INVESTIGATION ON ANTIMICROBIAL PROPERTY OF CERTAIN PLANT PRODUCTS AGAINST FEW PHYTOPATHOGENIC ORGANISMS

A dissertation thesis submitted to Nirma University in Partial fulfillment for the Degree of

MASTER OF SCIENCE

IN

BIOTECHNOLOGY/MICROBIOLGY

Submitted by:

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With the guidance of

Dr. Vijay Kothari

Dedicated to
God and our family

Acknowledgment

"Success doesn't come to you? You go to it."
Marva Collins

First of all we gratefully acknowledge our family, for their encouragement, prayers, magnificent support, love and patience throughout our years of education. We also thank to god.

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Binjal A. Darji
Megha A. Doshi
Jaydeep B. Ratani

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ABBREVIATIONS

EtOH	Ethanol
HPLC	High performance liquid chromatography
MAE	Microwave assisted extraction
MBC	Minimum bactericidal concentration
MeOH	Methanol
MFC	Minimum fungicidal concentration
MH Agar	Mueller-Hinton agar
MHB	Mueller-Hinton broth
MIC	Minimum inhibitory concentration
NA	Nutrient agar
NCCLS	National committee for clinical laboratory standards
NI	No inhibition
OD	Optical density
PAE	Post antibiotic (antibacterial) effect
PEE	Post extract effect
PLE	Pressure liquid extraction
R_f	Retardation factor
SFE	Supercritical fluid extraction
SPE	Solid pressure extraction
TLC	Thin-layer chromatography
UAE	Ultrasonic assisted extraction
ZOI	Zone of inhibition

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Introduction

1. INTRODUCTION

"Eat leeks in March and wild garlic in May, and all the year after the physicians may play."

-Traditional Welsh rhyme

A large proportion of crops are lost due to plant pathogens each year, there is currently much interest in developing strategies to control plant pests. Major crop losses occur due to diseases and insects demanding serious attention towards food protection. *Xanthomonas campestris* causes 30% loss in rice production in Southeast Asia [Beattie, 2006]. International trade of fruits and vegetables has grown greatly in the past 20 years and is presently a multi-billion dollar business [Golan and Paster, 2008]. *Callasobruchus chinensis* L is devastating pests of various storage pulses throughout the world, causes 32.64% of damages to stored pulses as compared with vegetables and oil seeds (3%) [Kumar et al., 2011]. Fungal contamination and subsequent production of aflatoxin can occur in crops in the field, during harvest, postharvest operations and in storage. Food and Agricultural Organization (FAO) estimated 25% loss of world food crops, affected by mycotoxins [Dubey et al., 2011]. Fungal deterioration of storage seeds and grains is a major problem in the Indian storage system due to hot humid climate. *Aspergillus* sp. is most common fungal species that can produce mycotoxins in food and feed stuffs. Among all mycotoxins, particularly aflatoxin B_1 (AFB_1) is the most toxic form for mammals and cause damage as toxic hepatitis, hemorrhage, immunosuppression, hepatic carcinoma, etc [Reddy et al., 2009]. AFB_1 has been classified as a class II human carcinogen by the International Agency for Research on Cancer [IARC, 1993].

In past few decades, a worldwide increase in reports of multi-drug resistant microbial strains, increased usage of chemical pesticides to control plant infections, has added new aspects to be focused. "India ranks fourth in Asia and tenth in the world in plant diversity" said by N.N. Singh (vice chancellor, Birsa Agricultural University) [http://timesofindia.indiatimes.com/home/environment/flora-fauna/India-ranks-10th-in-world-in-plant-diversity/iplarticleshow/7088101.cms]. The usage of synthetic compounds to control pests has caused several problems such as, contamination of soil and ground water, toxicity towards non target species including humans, disturbance of ecosystem, etc. Biochemical pesticides include plant extract, pheromones, plant hormones, natural plant derived regulators, enzymes, etc. [Chunxue et al., 2010].

The research on natural products and compounds derived from natural products has accelerated in recent years due to their importance in drug discovery. Natural products from plant source may be used directly or considered as a precursor for developing better molecules. From centuries, the use of phytochemicals in food preservation and improvement of qualities of certain traditional foods has been in practice. Isolation of several phytochemicals is successfully achieved with advance in separation technology. Different compounds isolated from plants such as dimethyl pyrrole, hydroxydihydrocornin-aglycones, indole derivatives, etc., are reported to have antifungal activities [Arif et al., 2009]. Oil as plant component has been used for post harvest protection of crop [Davidson and Naidu, 2000]. The isoquinoline alkaloid emetine obtained from *Cephaelis ipecacuanha* and related species, has been used for many years as amoebicidal drug and also for the treatment of abscesses due to the spread of *Escherichia histolytica* infections [Ciocan and Bara, 2007].

Therefore, alternative disease management using natural compounds and other resistance types needs to be considered to inhibit the growth of plant pathogens.

Objectives:

1. To screen certain plant extracts/pure phytochemicals for their activity against various plant pathogenic microbes.
2. To determine Minimum inhibitory concentration (MIC), Minimum bactericidal concentration (MBC), and Minimum fungicidal concentration (MFC) of potential extracts against susceptible microbes.
3. To study the effects of plants extracts on aflatoxin production by *Aspergillus parasiticus*.

Review of literature

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2.1. Synthetic pesticides and its challenges

The usage of synthetic pesticides has increased for the control of plant disease due to its effectiveness in controlling phytopathogens. The unrestrained use of these chemicals, under the adage, "if little is effective, a lot more will be powerful" has played ravage with human and other life forms, environment, etc. Due to non-biodegradable nature of chemical pesticides they get accumulated at each trophic level of food chain. Since humans occupy the top level in any food chain, so the maximum amount of harmful chemical pesticides gets accumulated in our bodies. Thus accumulation of such chemicals in living bodies at each trophic level of food chain is called biomagnifications. [http://www.preservearticles.com/2012010219581/essay-on-accumulation-of-harmful-chemicals-in-food-chains.html].

