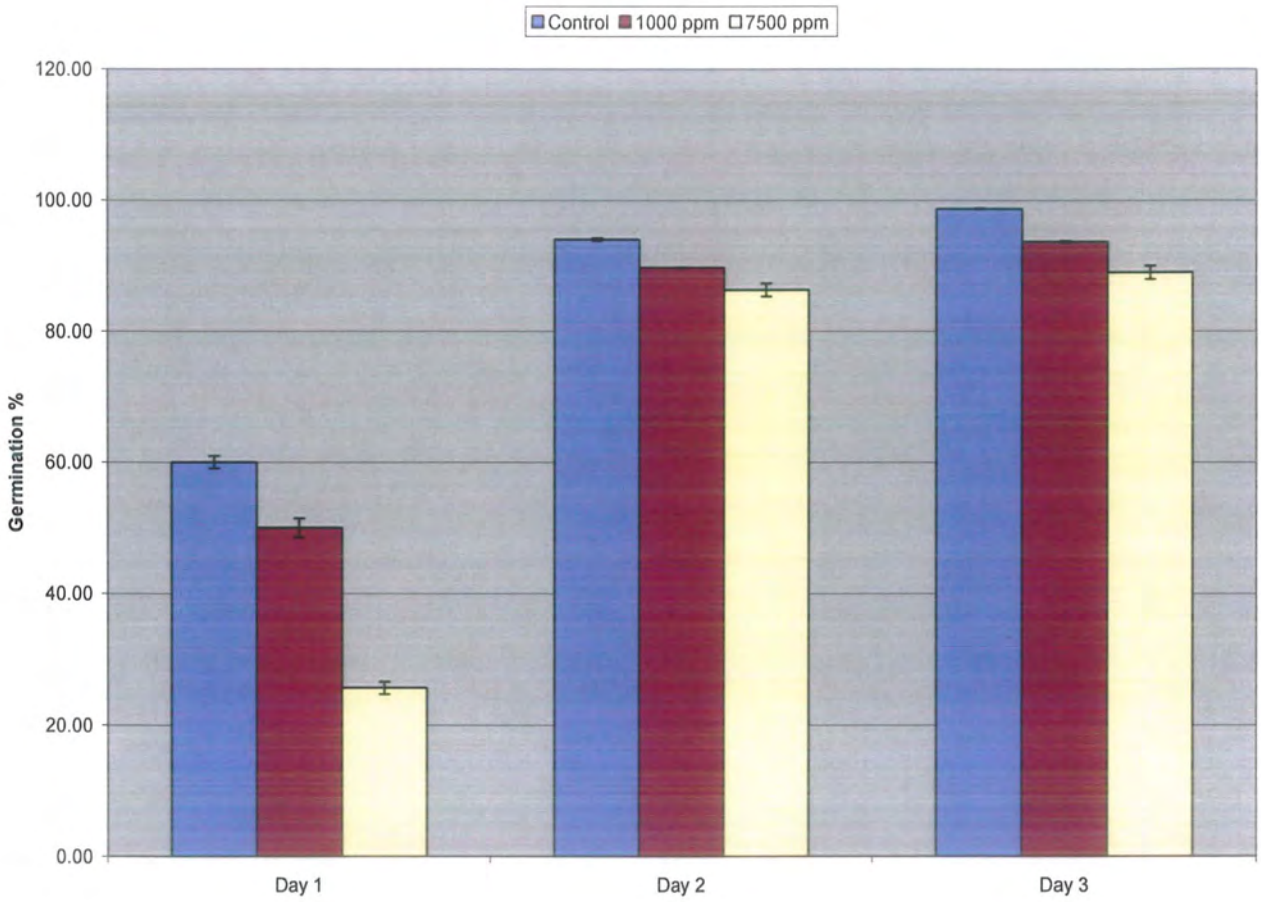


Fig. 3.9: Effect of different concentrations of the plant extract and incubation period interaction on germination % of radish seeds.

3.5: *Lemna* bioassay for inhibitors and promoters of plant growth

The lemna bioassay is quick measurement of phytotoxicity of the materials. Methanolic extract of *Thymus serpyllum* L were tested for measurement of phytotoxicity. Phytotoxic activity of plant extract was observed.

The analysis was carried out in simple completely randomized design with different plant extract concentrations. Percentage growth regulation was observed after 7 days (Fig. 3.11). Methanolic extract of *Thymus serpyllum* L. had a highly significant ($P<0.05$) impact on percent growth regulation (Table 3.9). Mean values of percentage growth regulation were lower in control as compared to extract treated. *Lemna minor* untreated with extract showed 0% growth regulation while extract at 10ppm, 100ppm and 1000ppm treated showed above 30%, 36% and 48% growth regulation respectively. (Table 3.10 and Fig. 3.11).

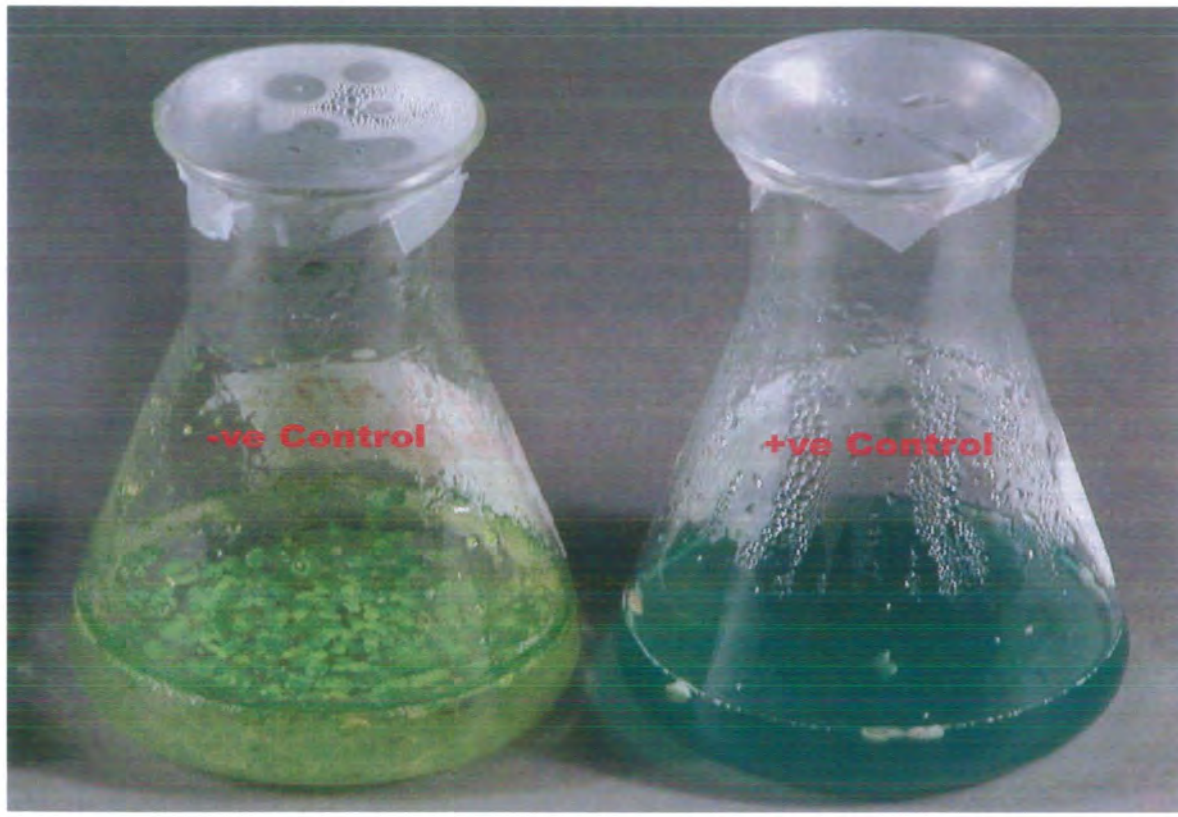
Table 3.9: Analysis of variance for factors affecting growth regulation of *Lemna minor*

Source of variation	df	Sum of Squares	Mean Square	F Value	P-value
Concentrations	3	6534.917	2178.306	281.072	0.0000
Error	8	62.000	7.750		
Total	11	6596.917			

Coefficient of Variation = 2.94%

Table3.10: Effect of the extract concentration on mean number of fronds (α = 0.05).