2.1.1 Effect of pesticides

The credits of pesticides include enhanced economic potential in terms of increased production of food & fibre, amelioration of vector-borne diseases and then their debits have resulted in serious health implications to man & his environment [Aktar et al., 2009]. The World Health Organization (WHO) estimated that 200,000 people are killed worldwide, every year due to pesticide poisoning [Dubey et al., 2011]. During pesticide spraying, it can enter to body through skin contact and inhalation of aerosols. Among side effects of pesticide are hormonal disruption, cancer, neurotoxicity, birth defects, etc.

2.1.2 Risk associated to pesticides

Acephate, dithiocarbamates, DDT, endosulfan, thiabendazole, triazophos, methidathion, etc are common pesticides used to spray on agricultural field. Over 100 people died after consuming wheat flour contaminated with parathion (herbicide), this was first report of poisoning due to pesticide in India (1958). Herbicides are formulated to kill particular plant but few herbicides get volatilized off treated plants and cause sub-lethal effects on non-target plants like phenoxy herbicides,2,4-D, glyphosate can severely reduce seed quality [Aktar et al., 2009]. Leakage of pesticides from soil to water leads to contamination in rivers, lakes, aquatic vegetation, etc. Also over spraying of pesticides on plants causes severe reduction of normal flora of soil and cause impact on fertility of soil. Majority population affected includes agricultural farm workers, formulators, workers, sprayers, etc.

2.1.3. Advantage of plant products over chemical pesticides

The plant product used as pesticide is referring to biochemical pesticide. They are generally less toxic to the user and to non-target species, making them desirable and sustainable tools for disease management. They are much cheaper than chemical pesticide & ecofreindly.

2.2. Major Phytochemicals

In late 1990s the use of plant products as therapeutic agent was in high popularity. In 1996 there was increase in sales of botanical medicines by 37% over 1995. Phytochemicals serve as a plant defense mechanism against infection by microorganisms, insects, etc [Cowan, 1999]. Different parts of plant contain active compounds like, roots of ginseng plants contain saponins, eucalyptus leaves has tannins [Sharma and Arora, 2006].

2.2.1. Phenols and Polyphenols

Majority of plants have ability to synthesize aromatic substances like phenols, oxygen-substituted derivatives. A phenolic compound has a single substituted phenolic ring, C_3 side chain at a lower level of oxidation. Few common phenolic compounds are caffeic acid from tarragon and thyme, cinnamic acid from brassica oil seeds, coumarin from spices, sesamol from sesame oil, quinnones, tannins, flavonoids, etc [Davidson and Naidu, 2000]. The hydroxylated phenols, catechol and pyrogallol are toxic to microorganisms, as catechol has two OH^- groups and pyrogallol has three [Sharma and Arora, 2006]. Amentoflavone from *Selaginella tamariscina* have antifungal activity with IC_{50} 18.3 µg/mL [Arif et al., 2009]. Quercetin is the most abundant of the flavonoids found in lotus leaves as a component that may be a potential antibacterial agent [Mingyu and Zhuting, 2008].

2.2.2. Terpenoids

The fragrance of plants is carried in essential oil fraction. Terpenoids share origins with fatty acids, synthesized from acetate units [Cowan, 1999]. An antimicrobial diterpene 8 from *Alpinia galanga* synergistically enhanced the antifungal activity of quercetin and chalcone against *Candida albicans* [Arif et al., 2009]. These compounds are based on isoprene structure, they occur as diterpenes, triterpenes, and tetraterpenes (C20, C30, and C40) and sesquiterpenes (C15). The sesquiterpene isolated from dichloromethane extract from the roots of *Vernonanthura tweedieana* was effective against *Trichophyton mentagrophytes*. Clerodane

diterpenes isolated from fruit pulp extract of *Detarium microcarpum* showed inhibition of growth of the plant pathogenic fungus *Cladosporium cucumerinum* [Abad et al., 2007].

2.2.3. Essential oils

The fragrance of plants is carried in essential oil fraction. Antifungal activity of essential oil isolated from Eucalyptus against phytopathogenic fungi *Pythium ultimum, Rhizoctonia solani* and *Bipolaris sorokinian* [Katooli et al., 2011]. Essential oil from oregano showed antibacterial activity against phytopathogens *P. marginalis, P. syrinagae and Xanthomonas vesicatoria* [Vasinauskiene et al., 2006]. The antifungal activities of the essential oil from *Agastache rugosa* and its main component, estragole, combined with ketoconazole, were reported to have significant synergistic effects [Arif et al., 2009].

2.2.4. Alkaloids

A natural compound isolated from medicinal plants. It is a heterocyclic nitrogen compounds. Morphine isolated from opium poppy *Papaver somniferum* in 1805, was first medically useful example of alkaloid. A quinolinone alkaloid from leaves of *Melochia odorata,* were reported to exhibit antifungal activities against a broad spectrum of pathogenic fungi [Arif et al., 2009]. Pyrrolizidine alkaloids from *Heliotropium subulatum* extracts showed antimicrobial activity against both fungal and bacterial species [Craig, 1998].

2.2.5. Saponins

These are glycosylated compounds. It is stored in plant cells as inactive forms but in presence of pathogen it gets converted in to biologically active antibiotics by enzymes. It is divided into three major groups, a triterpenoid, a steroid, or a steroidal glycoalkaloid [Arif et al., 2009]. Triterpenoid saponins are found primarily in dicotyledonous plants but also in some monocots, whereas steroid saponins occur mainly in monocots, steroidal glycoalkaloids are found primarily in members of the family Solanaceae, which includes potato and tomato, but also in the Liliaceae [Osbourn, 1996].

2.2.6. Peptides and proteins

In 1942, it was first reported that peptides can be inhibitory to microorganisms. The vulgarinin purified from the seeds of *Phaseolus vulgaris L* displayed antifungal property against few plant pathogenic fungi *Fusarium oxysporum, Mycosphaerella arachidicola, Physalospora piricola* and *Botrytis cinerea* [Abad et al., 2007].