Concentration(ppm)	Mean number of fronds ± S.E.	Ranked order
Control	132.33± 2.60	A
10	92.66± 1.45	B
100	84.00± 1.15	C
1000	69.33± 0.33	D
LSD value	**5.242**	



a b

Fig. 3.10: *Lemna minor* growth

a) -ve control

b) +ve control (with standard drug paraquate)

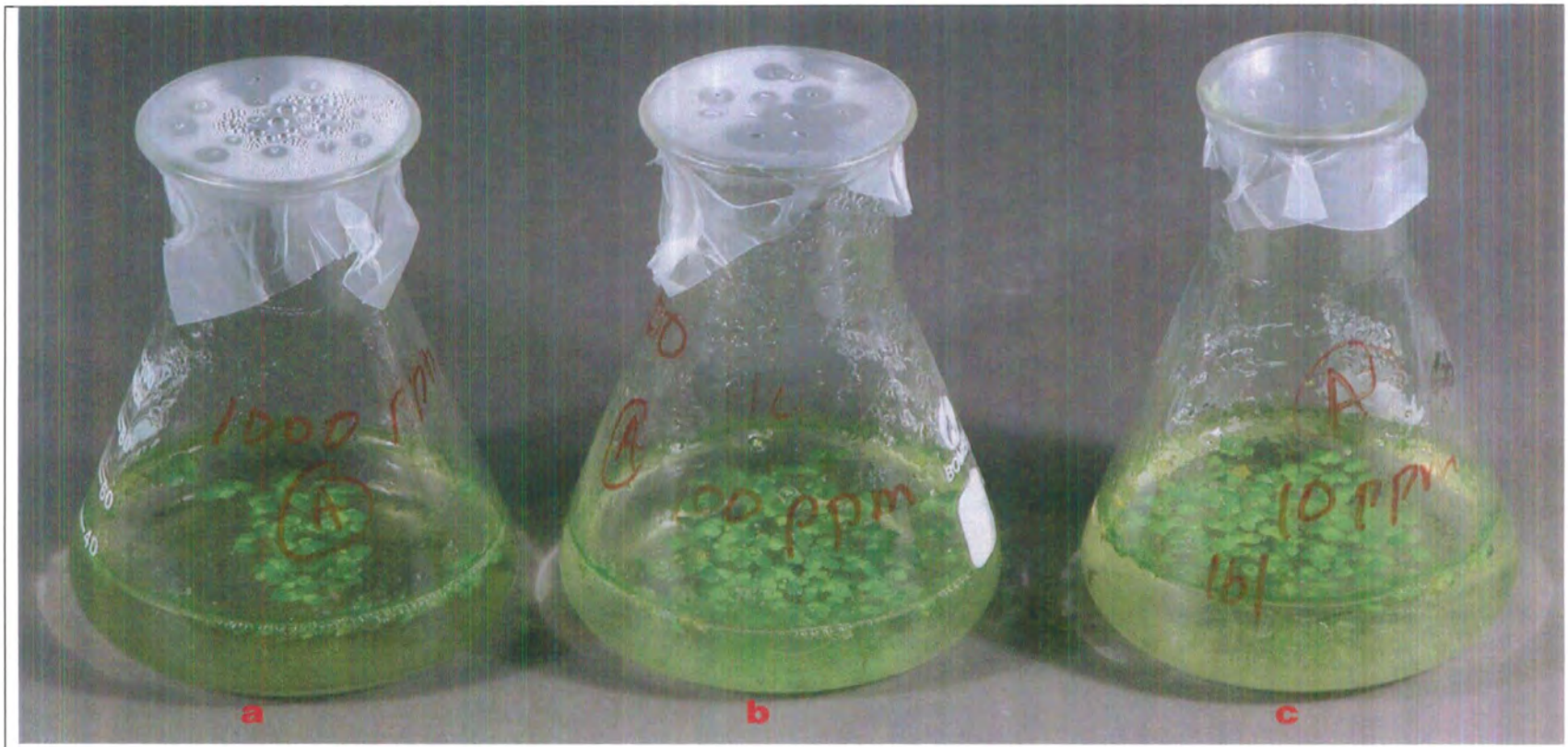


Fig. 3.11: Growth inhibition of *Lemna minor* after 7 days of incubation

a) Treated with1000 ppm concentration of the plant extract.

b) Treated with100 ppm concentration of the plant extract.

c) Treated with10 ppm concentration of the plant extract. .

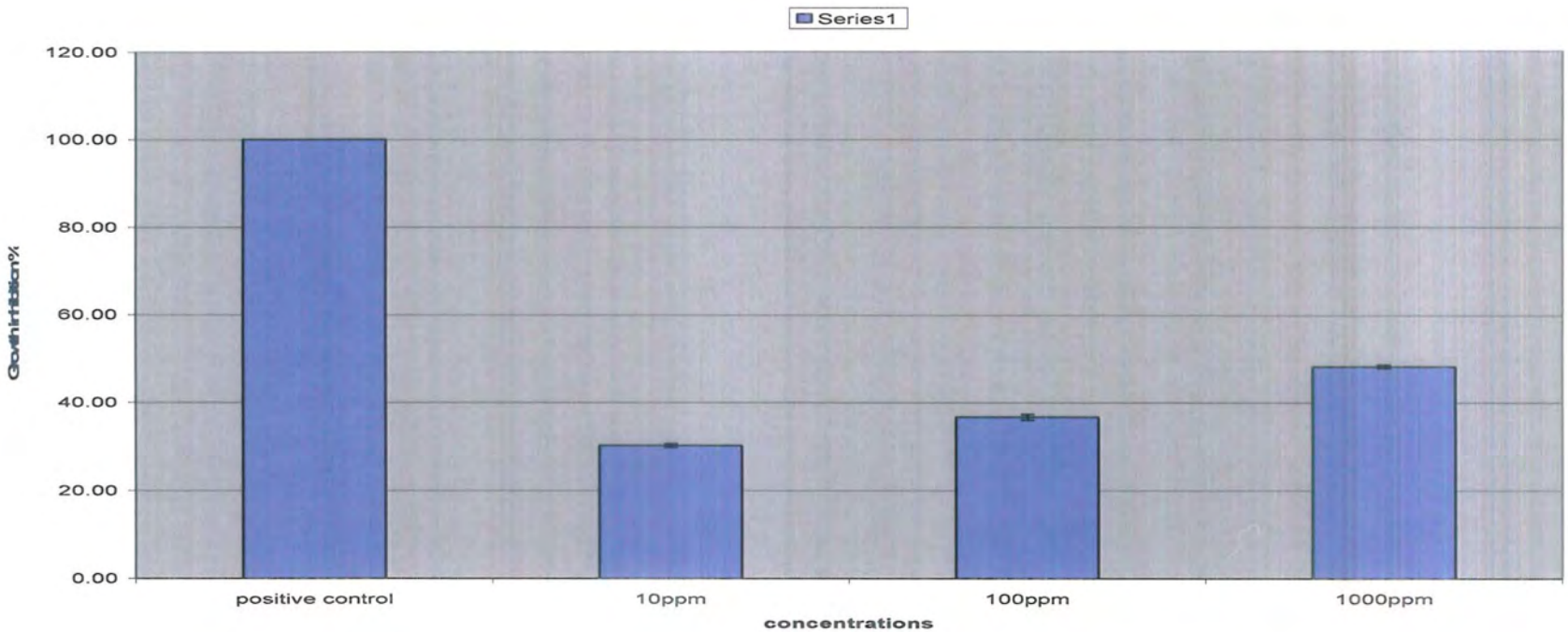


Fig. 3.12: Effect of different concentrations of the plant extract on % growth inhibition of *Lemna minor* L.

3.6. Antibacterial Assay

Methanolic extract of *Thymus serpyllum L.* was used against eight stains of bacteria. Three were gram positive, *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis* (ATCC 6633) *and Micrococcus luteus* (ATCC 10240) and five were gram negative, *Escherichia coli* (ATCC 15224), *Salmonella setubal* (ATCC 19196), *Pseudomonas picketii* (ATCC 49129), *Bordetella bronchiseptica* (ATCC 4617), and *Enterobacter aerogens* (ATCC 13048). Zones of inhibition were measured (fig. 3.13). All dilutions were made in dimethyl sulphoxide (DMSO). This solvent has no inhibitory effects on growth of bacteria. Zone of inhibition of Cefixime 2mg/mL (positive control) was between 15 to 38.5mm while Roxithromycin 2mg/mL (positive control) showed zone of inhibition between 15 to 38.00mm for the different bacterial strains.

Eight concentrations (max. 15mg/ml and min. 1mg/ml) of the extract were tested against the eight strains of bacteria. Our results showed no antibacterial activity against all the eight strains of bacteria (Fig. 3.13).

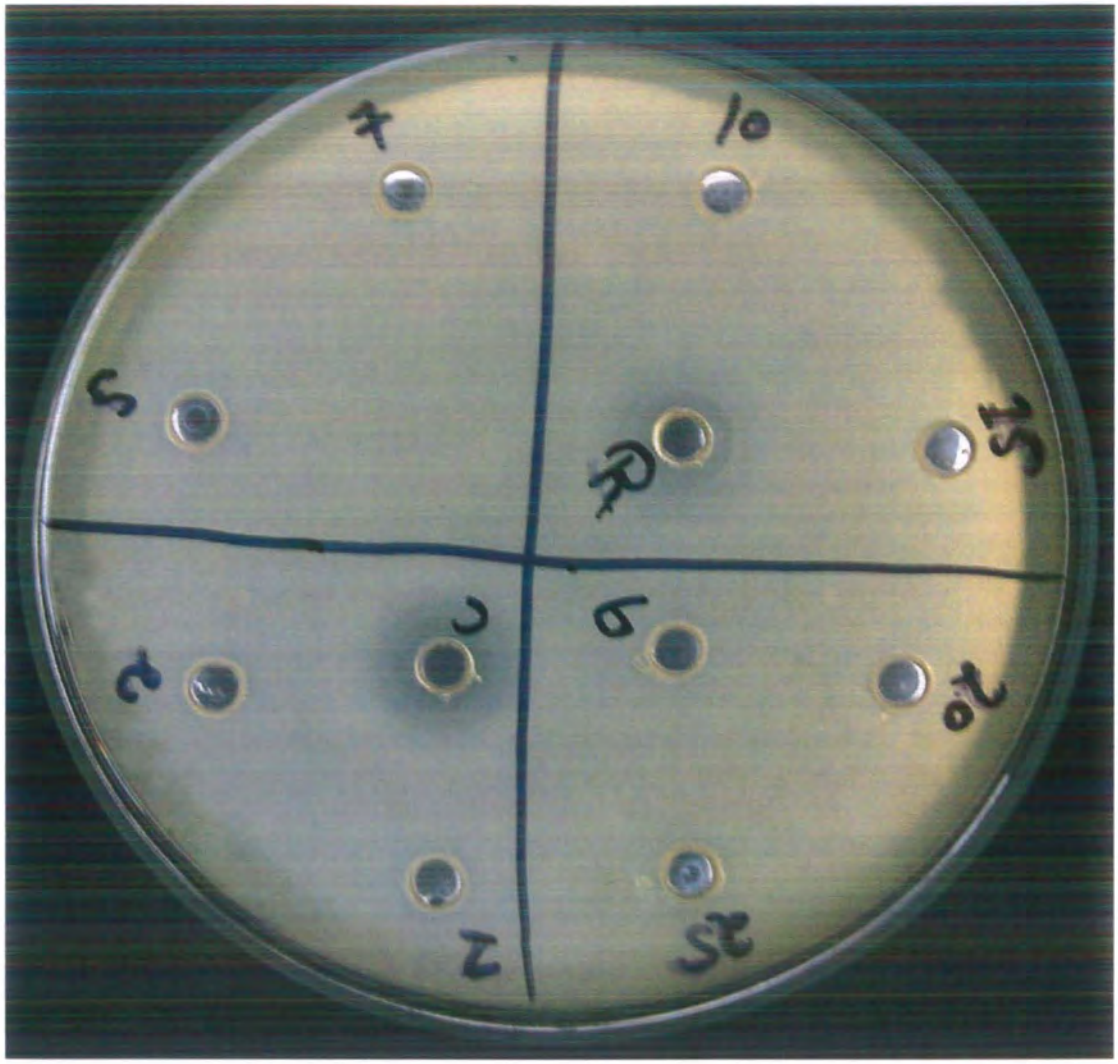


Fig. 3.13: Antibacterial assay showing no activity of the methanolic plant extract.

CHAPTER 4

DISCUSSION

DISCUSSION

Bioassay permits convenient and rapid evaluation of various plant parts, ontogenic and seasonal variations within individual plant and highly bioactive genotype within the intra-specific variations. The crude botanical extracts, containing mixture of bioactive compounds are usually effective and these procedures may permit their conventional standardization. Genus *Thymus*, includes 350 species world widely distributed and is known for its bioactive secondary metabolites and essential oils used for flavoring, fragrance and medicinal purpose (Demissew, 1993). One of its specie *Thymus serpyllum* L. was considered for antifungal, cytotoxic, antitumor, antimicrobial and phytotoxicity assay carried out in the present study.

For extraction methanol was selected as solvent on the bases of previous reports suggesting that it is a better solvent than water or chloroform. Chandrasekhar and Venkatesalu (2004) proposed that the methanolic extract had higher antifungal and antimicrobial activity than that of aqueous extract which may be due to solubility of the different constituents in different solvents having antimicrobial activity. Vlachos *et al.* (1996) also concluded that methanol was the most effective solvent for the extraction of antibacterial compounds from the selected seaweeds.

4.1: Antifungal assay

Although Pakistan possesses a rich tradition in the use of medicinal plants and an outstanding floral diversity of vascular plants, little research has been done to isolate and characterize phytochemical leads for therapeutic use. The antifungal activities of numerous herbs and spices have been reported by various workers and proved to be at least partially due to their essential oils (Thompson,1989; Razia *et al.*,1995).

The antifungal activities shown by plants may be toxinogenesis inhibition (Hitokoto *et al.*, 1980), lethal effect (Hajji *et al.*,1993; Tantoui-Elaraki *et al.*,1993), growth inhibition (Bullerman *et al.*,1877; Hmamouchi *et al.*,1990) or growth retardation (Es-safi *et al.*,1991). Fungal reproduction consists of three steps: spore germination, mycelium formation and sporulation. Different workers have reported the effects of spices and members of the Umbelliferae on mycelial growth, sporulation and spore germination (Rahman and Gul, 2000). The present study deals with an investigation of the antifungal activities of methanolic extrat of *Thymus serpyllum* L. as inhibitor of mycellial growth and spore formation of ten fungus strains .

Mycotoxic effects of plant extract on four species of *Aspergillus* (*A. flavus, A. niger, A. flavus green* and *A. fumigatus*), two species of *Fusaroum* (*F. moniliforme* and *F. solani*), two species of *Candida* (*C. albicans* and *C.galabarata*), one *Mucar specie* and one *Alternaria specie* were investigated. Moderate activity was seen against *Fusarium moniliforme, Fusarium solani, Alternaria specie, Candida albicans, Candida glabarata* and while low activity was seen against *Mucor specie* and *Aspergillus flavus* (Fig. 3.2). Spore formation inhibition was observed in the case of *A. niger, A. flavus g* and *A. fumigatus* when exposed to the methanolic extract (Fig.3.1).