Table 2.1: Class and subclass of natural antimicrobial compounds

Class	Subclass	Plant source	Activity	Reference
Phenols and polyphenols	Tannins , salicylic acid	*Gaullher procumbens, Rhammus purshiand, Anacardum pulsatilla*	Antifungal	Arif et al., 2009
	Polyisoprenylated	*Cuban propolis*	Antimicrobial	
	Amentoflavones	*Selaginella tamariscina*	Antifungal	
	Prenylated	*Fsafetida foetida*	Antifungal	
	Phloretin	*Malus sylvestris*	Antimicrobial	Cowan, 1999
	Quercetin	*Quercus rubra*	Antivirus	
	Coumarins	*Carum carvi*	Antibacterial	
	Anthemic acid	*Matricaria chamomilla*	Antibacterial	
	Tannin	*Eucalyptus globules*	Antibacterial	
	Catechin	*Camellia sinensis*		
	Galangin	Propolis	Antifungal	Cushnie and Lamb, 2005
	7-hydroxy-3,4-flavan	*Terminalia bellerica*		
	Ostruthin	*Peucedanum ostruthium*	Antimycobacterial	Schinkovitz et al., 2003
	Crassinervic acid	*Piper sp.*	Antifungal	Abad et al., 2007
	Biochanin A , dihydrobiochanin A	*Swartzia polyphylla*		
Terpenoids	Eugenol	*Syzygium aromaticum*	Antimicrobial	Cowan, 1999
	Sesquiterpene	*Vernonanthura tweediean*	Antifungal	Abad et al., 2007
	Drimenol, drimenal, viridiflorol, gymnomitrol, chloroisopiagiochin D.	*Bazzania trilobata*		
	Costunolide and dehydrocostunolide	*Centaurea sp.*		
Alkaloids	Cocaine	*Erythroxylum coca*	Gram-negative and positive cocci	Cowan, 1999
	Colchicine	*Gloriosa superba*	Antimicrobial	
Saponins	stigmasta-4-en-3-one, β-sitosterol	*Morinda citrifolia*	Antimycobacterial	Okunade, et.al. 2004
	physalin B	*Physalis angulata*		
	cayenne pepper	*Capsicum frutescens L.*	Antifungal	Abad, et.al., 2007
Essential oils	linalool and linalyl acetate	*Lavandula angustifolia*	Antifungal	Abad, et.al., 2007
	borneol, camphene, camphor, α-pinene verbenone, bornyl acetate	*Rosmarinus officinalis*		
Peptides and Polypeptides	Angularin	Adzuki bean	Antifungal	Arif et al., 2000
	Purothionin	*Triticum aestivum*	Antibacterial	Pelegrini et al., 2011
	α- and β-basrubrins	*Basella rubra*	Antifungal	Wang et al.,
	Snakin	Potato tubers	Antibacterial, antifungal	Butu and Butu., 2011
	Defensin	*Trigonella foenum-graecum L*	Antifungal	Abad et al., 2007
	Vulgarinin	*Phaseolus vulgaris L*	Antifungal	

2.3. Plant Material

Medicinal herbs are significant source of synthetic and herbal drugs. Isolated active compounds are used for applied research. Herbs like turmeric, fenugreek, ginger, garlic, holy basil, etc are integral part of ayurvedic formulations [Ahmed, 2010]. Medicinal herbs are considered to be natural factory producing natural products having antimicrobial, antiviral activities, etc. Plants comprise several active components as described in section 2.2. Different parts of plant like leaves, roots, bark, fruit and seeds contain different active ingredients, few may be toxic, others may be harmless. For example fruit capsules of *P. somniferum* produce powerful drugs while seeds do not contain alkaloids [Wyk and Wink, 2004]. Phytochemicals have astronomical usage like therapeutic materials for humans, animals, plants ailments, treatment of challenging diseases like cancer, asthma, diabetes, fungal infections, biopesticides, preservatives, etc. Failure of chemical pesticides in controlling pest and increasing resistant strain of microorganisms has coerced to search novel source of natural compounds. In India and Africa the development of biopesticides is specially advocated to develop their own natural resources in crop protection [Agrawal and Pandey, 2011]. Along with research on emerging natural plant products, phytochemicals industry is also growing with tremendous pace. Antibiotics may be more useful than synthetic chemicals in the control of plant diseases due to following reasons: applied selectively in low concentrations, easily broken down by soil microorganisms, etc. But application of antibiotics on fields in uncontrolled manner might develop resistance in organisms. Different antibiotics used to control plant pests are blasticidin, mildomycn, polyoxin, prumycin, cycloheximide, kasugamycin, validamycin and tetranactin. Streptomycin is used to combat plant disease caused by *Pseudomonas* sp. and *Xanthomonas oryzae* [Crueger and Crueger, 1989].

The information regarding selected seeds is given below.

2.3.1. *Tamarindus indica*

Family: Leguminosae

Common name: Imli

The plant is extensively grown in all over the Bangladesh, is widely used all over tropical Africa, Sudan, India, Pakistan for different purposes. Different parts of this plant are used in the indigenous systems of medicine for the treatment of a variety of human ailments. Ara and Islam, (2009), reported the presence of alkaloids, glycosides, flavonoids and saponins in

ethanolic extract of *T. indica* seeds and its antibacterial activity against *Shigella dysentriae* and *Staphylococcus aureus*. Methanolic extract of tamarind seeds has high concentration of flavonoids, tannins, and steroids. Tamarind fruit pulp is used for seasoning, as a food component, to flavor confections, curries and sauces and is a main component in juices and certain beverages. The major industrial product of tamarind seed is the tamarind kernel powder (TKP) which is an important sizing material used in the textile, paper and jute industries. Tamarind seed kernels have a relatively high antioxidant activity and phenolic content [Caluwe et al., 2010].

2.3.2. *Syzgium cumini*

Family: Myrtaceae

Common name: Jamun

Different parts of this plant, such as seeds, bark, fruit, and leaves are used to treat diabetes mellitus in many countries [Oliveira et al., 2007]. Fruit of *S. cumini* contains mallic acid, a small quantity of oxalic acid, gallic acid and tannins account for astringency of the fruit. Seeds contain flavonoid such as rutin, quercetin and 11ß-sitosterol. Stem of *S. cumini* tree contains betulinic acid, ß-sitosterol, friedelin, epi-friedelanol and eugenin. The plant possess antidiabetic, anti-inflammatory, antiallergic, gastroprotective, antiviral, antibacterial activity, etc [Jadhav et al., 2009]. *Escherichia coli* and *Vibrio cholera* were inhibited by methanol extract of *S. cumini* seeds at 1100 µg/mL and its ethanol extract at 2500 µg/mL. The HPLC and TLC of *S. cumini* (MeOH) seed extract confirmed the presence of quercetin and gallic acid [Kothari et al., 2011].