Kieren *et al.* (1998) reported that Fluconazole-resistant *C. species*, a cause of oropharygeal candidiasis in patients with human immunodeficiency virus infection has recently emerged as a cause of candidiasis in patients receiving cancer chemotherapy and marrow transplantation. The diminishing biological activity of natural products against fungal plant pathogens is a problem throughout the world (Agrios, 1997). Our results show that even crude plant extracts can be used to protect plants against damages inflicted by fungal infections. Various studies have shown a similar strategy to use crude plant extracts against plant pathogens for broad community uses (Smith *et al.*, 1991).

Plaza *et* al., (2004) reported that the essential oil of *Thymus serpyllum* L. have the potential instead of synthetic fungicides to control fungus on citrus fruits. Shahidi, (2004) reported that Methanolic plant extract of thyme have antifungal activity. Our results agree with these reports showing varying levels of activity against all the ten test fungi. This suggests that it has a broad spectrum of activity, although the degree of susceptibility could differ between different organisms. The plant material appears to be attractive material for antifungal drug development. There is urgent need to develop new antifungal agents. Almost all of the antifungal agents, which are currently in use are relatively expensive and have toxics side effects (Morens *et al.*, 2004).

4.2: Cytotoxicity assay

Cancer is a big challenge to the world as suitable remedy is very costly and even impossible in some cases. On the other hand the conventional chemotherapeutic agents are growing resistance (Kelland, 1993). Microbial infections are also creating health hazards for multi drug resistance bacteria. Scientists are now engaged to find potent remedy from cancer and other infectious diseases through the discovery of new and effective chemotherapeutic agents from plants, microbes and other suitable sources. In the continuation of this search we have studied the cytotoxic effects of crude extract of aerial part of *Thymus serpyllum* L. found in Pakistan.

Brine shrimp lethality bioassay was used for preliminary assessment of toxicity of methanolic plant extract. Results showed that extract had ED_{50} at 466ppm whereas 19.3% death was observed at concentration of 10 ppm. It showed that plant extract had cytotoxic activity that could be further evaluated for pharmacological activity. Brine shrimp assay is suggested to be a convenient probe for the pharmacological activities in plant extracts (Meyer *et al.*, 1982). Therefore plant extract seems to contain important pharmacological compound. Other plant extract concentrations have shown 27% and 50% death at 100ppm and 1000ppm, respectively. Brine shrimp lethality bioassay is a primary assay to detect cytotoxic property of plant extract and further studies are required to establish the cytotoxicity of plant extracts against human cancer lines however on the basis of our results we can predict that methanolic plant extract will give better results on cancer cell lines.

4.3: Antitumor assay

Crown gall disease is a chronic disease problem that affects many perennial fruit, nut, and ornamental crops. It is a neoplastic disease of plants, in which autonomous plant tumor cells are produced from normal, wounded plant cells by the action of bacteria born tumor-inducing Ti plasmids. The induction of plant tumors is known as crown galls. The causative agent of this disease is the gram negative *Agrobacterium tumefaciens* (Galsky *et al.*, 1980) originates from the transfer of T-DNA into the plant genome and its expression (Thomashow *et al.*, 1980; Stachel *et al.*, 1985). Crown gall tumor assay (CGTA) is a low cast, fast turn-around procedure that indicates antitumor activity of test compounds by their inhibition of formation of characteristic crown galls induced in wounded potato tissue by the organism *Agrobacterium tumefaciens.*

This bioassay is sensitive bench top antitumor assay for chemicals that disrupt the cell cycle regardless of their mode of action (Coker *et al.*, 2002). This test discriminates between active and inactive compounds in predicting their in vivo antitumor activity but not necessarily in a direct linear effect (Mc Laughlin *et al.*, 1993). A degree of initial familiarization is required in identifying tumors from possible artifacts, and a set of criteria for recognition of valid tumors is set up (Kerr, 1999). Such criteria includes the definition of isolated buff colored lumps, distinctly bordered lumps in streaks, raised streaks without clear lumps, and any lumps appearing on the top surface of the potato discs as acceptable tumors. It has been suggested that tumor inhibition values of more than 20% in two or more independent assays may be considered worthy of further investigation (Ferrigini, 1982).

Methanolic extract of *Thymus serpyllum* L. were tested for anti-tumorigenic activity against *Agrobacterium tumefaciens* induced tumors on potato discs and anti tumor activity was observed. This study was conducted in factorial experiment to see the effect of different concentrations and incubation time on tumor formation (Fig. 3.4). Result showed that concentrations ($P<0.05$) and incubation period ($P<0.05$) had a highly significant impact on tumors formation (Table 3.3). Percent inhibition at 1000ppm, 100ppm and 10ppm concentration were 82%, 63% and 40 %, after 12 day's and 72%, 61% and 43%, respectively after 21 day's incubation period.

Plant extract that are active during this test are examined for the ability to effect bacterial growth of *Agrobacterium tumefaciens* by the standard agar well diffusion method (Perez *et al.*, 1990). Result showed that plant extract had no effect on the viability of the bacterium. The attachment of the bacterium to a tumor binding site is complete within 15 minutes following inoculation (Gologowski and Galsky, 1978) and other tests have shown no effect on bacterial viability or on the attachment process (Galsky *et al.*, 1980).

The inhibition of crown gall tumors on discs of potato had shown an apparent correlation with compounds and plant extracts known to be active in the 3PS (murine leukemia) anti-tumor assay (Ferrigini, 1982). Mc Laughlin *et al.*, (1998) reported that crown gall tumors on potato discs could routinely be employed as a comparatively rapid, in expensive, safe, animal-sparing, and statistically reliable prescreen for *in vivo* 3PS antitumor activity. Mc Laughlin *et al.* (1998) have since used this assay to detect and isolate several dozen novels, antitumor compound from various plant species.

Ferrigini *et al.* (1982) reported that a combination of simple brine shrimp lethality and crown gall bioassays both can be used to detect and to isolate plant antitumor substances. Researchers working on the development of new anticancer drugs at the National Cancer Institute (NCI) in the United States had reported positive correlation between lethality to brine shrimp and antitumoral activity (Anderson, 1991). Our results of antitumor assay as well as cytotoxicity assay show that this extract is highly effective as an antitumor agent.

4.4: Radish seed bioassay

Radish *(Raphanus sativus* L) seeds have been used in general toxicity studies because of their sensitivity to phytotoxic compounds (Einhilling and Rasmussen, 1978) and are a standard assay in allelopathic studies (Patterson, 1986). The allelochemicals present in higher plants as well as in medicinal plants can be directly used for weed management on the pattern of herbicides. Their bioefficiency can be enhanced by structural changes or the synthesis of chemicals analogues based on them (Singh *et al.*, 2003).

Methanolic plant extract were tested for phytotoxic activity against radish seed germination. Two different parameters were determined, namely root length and percent of seed germination. Phytotoxic activity of plant extract was observed. This study was conducted in factorial experiment to see the effect of different concentrations and incubation time on seedling root length and percent seed germination (Fig.3.6 and Fig.3.8). Result showed that different plant extract concentrations ($P<0.05$) and incubation period ($P<0.05$) had a highly significant impact on seedling root length and percent seed germination (Table 3.7and Table 3.9).

These results are in agreement to a previous study where Dudai *et al.* (1999) reported that seed germination of several species was strongly inhibited by essential oil from thyme when applied at concentration 20 to 80 ppm.