2.3.3. *Phoenix sylvestris*

Family: Arecaceae

Common name: Dates

Phoenix sylvestris Roxb. is gregarious in many parts of India. It is an ornamental tree and can also be used as an avenue plant The fruit is cooling, oleaginous, cardiotonic, fattening, constipative, good in heart complaints, abdominal complaints, fevers, vomiting and loss of consciousness. The juice obtained from the tree is considered to be a cooling beverage. The roots are used to stop toothache. The fruit pounded and mixed with almonds, quince seeds, pistachio nuts and sugar, form a restorative remedy. The central tender part of

the plant is used in gonorrhea [Parmar and Kaushal, 1982]. Ethanolic extract of *P. sylvestris* showed antibacterial activity against *Salmonella paratyphii A* and *Staphylococcus epidermidis* [Kothari, 2011].

2.3.4. *Manilkara zapota*

Family: Sapotaceae

Common name*:* Sapodilla, cheeku

M. zapota is a species of the lowland rainforest. Trees grow well in a wide range of climatic conditions from wet tropics to dry cool subtropical areas. The seed kernel (50% of the whole seed) contains 1% saponin and 0.08% sapotinin. Immature sapodillas are rich in tannin (proanthocyanadins) and very astringent. Ripening eliminates the tannin except for a low level remaining in the skin. It is highly drought-resistant, can stand salt spray, and approaches the date palm in its tolerance of soil salinity. The antimicrobial potential of *M. zapota* has been reported against different pathogenic bacteria and fungi eg., *Salmonella typhi, S. dysenteriae, Shigella sonnei, Shigella shiga, Aspergillus flavus, Fusarium* spp*, Aspergillus fumigatius, C. albicans, Vasianfactum sp.* This plant has antioxidative property and its fruit is preventive against biliousness and attacks of fever where as seeds are diuretic [Osman et al., 2011]. Acetone extract of *M. zapota* seeds was found to have significant antibacterial activity against *V. cholera and Pseudomonas oleovorans*, this extract showed positive results for alkaloids, phenols and flavonoids tests [Kothari and Seshadri, 2010].

2.3.5. *Annona squamosa*

Family: Annonaceae

Common name: Custard apple

The fruit is juicy and creamy white, it may contain up to 40 black seeds. These seeds are poisonous. The peelings and pulp contain oil that is useful in flavouring, the bark and leaves contain annonaine, an alkaloid. In tropical America, a decoction of the leaves is used as a cold remedy and to clarify urine, root is used in treatment of dysentery [http://www.flowersofindia.net/catalog/slides/Sugar%20Apple.html]. The plant contains glycoside, alkaloids, saponins, flavonoids, tannins, phenolic compounds, phytosterols. The ethanolic extract of leaves and stem is reported to have anticancer activity, flavonoids

isolated from aqueous extract of *A. squamosa* Linn. has been showed antimicrobial activity, the pure compound annotemoyin-1 isolated from the chloroform extract of the seeds of *A. squamosa* Linn was evaluated for its pesticidal activity [Pandey and Barve, 2011].

2.4. Test microorganisms

2.4.1. *Pseudomonas syringae*

It is a rod shaped gram-negative bacterium with polar flagella. It is a plant pathogen which can infect a wide range of plant species. It was first isolated from lilac tree (*Syringa vulgaris*). *P. syringae* also produce *Ina* (Ice nucleation activity) proteins which cause water to freeze at fairly high temperatures, resulting in injury to plants. The ability to cause disease on well-characterized host plants includes *Arabidopsis thaliana*, *Nicotiana benthemiana* and tomato. Syringomycin, syringotoxin, and syringostatin are produced by strains of *P. syringae* pv. *syringae*, these molecules are lipodepsipeptide that are toxic to wide range of plants [Bultreys and Gheysen, 1999]. *P. syringae* is a worldwide phytopathogenic microorganism mainly adapted to plant species, both monocotyledon and dicotyledon. *P. syringae* pv. *papulans*, *P. syringae* pv. *syringae* are streptomycin resistant [Han et al., 2003]. The most common symptoms of *P. syringae* include leaf spots and necrosis, fruit specks and scabs, flower wilting, twig die-back, branch and trunk cankers and, in particular circumstances, plant death. *P. syringae* pv. *actinidiae* is the causal agent of bacterial canker of kiwigreen (*Actinidia deliciosa*) [Marcelletti et al., 2011]. *P. syringae* pv. *syringae* causes bacterial canker of plum, annual tree mortality rates as high as 30% in Germany. Apical necrosis of mango caused by *P. syringae* pv. *syringae* [Kennelly et al., 2007]. *P. syringae* pv. tomato, the casual agent of bacterial speck of tomato [Bashan and Bashan, 2002]. *P. syringae* pv. *syringae* cause bacterial canker of stone fruit trees and blight of wheat and barley [Aldaghi et al., 2010].

2.4.2. *Pseudomonas marginalis*

P. marginalis is gram-negative rod. Han et al, (2003), reported that strains of *P. marginalis* (BJW1, HMY3, HOM5, NSS2) isolated from Korea were streptomycin resistant. *P. marginalis* cause soft rot of onion bulbs stored under low temperature [Kim, 2002]. Bacterial soft rot is a problem in broccoli production in areas of Australia, Canada,

Ireland and the United Kingdom. 30 to 100% losses of crop in some fields, additional losses may occur in cold storage [Canaday, 1991].

2.4.3. *Pectobacterium caratovorum*

It is a gram negative rod. It is also known as *Erwinia caratovorum* [Janes, 2005; Cui and Harling, 2006]. It is the causal agent of soft rot and blackleg of potato, lead to severe losses to the potato seed [Haan et al., 2008]. In South Africa during the 2006/2007 potato growing season, outbreaks of blackleg occurred, caused severe economic losses in commercial potato production fields [Merwe et al., 2008]. It is streptomycin resistant.

2.4.4. *Agrobacterium tumefaceins*

A. tumefaciens is a gram-negative rod, a soil-borne, nonsporulating, motile, phytopathogenic bacterium. It mainly causes 'crown-gall' disease in many dicotyledonous plants [Anand and Mysore, 2006]. It causes economic damage to plants with agricultural importance such as walnuts, tomatoes and roses [http://microbewiki.kenyon.edu]. *A. tumefaciens* was the causal agent of crown gall disease in Paris daisy. It induces tumors on wound sites of stems, roots [Escobar and Dandekar, 2003].

2.4.5. *Xanthomonas campestris*

X. campestris is a gram-negative, rod shaped. It causes black rot of crucifers which is major disease in Kashmir valley. Crucifers include cabbage, radish, turnip, cauliflower, mustard, etc. [Bhat et al., 2010], also infects to weeds like *A. thaliana* [http://www.ebi.ac.uk/2can/genomes/bacteria/ *X. campestris*.html]. *X. campestris* cause > 50 % loss of crucifers in India [Singh et al., 2011]. *X. campestris* ferments a stabilizing agent called xanthan gum that is used in many everyday products. It can live in the soil for over a year and spread through overhead irrigation and surface water [http://microbewiki.kenyon.edu/index.php/*Xanthomonas_campestris*]. *X. campestris* pv. *translucens* has ice-nucleating activity and cause frost injury. *X. campestris* pv. *vesicatoria* was found to be streptomycin resistant [Han et al., 2003]. *X. campestris* pv. *campestris*, a causal organism of black rot of cabbage and cauliflower [Bhardwaj and Laura, 2009]. *Artemisia nilagirica* (hexane) extract exhibits antibacterial activity against *X. campestris* (MTCC 2286) [Ahameethunisa and Hopper, 2010]. Citrus canker (*X. campestris* pv. *citri*) is a

bacterial disease causing lesions on the leaves, stems and fruit of citrus trees [Spreen et al., 2003].

2.4.6. *Aspergillus parasiticus*

A. parasiticus is a mold, producing mycotoxins on plants such as peanuts, figs, stone fruits, etc [Golan, 2008]. *A. parasiticus* is able to produce aflatoxin in food and feedstuffs which have been known to be potent heptocarcinogens in animals and humans [Golan, 2008]. Aflatoxin is secondary fungal metabolites isolated from contaminated meals and animal feeds, it strongly fluorescent in ultraviolet light that facilitates its detection [Wogan, 1966]. Aflatoxins can contaminate corn, cereals, sorghum, peanuts and other oil seed crops [Verma, 2004]. *A. parasiticus* produces aflatoxin G_1 and G_2, in addition to B_1 and B_2 [Hedayati et al., 2007]. The extract of *Azadirachta indica* was observed to be a good inhibitor of both growth and toxin production of *A. parasiticus* [Bhatnagar and McCormick, 1988]. The aqueous extract of *Terminalia chebula* was effective on inhibiting 74 % growth and 54 % aflatoxin production by *A. parasiticus* at concentration of 25% [Gali et al., 2010].

2.5. Extraction of Plant material

Extraction, involves the separation of biologically active component from plant using selective solvents through standard extraction procedures. The objective behind standardized extraction procedures is to obtain therapeutically effective compounds from the plant and at the same time elimination of undesired compounds with the help of selective solvents.

2.5.1. Microwave assisted extraction (MAE)

Usually dried plant material is used for extraction in most cases, but the plant cells contain minute traces of moisture that serves as the target for microbial heating. Hence, the extraction principle of MAE is when the moisture is heated up inside the plant cell due to the microwave effect; it evaporates and generates tremendous pressure on the cell wall due to the swelling of the plant cell. This pressure push the cell wall from inside and ruptures it, facilitating leaching out of the active constituents from the ruptured cells to the surrounding solvent thus improving the yield of phytoconstituents (Heemken et al., 1997). Higher temperature attained by microwave radiation can hydrolyses ether linkages of cellulose, and can convert it into soluble fractions within one to two min. This higher temperature attained

by the cell wall, during MAE, enhances the dehydration of cellulose and reduces its mechanical strength which in turn helps solvent to have an easy access easily to compounds inside the cell (Pare and Lapointe, 1990). There are multiple factors that affect efficiency of MAE (Llompart et al., 1997; Proestos and Komaitis et al., 2007), such as:

- Solvent nature and volume
- Extraction time
- Microwave power
- Matrix characteristics
- Temperature, etc

Numerous extraction methods are available, the simplest being cold extraction which allows most compounds to be extracted, although some may have limited solubility in the extracting solvent at room temperature. The other methods used for extraction of plant natural products are supercritical fluid extraction (SFE), extraction using carbon dioxide, ultrasonic assisted extraction (UAE), pressurized liquid extraction (PLE), solid-phase extraction (SPE) and Soxhlet extraction (Heemken, Theobald and Wenclawiak, 1997; Huie, 2002). The MAE, UAE and microwave assisted extraction continuous heating (MAEC) were not as good as soxhlet with respect to extraction efficiency but this methods were better for providing flavonoids extraction, antibacterial activity, etc. [Kothari et al., 2012].

The aqueous and alcoholic extracts of *Coffea robusta* obtained by MAE were reported to have radical scavenging activity > 75% [Upadhyay et al., 2011]. The MAE increase antibacterial activity against *E. coli* and *S. aureus* of Teak leaves (*Tectona grandis*) crude extract when tested *in-vitro* [http://elinacynthiaherbalgarden.blogspot.in/2012/02/antibacterial-activity-of-golden-teak.html]. MAE was applicable for extraction of bound phenolic acids from bran and flour [Chiremba et al., 2012]. MAE was identified as the best extraction process for *Cylindrotheca closterium* pigments as it combined rapidity, reproducibility, homogeneous heating and high extraction yields [Pasquet et al., 2011].

The advantages of MAE over these extraction methods are as follows:

- Significant reduction of extraction time
- Reduced solvent usage

• Improved extraction yield (Hawthorne and Miller, 1994)
• Provision for automation
• Suitable for thermolabile constituents, etc. (Huie, 2002; Buchholz and Pawliszyn, 1994)
• Low financial investment
• Presence of water does not cause any blockage as in other techniques (Barnabas et al., 1994).