4.5: Lemna bioassay

The search for biodegradable herbicides may be extended to include natural compounds (allelochemicals) and this is a simple screen for such activity. Some natural substances stimulate frond proliferation, and the assay may be useful to detect new plant growth stimulants. Usnic acid was shown to be potent herbicide (FI50 =0.91ppm). Hippuric acid shown to be a potent (FP50 =125ppm) plant growth stimulants this unexpected activity of this common urinary constituent had never before been documented (Mc Laughlin *et al.*, 1998). The commercial need for such natural, biodegradable herbicides and plant growth stimulants may some day be filled with natural products detected by this simple and convenient *Lemna* bioassay.

The results of *Lemna minor* bioassay for phytotoxic activity of the plant extract are shown in table 3.9. This study was conducted in simple completely randomized design to see the effect of different concentrations on percent growth inhibition (Fig. 3.11). Result showed that concentrations ($P<0.05$) had a highly significant impact on frond formation (Table 3.9). The extract displayed moderate percent growth regulation in the highest tested concentration (1000ppm) and caused (48%) inhibition of growth of *Lemna minor*. It also exhibited a low (36% and 30%) activity at concentration of 100ppm and 10ppm, respectively. Our results clearly

indicated the involvement of chemical factors in the growth inhibitory effects of *Thymus serpyllum* L. against other plants such as *Lemna minor* L.

There are no previous reports on the growth inhibitory effect of methanolic extract of *Thymus serpyllum* L. but our results agree with Tworkoske (2002) that essential oil from thyme were the most phytotoxic and caused electrolyte leakage resulting in cell death. These data suggest that the plant extract could be used to inhibit the emergence of weeds. The primary goal of this project is to observe the growth inhibitory effect of plant extract under controlled laboratory conditions and to study the effect of the herbicidal activities of the plant extract.

4.5: Antibacterial assay

Antibacterial assay of crude extract of *Thymus serpyllum* L show that methanolic extract of aerial part has no antibacterial activity against all the eight strains (three were gram positive, which were *Staphylococcus aureus, Bacillus subtilis* and *Micrococcus luteus* and five were gram negative, which were *Escherichia coli Salmonella setubal, Pseudomonas picketii, Bordetella bronchiseptica,* and *Enterobacter aerogens*) tested.

Our results agree with Shahidi *et al.* (2004) that methanolic extracts of thymus species shows no activity against gram negative (*Escherichia coli, Pseudomonas picketii, Bordetella bronchiseptica)* and gram positive (*Staphylococcus aureus* and *Micrococcus luteu)* bacterial strains. Nevas *et al.* (2004) reported that thyme showed broad antibacterial activity by inhibiting the growth of both gram-positive and gram-negative bacteria. In these experiments only methanol was used as solvent, so may be maximum number of compounds can not be extracted and tested for their biological activities. Plant extract have no activity against different strains of bacteria, therefore, it can be deducted that the compound/s in the aerial part, which was/were active against these bacterial strains, were not soluble in methanol.

Conclusion

The obtained findings clearly indicated that methanolic extract of *Thymus serpyllum* L. showed significant activity in brine shrimp lethality assay and antitumor assay. These findings provide the evidence for a very strong positive correlation between these two assays. Plant extract also exhibited significant activity in phytotoxicity assays so there is need to purify the active contents of this plant which will open new horizon in field of the herbicides. Plant extracts were not effective against eight bacterial strains. In future, characterization of crude methanolic extract of *Thymus serpyllum* L. may lead to findings of biologically active compounds.

CHAPTER 5

REFERENCES

REFERENCES

Adam K, Sivropoulou A, Kokkini S, Lanaras T, Arsenakis M (1998). Antifungal activities of *Origanum vulgare* subsp. *hirtum*, *Mentha spicata*, *Lavandula angustifolia*, and *Salvia fruticosa* essential oils against human Pathogenic fungi. J Agric Food Chem 46:1739-1745

Adilson S, Ana Lucia MM, Camila D, Figueira GM, Marta CT, Duarte V, Lucia GR (2004). Composition and antimicrobial activity of essential oils from aromatic plants used in Brazil. Brazilian Journal of Microbiology. 35:275-280

Agirose GN (1997). Plant disease caused by prokaryotes: bacteria and mollicutes. Plant Pathology. Academic Press, San Diego. pp 407-470

Anaissie EJ, Bodey GP, Rinaldi MG (1989). Emerging fungal pathogens. Eur J Clin Microbiol Infect Dis 8:323-330

Anaissie E, Kantarjian H, Ro J, Hopfer R, Rolston K, Fainstein V, Bodey G (1988). The emerging role of *Fusarium* infections in patients with cancer. Medicine (Baltimore). 67:77-83.

Anderson J (1991). A blind comparison of simple bench-top bioassay and human tumor cell cytotoxicities as antitumor prescreens. Phytochemical Analysis. 2: 107-111

Arzu UT, Camper ND (2002). Biological activity of common mullein, a medicinal plant. Journal of Ethno pharmacology. 82:117-125

Badaway AS, Kappe T (1997). Potential antineoplastics. Synthesis and cytoxicity of certain 4-chloro-3-(2-chloroethyl)-2-methylquinolines and related derivatives. European Journal of Medicinal Chemistry. 32: 815-822

Bagamboula CF, Uyttendaele M, Debevere J (2003). Antimicrobial effect of spices and herbs on *Shigella sonnei* and *Shigella flexner*. J Food Prot 66:668-673

Bauer K, Garbe D, Surburg H (1997). Common fragrance and flavor materials Preparation, properties and uses, Wiley-VCH, New York. pp 214

Blum U (1995). The value of model plant-microbe-soil system for understanding processes associated with allelopathic interactions. In: Allelopathy, Organisms, Processes and Applications. eds. Inderjit, Dakshini and Einhellig. ACS Symposium Series 582. Washington DC. pp 127-131

Blum U, Shafer SR (1998). Microbial populations and phenolic acids in soil. Soil Biology and Biochemistry. 20:793-800

Blumenthal M, Goldberg A, Brinckmann J (2000). Herbal Medicine, Expanded Commission E. Monographs, 1st ed. Integrative Medicine Communications, Newton, MA.

Broucke CVD (1983). The therapeutic of *Thymus species*. Fitoterapia. 4:171-174

Buffington J, Reporter R, Lasker BA, McNeil MM, Lanson JM, Ross LA, Mascola L, Jarvis WR (1994). Investigation of an epidemic of invasive aspergillosis: utility of molecular typing with the use of random amplified polymorphic DNA probes, Pediatr. Infect Dis J 13:386-393

Bullerman LB, Lieu FY, Seier SA (1977). Inhibition of growth and aflatoxin production by cinnamon and clove. J Food Sci. 42:1107-1109

Burt SA, Reinders RD (2003). Antibacterial activity of selected plant essential oils against *Escherichia coli*. Appl Microbiol 36:162-167

Cabo J, Crespo ME, Jimenez J, Navarro C, Risco S (1982). Seasonal variation of essential oil yield and composition of *Thymus hyemalis*. Planta Medica. pp 380-382

Chandrasekaran M, Venkatesalu V (2004). Antibacterial and antifungal activity of *Syzygium jambulanum* seeds. Plant physiology. 91:105-108

Choudhary MI, Shahwar D, Parveen Z, Jabbar A, Ali I, Rahman A (1995) Antifungal steroidal lactones from *Withania coagulance*. Phytochemistry. 40(4):1243-1246

Coker PS, Redecke J, Guy C, camper ND (2003). Potato disc tumor induction assay: A multiple mode of drug action assay. Phyto medicine. 10:133-138

De Feo V, De Martino L, Quaranta E, Pizza C (2003). Isolation of phytotoxic compounds from tree-of-heaven (*Ailanthus altissima swingle*). J Agric Food Chem. 51(5):1177-1180

De Hoog GS, Guarro J, Gene J, Figueras MJ (2000). Atlas of Clinical Fung, Centraal bureau voor Schimmelcultures, Utrecht, The Netherlands. 29(1):67-76

Deacon J (2005). Biology and Control of Crown Gall (*Agrobacterium tumfaciens*). The Microbial World. The University of Edinburgh.