2.6. Antimicrobial susceptibility testing

2.6.1. Antibacterial susceptibility testing

2.6.1.1. Broth dilution assay

In the broth dilution method, various concentrations of an antimicrobial drug are inoculated with a standard suspension of test bacteria. Following an overnight incubation at 30°C, the MIC is determined by observing the lowest concentration of the drug that will inhibit visible growth of the test bacteria. This method can be done by using the test tube dilutions (macrodilution) or the plastic microdilution tray 96 well plates (microdilution) [Mendoza and M.D, 1998]. The advantages of the microdilution procedure include the generation of MICs, the reproducibility, economy of reagents, space that occurs due to the miniaturization of the test and assistance in generating computerized reports if an automated panel reader is used [Turnidge et al., 2003].

2.6.1.2. Minimum inhibitory concentration (MIC)

MIC is the concentration of an agent which inhibits 80% growth of the initial growth of an organism. MIC for each dilution is determined in triplicate by a microtitre modification of the method. Microtitre trays containing 96 wells are used. Each well is filled with Mueller-Hinton broth (MHB), and serial twofold dilutions of the drug are performed (Turnidge et al., 2003). Bacterial strains are suspended in normal saline after overnight growth on agar medium and added in an equal volume to the wells containing MHB. The final inoculum size is according to 0.5 Macfarland turbidity standards. MIC endpoints are read as the lowest concentration of antibiotic with no turbidity. It can be performed in microbroth as well as macrobroth dilution methods. It is a quantitative test and can be further tested for the exact bactericidal concentration of the agent by MBC. Microbroth dilution method is preferred over

macrobroth dilution method because the volume of extract required is small, easy to operate and multiple extracts can be tested against multiple no. of organisms at a single time.

2.6.1.3. Minimum bactericidal concentration (MBC)

It is defined as the lowest concentration of antimicrobial agent needed to kill 99.9% of the initial inoculum after incubation for 24 h under a standardized set of conditions and is determined by noting the presence or absence of a $^{3}\log_{10}$-unit decrease in the CFU per milliliter [Pfaller et al., 2004]. MBC for each strain is determined by subculturing 20 µL from each microtitre well to drug-free nutrient agar plates. MBC endpoints are read as the lowest dilution of drug with no growth (>99.9% killing) after overnight incubation at 35°C [Collins et al., 2001]. Numerous biological and technical factors exist that can interfere with the performance of various assays and make the interpretation of the results quite difficult [Pfaller et al., 2004]. That includes

(i) Persisters: The bactericidal activities of certain antimicrobial agents are directly related to the bacterial growth rate. Lower the rate of growth, the slower the bactericidal effect.

(ii) Insufficient contact: Organisms in broth are generally considered to be fully exposed to antimicrobial agent in solution. It depends on how the inoculums was added, composition of tube or well, splashing at the time of inoculation and incubation, adherence to plastic tubes, etc. Delivering the inoculums below the surface of the broth test medium is recommended.

(iii) Antimicrobial agent carryover: The determinations of survivors by plating an aliquot from broth containing wells /tubes onto an agar surface, resulting in continued inhibition of growth on agar plates and overestimation of the killing effect. Such problem is more at higher concentrations of antimicrobial agents and with large volume transfers.

(iv) Paradoxical effect: The paradoxical effect is manifested when the proportion of surviving cells increases significantly as the concentration of the antimicrobial agent increases beyond the MBC, this effect is thought to occur in vitro when the growth of the organism is slowed to the extent that the lethal action of the drug is circumvented.

2.6.1.4. Synergistic activity of extracts

Synergy can be defined as interactions of constituents within a total extract of a single plant part, as well as between different plant extracts in a formulation (Williamson, 2004). Generally synergistic activity of extracts is being reported in relation with the antimicrobial drug i.e., the activity of the extract increases in combination with the antibiotic and vice versa (Nascimento et al., 2000; Adwan et al., 2002). When two different seed extracts are combined or same seed powder is reconstituted in different solvents and then combined can also give inhibition in the antibacterial assays. There is a possibility that two different components may act in synergy to give an enhanced activity than they give individually at the same concentration. Also, the potency of a lesser potent extract can increase when combined with another extract. Thus, synergistic interactions are of vital importance in phytomedicines, to explain difficulties in always isolating a single active ingredient, and explain the efficacy of apparently low doses of active constituents in a natural product. The combination of methanolic extract of *Tectona grandis* leaves with tetracycline leads to decrease in MIC value as compare to MIC value of individually against *Salmonella typhimurium, Klebsiella pneumonia* [Purushotham et al., 2010]. Combined antibiotic therapy has been shown to delay the emergency of bacterial resistance and may also produce desirable synergistic effects in the treatment of bacterial infection. Drug synergism between known antibiotics and bioactive plant extracts is a novel concept and could be beneficial (synergistic or additive interaction) or deleterious (antagonistic or toxic outcome) [Adwan and Mhanna, 2008].

2.6.2. Antifungal susceptibility testing

Antifungal activity was evaluated on the toxinogenic fungal strains using RPMI 1640 broth according to the manual of clinical microbiology [Turnidge and Jorgensen, 2003]. Broth dilution method for MIC determination described in NCCLS document M38-A for *Aspergillus*. Minimum fungicidal concentration (MFC) was defined as the lowest drug dilutions that yielded 99 to 99.5% killing. In vitro antifungal susceptibility testing is influenced by a number of technical variables, including slower growth rate, inoculum size and preparation, solubility of test agent, morphological variation or complexity, medium formulation and pH, duration and temperature of incubation, MIC endpoint determination [Pfaller et al., 2004]. The micorbroth dilution test has become the most widely used technique for antifungal susceptibility testing as describes in NCCLS document M27-A [Ingroff and

Pfaller, 2003]. Methanolic extract of *Syzygium jambolanum* seeds showed antifungal activity in range of 31.25 to 250 µg/mL and exhibited MFC at a concentration of 500 µg/mL [Chandrasekaran and Venkatesalu, 2004]. Aflatoxin produced by *A. parasiticus, A. flavus, A. nominus* [Golan, 2008]. Aflatoxin belongs to a family of compounds with difuranocoumarins. Aflatoxins B_1, B_2, G_1, and G_2 (AFB_1, AFB_2, AFG_1, and AFG_2) are the four major aflatoxins. Aflatoxin M_1 is a hydroxylated derivative metabolized from aflatoxin B_1 by cows and secreted in milk. *A. parasiticus* produces G_1 and G_2 in addition to aflatoxins B_1 and B_2. Postharvest aflatoxin contamination can be problematic if grain storage is poorly managed. *A. parasiticus* synthesize aflatoxin by oxidative stress [Guo et al., 2009].

2.7. Time required to kill

Determination of the killing of a bacterial isolate over time by one or more antimicrobial agents under carefully controlled conditions is known as the time-kill method. This is a broth-based method where the rate of killing of a fixed inoculum (usually 5×10^5CFU/ml) is determined by sampling control (organism, no drug) and antimicrobial agent-containing tubes or flasks at intervals (usually 0, 2, 4, 6, 8, 10 to 12, and 24 h of incubation) are spreaded on to agar plates. Determine the decrease in percent of growth in experimental plates as compare to controls. The time-kill method has been used widely for the evaluation of new antimicrobial agents and allows the determination of whether an agent produces concentration-dependent killing (the percent of killing increases with increased drug concentrations) or time-dependent killing (the killing continues only as long as the concentrations are in excess of the MIC) [Pfaller et al., 2004]. Time-kill studies are useful in determining tolerance to the lethal activity of antibacterial agents. An additional biological issue that may affect bactericidal testing is the so-called post-antibacterial effect (PAE) (appendix A). PAE is the suppression or slowing down of bacterial growth after a short exposure to antimicrobial agent, which is then eliminated. Study of PAE is important for those antimicrobial agent-microorganism combinations with which a prolonged PAE is obtained and also it reduces the cost and toxicity of powerful antimicrobial agents [Dominguez et al., 2001]. Agents exhibiting a PAE require extended incubation following subculture in either time-kill determinations [Pfaller et al., 2004].

2.8. Characterization of crude extract

Once a plant constituent has been isolated and purified, it is necessary first to determine the class of compound and then to find out in which particular class it lies. The class of compound is usually clear from its response to color tests, its solubility, its R_f and its UV spectral characteristics. Complete identification within the class depends on measuring other properties (melting point, boiling point, optical rotation, etc.) and then comparing these data with those in the literature.

2.8.1. Thin layer chromatography (TLC)

2.8.1.1. Analytical TLC

Thin-layer chromatography is useful in fractionation as a final process for purification of comparatively small amounts of almost pure compounds. This method is used very widely for qualitative analysis and the system selection entails choosing the mobile phase, the stationary phase and the detection method. The thin-layer plates are available commercially as ready-made products with stationary phase as silica, alumina or polyamide. Sorbent-coated plates often incorporate a fluorescent indicator (F_{254}) so that natural products that absorb in short wavelength UV light (254 nm) will appear as dark spots on a green background. Under long wavelength UV, certain compounds may emit a brilliant blue or yellow fluorescence (Heinrich et al., 2005). Both UV absorbance and fluorescence properties may be used to monitor the separation of compounds on a TLC plate. Natural products that are not UV-active will need development using a suitable spray reagent such as vanillin-sulphuric acid, Dragendorff"s reagent, phosphomolybdic acid or antimony trichloride where separated compounds are visualized as colored bands. The bands containing pure natural product are scraped off the plate and the natural product is desorbed from the sorbent. This desorption may be carried out by placing the compound-rich sorbent into sintered glass funnel and washing with a suitable solvent followed by collection and concentration of the filtrate. The purified 'band' should then be assessed for purity by analytical TLC.

2.8.1.2. Preparative TLC

Preparative TLC has a great use and loadings of 1-100 mg can readily produce enough purified material for biological assays and structure elucidation. Preparative plates are available as precoated plates of 1-2 mm thickness in silica or alumina. The scale-up from analytical to preparative mode is crucial, as an increase in the sample load may drastically change the separation of the components. A number of secondary metabolites such as anthraquinones, phenols, etc. have been isolated and characterized using TLC (Shahidi et al., 2007; Wettasinghe et al., 2001; Rai and Shok, 1981). Identification of the fractionated compound(s) may be achieved using a combination of compatible techniques viz. HPLC, IR, NMR, etc.

Materials and methods

3. MATERIALS AND METHODS

3.1. Plant materials

The plants were selected on the basis of earlier reported antimicrobial activity of their other parts like pulp, stem, leaf, fruits, etc. The seeds of five plants *Manilkara zapota, Annona squamosa, Phoenix sylvestris, Syzygium cumini* and *Tamarindus indica* were collected during months of October to December, 2011 from the fruits purchased from local market of Ahmedabad city. Seeds were carefully examined for any visible contamination for their further use. They were thoroughly washed in tap water, air-dried and stored in an air-tight container at room temperature in dark to avoid photo-oxidation. The seeds were checked at regular intervals for any physical damage.

3.2. Test microorganisms

Five phytopathogenic bacterial strains and one fungal strain were selected, based on total agricultural (pre-harvest, post-harvest, transport, storage, etc.) losses and their geographical distribution. *Pseudomonas syringae, Pseudomonas marginalis, Agrobacterium tumefaceins, Xanthomonas campestris, Pectobacterium caratovorum*, and *Aspergillus parasiticus* were obtained from Microbial Type Cell Culture (MTCC), Chandigarh (table 3.1).

The bacterial strains were sub-cultured once in every month on nutrient agar slants and fungi on potato dextrose agar slants and then stored at 4°C. Further, they were maintained by preparing paraffin and glycerol stocks and stored at 4-8°C and -20°C, respectively.