Demissew S (1993). The Genus *Thymus* (*Labiatae*) in Ethiopia. Opera Bot. 121: 57-60

Deshpande SD, Koppikar GV (1999). A study of mycotic keratitis in Mumbai. Indian J. Pathol Microbiol. 42:81-87.

Dudai N Poljakoff-Mayber A, Mayer AM, Putievsky E, Lerner HR (1999). Essential oils as allelochemicals and their potential use as bioherbicides. Journal of Chemical Ecology. 25:1079 –1089.

Duke SO, Dayan FE, Rimando AM (1998). Natural products as tools for weed management. Proc. J Weed Sci Suppl. 2:1-11

Dursun N, Liman N, Ozyazgan I, Gunes I, Saraymen R (2003). Role of thymus oil in burn wound healing.J Burn Care Rehabil.24:395-339

Dutkiewicz J. (1997). Bacteria and fungi in organic dust as potential health hazard. An Agric Environ Med. 4: 11-16

Einhellig FA, Rasmussen JA (1978). Synergistic inhibitory effect of vanillic and p-hydroxybennzoic acids on radish and grain sorghum. Journal of chemical ecology. 4:425-436

Einhellig FA, Leather GR, Hobbs LL (1985). Use of *Lemna minor* L. as a bioassay in allelopathy. J Chem Ecol. 11: 65-72

Ellof JN (1998). Which Extractant should be used for the screening and isolation of antimicrobial components from plants? Journal of Ethno pharmacology. 60:1-8

Es-safi N, Hmamouchi M, Tantaoui-Elaraki A, Agoumi A (1991). Influence of the incubation to activate antimicrobial activity of essential oils. Al biruniya, Rev Mar. Pharm. 7:113-133

Evans CE, Banso A, Samuel OA (2002). Efficiency of nupe medicinal plants against Salmonella typhi: an in vitro study. J Ethno pharmacology. 80:21-24

Fabio A, Corona A, Forte E, Quaglio P (2003). Inhibitory activity of spices and essential oils on psychrotrophic bacteria. New Microbiol. 26:115-120.

Fan M, Chen J (2001). Studies on antimicrobial activity of extracts from thyme. Wei Sheng Wu Xue Bao. 41:499-504

Farina C, Vailati F, Manisco A, Goglio A (1999). Fungaemia survey: a 10 year experience in Bergamo, Italy. Mycoses. 42:543-548

Fatope MO (1993). Screening of higher plants reputed as pesticide using the brine shrimp lethality assay. International Journal of Pharmacognosy. 31: 250-254

Fehrand D, Slenzhorn G (1979). Studies on the shelf life of peppermint leaves, rosemary leaves and thyme. Pharm Ztg, 124: 2342-2349

Ferrigini R, Putnam JE, Andersen B, Jacobsen LB, Nichols DE, Moore DS, McLaughlin, JL (982). Modification and evaluation of the potato disc assay and antitumor screening of *Euphorbia*ceae seeds. Journal of natural products. 45:679-686

Fetrow CW, Avila JR (1999). Professional's handbook of complementary and alternative medicines. Springhouse Corporation, Springhouse, PA.

Finny DJ (1971). Probit. analysis 3rd edition, Cambridge university press, Cambridge, UK. pp 18, 37, and 77.

Fridkin SK, Jarvis WR (1996). Epidemiology of nosocomial fungal infections. Clin Microbiol. Rev. 9:499-511

Fridman M, Henika PR, Mandrell RE (2002). Bacterialcidal activities of plant essential oil and some of their isolated constituents against *Campylobacter Jejuni, Escherichia coli, Listeria monocytogenes* and *Salmonella enterica*. J Food Prot. 65:1545-1560

Galasky AB, Wilsey JP, Powell RG (1980). Crown gall tumor disc bioassay, A possible aid in detection of compounds with antitumor activity. Plant physiology. 65:184-185

Geoffrey AC, Cindy KA, John MP (1994). Recent studies on cytotoxic, anti-HIV and antimalarial agents from plants. Pure and Appl Chem. 66:2283-2286

Glogowski W, Galsky AG (1978). *Agrobacterin tumefaciens* site of attachment as a necessary prerequisite for crown gall tumor formation on potato discs. Plant physiology. 61:1031-1033

Goldblum D, Frueh BE, Zimmerli S, Bohnke M (2000). Treatment of postkeratitis fusarium endophthalmitis with amphotericin B lipid complex (In Process Citation). Cornea.19:853-856

Gouyon PH, Vernet P, Guillerm JL, Valdeyron G (1986). Polymorphisms and environment: the adaptive value of the oil polymorphisms in *Thymus vulgaris* L. Heredity. 57:59–66

Gouyon PH, Vernett P, Guillerm JL, Valdeyron G (1988). Polymorphisms and environment the adaptive value of the oil. Polymorphisms in *Thymus vulgaris,* Heredity. 57:59-66

Guarro J, Nucci M, Akiti T, Gene J, Barreiro MDC, Goncalves RT (2000). Fungemia due to *Fusarium sacchari* in an immunosuppressed patient. J Clin Microbiol. 38:419-421

Gupta AK, Summerbell RC (1999). Combined distal and lateral subungual and white superficial onychomycosis in the toenails. J Am Acad Dermatol. 41:938-944

Hajji F, Tantaoui-Elarki A, Fakih-Tetouani S (1993). Antimicrobial activity of twenty one *Eucalyptus* essential oils. Fitoterapia. pp 64-77

Hammer KA, Carson CF, Riley TV (1999). Antimicrobial activity of essential oils and other plant extracts. J Appl Microbiol. 86:985-990

Haroun EM, Mahmoud OM, Adam SE (2002). Effect of feeding Cuminum cyminum fruits, *Thymus vulgaris* leaves or their mixture to rats. Vet. Hum. Toxicol. 446:7-9

Hawksworth DL, (1991). The fungal dimension of biodiversity: magnitude, significance, and conservation. Mycological Research. 95:641-655

Hawksworth DL, Kirk PM, Sutton BC, Pegler DN (1995). Ainsworth and Bisby's Dictionary of the Fungi (8th Ed.). CAB International, Wallingford, United Kingdom. pp 616

Hedge IC (1990). Labiatae No.192 Royal Botanical Garden, Edinburgh.

Hitokoto H, Morozumi S, Wauka T, Sabai S, Kurata H (1980). Inhibitory effects of spices on growth and toxin production on toxigenic fungi. Appl Envir Microbiol. 39: 818-820

Hmamouchi M, Tantaoui-Elaraki A, Es-safi N, Agoumi A (1990). Evidence of antibcterial and antifungal properties of the essential oil. Plant. Med. Phytother. 24: 278-289

Hostettmann K, Marston A, Wolfender JL (1995). Phytochemistry of plants used in traditional medicine (K. Hostettmann, A. Marston, M. Maillard and M. Hamburger, eds.). Clarendon Press, Oxford. pp 17-45

Inderjit, Weiner J (2001). Plant allelochemical interference or soil chemical ecology? Perspectives in Plant Ecology. 4:3-12

Iwen PC, Davis JC, Reed EC, Winfield BA Hinrichs SH (1994). Airborne fungal spore monitoring in a protective environment during hospital construction, and correlation with an outbreak of invasive aspergillosis. Infect. Control Hosp. Epidemiol. 15:303-306

Janaki S, Vijayasekaram V (1998). Antifungal activities of *Aglaia roxbur ghiana* (W&A). MIQ, Var, Beddome.i.Biomedicine.18 (2):86-89

Juven BJ, Kanner J, Schved F, Weisslowicz H (1994). Factors that interact with the antibacterial action of thyme essential oil and its active constituents. J Appl. Bacteriol. 76:626-631

Kawaguchi S, Yoneyama K, Yokota T, Takeuchi Y, Ogasawara M, Konnai M (1997). Effects of aqueous extract of rice plants (*Oryza sativa* L.) on seed germination and radicle elongation of *Monochoria vaginalis* var. *plantaginea*. Plant Growth Regul. 23:183-189

Kelland LR (1993). New platinum antitumor complexes. Crit. Rev. Hematol. 15:191-219

Kerr PG, Langmoe RB, Yench R (1999). Maroon Bush an aboriginal medicinal plant of promises biomedical Research. Plant physiology. 1:3-15

Kieren AM, Lyons CN, Rustad T, Bowden RA, White TC (1998). Transient fluconazole resistance in *Candida species* is associated with increased mRNA levels of CDR. Antimicrobial agents and Chemotherapy. 42:2584-2589

Koneman EW, Allen SD, Dowell VR, Janda WM, Soomers HM, Winn WC (1988). Color atlas and text book of diagnostic microbiology, (3rd ed). pp 479-481

Kokkini S, Vokou D, Karousou R (1990). Essential oil yield of *Lamiaceae* plants in Greece. Fragrance and Flowers. 3:5-12

Kovacicova G, Spanik S, Kunova A, Trupl J, Sabo A, Koren P, Sulcova M, Mateicka F, Novotny J, Pichnova E, Jurga L, Chmelik B, Obertik T, West D, Krcmery VJ (2001). Prospective study of fungaemia in a single cancer institution over a 10-y period: Aetiology, risk factors, consumption of antifungals and outcome in 140 patients. Scand. J Infec Dis. 33:367-374

Labbe C, Castillo M, Connoly JD (1993). Mono and sesquiterpenoids from *Satureja gilliesii*. Phytochemistry. 34:441-444

Lattaoui N, Tantaoui-Elaraki A (1994). Comparativ kinatics of micrbial destruction by Essential Oils of *Thymus brousskonettii*, *Thymus zygist* and *Thymus satureioides*. J Essent Oil Res. 6:165-171

Lear, k. Act III, Scene – A reference to *Lemna minor*. (www.resk .org/earl /bwac/nature /aquatic/lemna.htm-24).

Leather GR, Einhellig FA (1985). Mechanisms of allelopathic action in bioassay. In: A. C. Thompson (ed.), The chemistry of allelopathy. Biochemical interactions among plants. Am. Chem. Soc., Washington DC. pp 197-205

Leather GR, Einhellig FA (1986). Bioassays in the study of allelopathy. The science of allelopathy. John Wiley and Sons, New York. pp 133-145

Lewis MA (1995). Use of freshwater in phytotoxicity testing, A Review Environ Pollut. 87(3):319-336

Liesegang TJ, Forster RK (1980). Spectrum of microbial keratitis in South Florida. Am. J Ophthalmol. 90:38-47

Linhart YB, Thompson JD (1999). Thyme is of the essence:biochemical polymorphism and multi-species deterrence. Evol.Ecol. 1:151–171

Linton A H (1983). Antibiotics: assessment of antimicrobial activity and Resistance. Russell AD and Quernel L B (eds.), Academic Press, London. pp 19-30

Loudon KW, Coke AP, Burnie JP, Lucas GS, Liu Yin JA (1994). Invasive aspergillosis: clusters and sources? J Med Vet Mycol. 32:217-224

Mackiewicz B, Skorska C, Dutkiewicz J, Michnar M, Milanowski J, Prazmo Z, Krysinska-Traczyk E, Cisak E (1999). Allergic alveolitis due to herb dust exposure. Ann Agric. Environ. Med. 6:167-170

Mayayo E, Pujol I, Guarro J (1999). Experimental pathogenicity of four opportunist *Fusarium species* in a murine model. J Med Microbiol. 48:363-366

Mc Laughlin JL, Chang CJ, Smith DL (1993). Simple bench top bioassay (Brine Shrimp and potato Discs) for the discovery of plant antitumor compounds. In: Human Medicinal Agents from plants, Asc symp series 534, ACS, Washington, DC

Mchlaughlin JL (1990). Bench tops bioassay for the discovery of bioactive compounds in higher plants. Brenena, pp 29

McLaughlin JL, Rogers LL (1998). The use of biological assays to evaluate botanicals. Drug Information Journal. 32:513–524

Mclaughlin JL, chang CJ, Smith DL (1991). "Bench-top" bioassay for the discovery of bioactive natural products: an update. In: Rhaman, AU (eds.) Studies in Natural Product Chemistry. Elsevier, Amsterdam.

Mensure O, Sezen T (1998). Drug Yield and Essential Oil of *Thymus vulgaris L.* as in Influenced by Ecological and Ontogenetical Variation. J of Agriculture and Forestry. 22:537-542

Meriçli-Ilisulu F, Tanker M (1986). The volatile oils of some endemic *Thymus Species* Grown in Southern Anatolia. Planta Medica. 2:340-345

Merino O, Martin MP (1990). Successional and temporal changes in primary productivity in two Mediterranean scrub ecosystems. Acta Ecologica. 1:103-112

Merzouk A, Ed-derfoufi F, Molero Mesa J (2000). Contribution to the knowledge of Rifian traditional medicine. II: Folk medicine in Ksar Lakbir district (NW Morocco). Fitoterapia. 71(3):278-307

Meyer BN, Ferrigini NR, Putnam JE, Jacobsen LB, Nichols DE, McLaughlin JL (1982). Brine shrimp: a convenient general bioassay for active plant constituents. Planta Med. 45:31-34.

Michael EK, Pharm, D(2001). Antifungal *Candida albicans* from human immunodeficiency Resistance among Candida species. Virus-negative patients never treated with azoles. Pharmacotherapy. 21:124-132

Mongelli E (1996). Screening of Argentine medicinal plants using the brine shrimp microwell cytotoxic assay. International Journal of Pharmacognosy. 34: 249-254

Morens DM, Folkers GK (2004). The challenge of emerging and re-emerging infectious diseases. Nature. 430:242-249

Moriarty AP, Crawford GJ, McAllister IL, Constable IJ (1993). Severe corneoscleral infection. A complication of beta irradiation scleral necrosis following pterygium excision. Arch Ophthal mol. 111:947-951

Navarez D, Olofsdotter M (1996). Relay seeding technique for screening allelopathic rice (*Oryza sativa*). Proc. 2nd Int. Weed Control Con Copenhagen, pp 1285-1290

Nevas M, Korhonen AR, Lindstrom M, Turkki P, Korkeala, H (2004). Antibacterial efficiency of finnish spice essential oils against pathogenic and spoilage bacteria. J. Food. Prot. 67:199-202

Newall CA, Anderson LA, Phillipson JD (1996). Herbal Medicines: A Guide for Health-Care Professionals. The Pharmaceutical Press, London, England.

New, Old and Forgotten Remedies. (2002). By Edward, Pollock Anshutz, presented by Dr. Robert Seror.

Oberlies NH (1998). Cytotoxic and insecticidal constituents of the unripe fruit of *Perseia Americana*. Journal of Natural Product. 61:781-785

Pannuti CS (1993). Hospital environment for high risk patients, *In* R. P. Wenzel (ed.), Prevention and control of nosocomial infections, 2nd ed. The Williams & Wilkins Co., Baltimore, Md. pp 365-384

Pannuti C, Gingrich R, Pfaller MA, Kao C, Wenzel RP (1992). Nosocomial pneumonia in patients having bone marrow transplant. Attributable mortality and risk factors. Cancer. 69:2653-2662

Parez H, Diaz F, Medina JD (1997). Chemical investigation and in vivo antimalarial activity of *Tabebuia ochracea* ssp. Neochrysantha. International Journal of Phrmacognosy. 35:227-231

Parra AL (2001). Comparative study of the assay of *Artemia salina* L. and the estimate of the median lethal dose (LD_{50} value) in mice, to determine oral acute toxicity of plant extract. Phyto medicine. 8:395-400

Patterson DT (1986). Allelopathy. In: Research Method in Weed Science. Camper ND Eds. pp 125-1126

Perez C, Pauli M, Bazerque P (1990). An antibiotic assay by the well agar method. Acta Biologica et Medica Experimentalis. 15:113–115

Piccaglia R, Marotti M. (1991). Composition of the essential oil of an Italian *Thymus vulgaris* L. ecotype. Flavour and Fragrance Journal. 6:241-244

Plaza P, Torres R, Usall J, Lamarca N, Vinasa I (2004). Evaluation of the potential of commercial post-harvest application of essential oils to control citrus decay. The Journal of Horticultural Science and Biotechnology. 6(79): 935-940

Pothier J, Galand N, Ouali M, Viel C (2001). Comparison of planar chromatographic methods applied to essential oils of wild thyme and seven chemotypes of thyme. Farmaco. 56(5-7):505-511

Putievsky E, Basker D (1977). Experimental cultivation of *Marjoram oregano* and *basil*. Journal of Horticultural Science. 52:181-188

Putnam AR (1986). Can it be managed to benefit horticulture? *HortScience* 21: 411-413

Putnam AR, DeFrank J, Barnes JP (1983). Exploitation of allelopathy for weed control in annual and perennial cropping systems. J. Chem. Ecol. 9:1001- 1010

Rahman A (1991) Studies in natural product chemistry, Netherlands, Elsevier Science Publisher

Rahman A (2003). International Workshop on the Development of Medicine from Plants. H.E.J. Comstech (CPC). Karachi, Pakistan

Rahman M, Gul S (2000). Inhibitory effects of pathogenic concerns on asexual reproduction of toxigenic fungi (Strains of *Aspergillus*). Punjab J. Biological Sciences. 3: 666-668

Razia RS, Uzma Z, Chaudhary SS, Ahmad H (1995). Antimicrobial activity of essential oil of *Schinus terebinthifolius, Cypress semervirens, Citrus limon, Ferula assafoetida.* Pak J Sci Ind Res. 38:35-36

Rhame FS (1989). Nosocomial aspergillosis: how much protection for which patients? Infect Control Hosp Epidemiol. 10:296-298

Rinaldi M (1983). Invasive aspergillosis. Rev. Infect. Dis. 5:1061-1075

Rolston KVI (2001). The spectrum of pulmonary infections in cancer patients. Curr Opin Oncol. 13:218-223

Romano C, Miracco C, Difonzo EM (1998). Skin and nail infections due to *Fusarium oxysporum* in Tuscany, Italy. Mycoses. 41:433-437

Sarubbi FA, Kopf HB, Wilson MB, McGinnis MR, Rutala WA (1982). Increased recovery of *Aspergillus flavus* from respiratory specimens during hospital construction. Am. Rev. Respir. Dis. 125:33-38

Putnam AR (1986). Can it be managed to benefit horticulture? *HortScience* 21: 411-413

Putnam AR, DeFrank J, Barnes JP (1983). Exploitation of allelopathy for weed control in annual and perennial cropping systems. J. Chem. Ecol. 9:1001- 1010

Rahman A (1991) Studies in natural product chemistry, Netherlands, Elsevier Science Publisher

Rahman A (2003). International Workshop on the Development of Medicine from Plants. H.E.J. Comstech (CPC). Karachi, Pakistan

Rahman M, Gul S (2000). Inhibitory effects of pathogenic concerns on asexual reproduction of toxigenic fungi (Strains of *Aspergillus*). Punjab J. Biological Sciences. 3: 666-668

Razia RS, Uzma Z, Chaudhary SS, Ahmad H (1995). Antimicrobial activity of essential oil of *Schinus terebinthifolius*, *Cypress semervirens*, *Citrus limon*, *Ferula assafoetida*. Pak J Sci Ind Res. 38:35-36

Rhame FS (1989). Nosocomial aspergillosis: how much protection for which patients? Infect Control Hosp Epidemiol. 10:296-298

Rinaldi M (1983). Invasive aspergillosis. Rev. Infect. Dis. 5:1061-1075

Rolston KVI (2001). The spectrum of pulmonary infections in cancer patients. Curr Opin Oncol. 13:218-223

Romano C, Miracco C, Difonzo EM (1998). Skin and nail infections due to *Fusarium oxysporum* in Tuscany, Italy. Mycoses. 41:433-437

Sarubbi FA, Kopf HB, Wilson MB, McGinnis MR, Rutala WA (1982). Increased recovery of *Aspergillus flavus* from respiratory specimens during hospital construction. Am. Rev. Respir. Dis. 125:33-38

Taumara J, Takebayashi T, Sugisawa H (1993). In vitro culture and the production of secondary metabolites. Biotech. Agric. Forestry. 21: 413-426

Tantoui-Elaraki A, Lattaoui N, Errifi A, Banjilali (1993). Composition and antimicrobial activity of the essential oil of *Thymus broussonettii*, *T zygis* and T. *satureioides*. J. Essent. Oil Res. 5: 45-53

Thomashow MF, Nutter R, Montoya AL, Gordon MP, Nester EW (1980). Integration and organization of Ti plasmid sequences in crown gall tumors. Cell. 19(3):729–739

Thompson DP (1989). Fungitoxic activity of essential oil components on food storage fungi. Mycologia. 81:151-153

Thompson JD (2002). Population structure and the spatial dynamics of genetic polymorphism in thyme. In: Thyme: the Genus *Thymus* (eds Stahl-Biskup, E. & Saez, F.). Taylor & Francis, London. pp 44–74

Thompson JD, Chalchat JC, Michet A, Linhart YB, Ehlers B (2003). Qualitative and quantitative variation in monoterpene cooccurrence and composition in the essential oil of *Thymus vulgaris* chemotypes. J Chem Ecol. 29(4):859-880

Thuille N, Fille M, Nagl M (2003). Bactericidal activity of herbal extracts. Int J Hyg Environ Health. 206:217-221

Tinland B, Hohn B (1995). Recombination between prokaryotic and eukaryotic DNA: Integration of *Agrobacterium tumefaciens* T-DNA into the plant genome. pp.209-229 In: Setlow JK (ed), Plenum Press, New York. Genetic Engineering

Tworkoski T (2002). Herbicide effects of essential oils. Journal of Weed Science. 4(50): 425-431

Ultee A, Gorris LGM, Smid EJ (1998). Bactericidal activity of carvacrol toward the food borne pathogen Bacillus cereus. J Applied Microbiology. 85(2): 211-218

Ultee A, Slump RA, Steging G, Smid EJ (2000). Antimicrobial activity ofcarvacrol toward *Bacillus cereus* on rice. J Food Prot. 63:620-624

Vernet P, Gouyon PH, Valdeyron G (1986). Genetic control of the oil content in *Thymus vulgaris* L. a case of polymorphism in a biosynthetic chain. Genetica.69: 227–231

Vlachos V, Critchely AT, Von HA (1996). Establishment of a protocol for testing antimicrobial activity in Southern African macro algae. Microbios. 88: 115-123

Weston LA (1996). Utilization of allelopathy for weed management in agro ecosystems. Agronomy Journal. 88:860-866

White GF, Marshall SJ, Krause D Blencowe DK (2004). Characterisation of Glycerol Trinitrate Reductase (NerA) and the catalytic role of active-site residues.Journal of Bacteriology. 186:1802-1810

Wichtl M (1994). Herbal Drugs and Phytopharmaceuticals. Stuttgart: Medpharm Scientific Publishers. Germany

A modern herbal by Mrs. M Grieve: www.botanical .com

Thyme: www.floridata/.com/refl/laur_nob.cfm.

Herbs and Oils: www.fortunecity.com/roswell/chaney/191/id120.htm.