Table 3.1: Test organisms

Name of organism	MTCC no.	Remarks (with inputs from MTCC catalogue)
Pseudomonas syringae	673	Soil from penguine rookery with algae (71, 15°C), Was collected from Antarctica, resistant to cefaclor and cefafroxii
Agrobacterium tumefaceins	431	Extremely good crown gall producer
Xanthomonas campestris	2286	Was originally collected from cabbage leaves bearing blackrot, resistant to cefaclor
Pectobacterium caratovorum	1428	Collected from potato. Production of asparginase and glutaminase, resistant to streptomycin, ampicillin, penicillin, cefuroxine, co-trimoxazole, cefaclor, cefafroxii, amikacin
Pseudomonas marginalis	2758	Antagonistic to postharvest pathogens of apples (37°C)
Aspergillus parasiticus	411	Aflatoxin production, transformation of sesqunlterpene lectone costunolide versiconal conversion of averufin in to aflatoxins, challenging organisms for testing inhibition of mycotoxin production, resistant to flucanazole and amphotericin B

3.3. Microwave Assisted Extraction (MAE)

Seeds were crushed twice in grinder (Maharaja whiteline bonus grinder) to coarse powder at knob 1 for 60 sec. In case of *P. sylvestris* knob 3 was used for at least 2 minutes. They were subjected to MAE (Kothari et al, 2009). The solvents used were: ethanol (50%), methanol and acetone. All solvents were from Merck, Mumbai, India.

The extraction was carried out in a domestic microwave oven (MW) (Electrolux EM30EC90SS). The heating and cooling time, microwave power and seed: solvent ratio was kept constant throughout the extraction process. A brown bottle of 250 mL (screw cape, Merck, Mumbai, India), containing 50 mL solvent, 1g of seed powder, mouth of bottle was closed loosely capped and kept in MW at constant power 720 W for extraction. Total extraction time and intermittent cooling periods were varied from solvent to solvent (table 3.2). The extraction was followed by macrofiltration, which was further subjected to centrifugation at 10,000 rpm for 15 min. Then the extracts were filtered using Whatman paper # 1 (Whatman International Ltd, Maidstone, England) to remove the fine particulate matter and were allowed to evaporate in petridishes at the temperature below the boiling

point of the respective solvent. Extracts were reconstituted in their respective solvents for disc diffusion assay and in dimethyl sulfoxide (DMSO) (Merck, Mumbai, India) for determination of minimum inhibitory concentration (MIC). Extraction and reconstitution efficiencies were calculated (appendices C) for different extracts which are shown in table 4.1. Extracts were collected in flat bottom glass vials (15 mL, Merck, India). The inner surface of the cap of the vial was covered with an aluminum foil to avoid leaching of filler components and their absorption by the extract (Houghton and Raman, 1998). They were then stored in refrigerator. Eight extracts of 5 different seeds were prepared and tested for the antibacterial and antifungal activity.

Table 3.2: Heating and cooling cycles for different solvents during MAE

Solvent	First heating time (s)	Cooling time (s)	Reheating time (s)	Total time (s)
Ethanol (50%)	40	40	10	70
Methanol	10	40	5	90
Acetone	25	40	10	120

3.4. Minimum inhibitory concentration (MIC)

3.4.1. Inoculum preparation

For inoculum preparation for bacteria, 24 h old culture was used. Two or three isolated colonies were taken and suspended in normal saline (0.85% NaCl). Then turbidity of the inoculum was adjusted to 0.5 MacFarland standard (10^8 CFU/mL) (appendix D).

For fungi, 48 h old culture was used. A spore suspension was prepared by washing slant with 0.85% NaCl. This suspension which contains spores was mixed for 15 s with a vortex mixer (Remi CM 101). The turbidity of the mixed suspension was measured with spectrophotometer at 530 nm and adjusted to a specific final optical density range (0.09-0.11 OD). So, inoculum size of suspension was 1.6×10^6 CFU/ml (Ingroff and Pfaller, 2003).

3.4.2. Macrobroth dilution method

Macrobroth dilution method was done according to NCCLS guidelines (Turnidge et al., 2003). Mueller-Hinton broth (MHB) (Himedia, Mumbai) was used for testing against bacteria and RPMI (HiMedia, Mumbai) broth against fungi. Extracts were reconstituted in DMSO and further dilutions were prepared. The volume of broth, extract, antibiotic (ampicillin, HiMedia, Mumbai), normal saline, solvent and inoculum was adjusted in such a way so that the extract gets diluted 100 times in each tube. For this, the total volume per tube was kept 1000 µL, tubes of growth control (750 µL broth + 250 µL inoculum), negative control (10 µL 1% v/v DMSO + 748 µL broth + 250 µL inoculums) as well as positive control (10 µL antibiotic + 748 µL broth + 250 µL inoculum) were also kept along with the absorbance controls (10 µL extract + 748 µL broth + 250 µL normal saline). The inoculum used (prepared from 24 h old culture), was already adjusted to 0.5 McFarland standard (1.0×108 CFU/mL). After vortexing the test tubes on a cyclomixer (Remi CM 101), they were kept for incubation at 30°C for 16-20 h. Visual observations were made and the OD was taken in a microtitre plate reader (BIORAD 680) at 655 nm. The % inhibition (I) was calculated according to the formula (appendix A).

3.4.3. Microbroth dilution method

Microbroth dilution method was also carried out according to NCCLS guidelines (Turnidge and Jorgensen, 2003). This assay was carried out in 96 well microtitre plates (HiMedia, Mumbai). These plates were flat bottom and untreated.

3.4.3.1. Microbroth dilution method against bacteria

MHB was used for testing against phytopathogenic bacteria. In case of *T. indica* (MeOH) and *P. sylvestris* (EtOH) extract, modified lactose minimal media (appendix B) was used because of precipitation of extract (Atlas, 2010). Extracts were reconstituted in DMSO and further dilutions were prepared. The volume of broth, extract, antibiotic (Ampicillin 10 (µg/mL), HiMedia, and Mumbai), normal saline, solvent and inoculum was adjusted in such a way so that the extract gets diluted up to 100 times in each well and hence the volume reaches to 200 µL. Also, the DMSO concentration was adjusted 1% v/v of the total well concentration. For this, the total volume per well was kept 200 µL. Wells of growth control (150 µL broth + 50 µL inoculum), negative control (2 µL DMSO + 146 µL broth + 50 µL

inoculum) as well as positive control (2 µL antibiotic + 146 µL broth + 50µL inoculum) were also kept along with the absorbance controls (2 µL extract + 146 µL broth + 50 µL normal saline). The inoculum used (prepared from 24 h old cultures), was already adjusted to 0.5 McFarland standard (10^8 CFU/mL). Wells of positive control, negative control as well as growth control were also kept along with the absorbance controls (as the extract was colored). The microtitre plates were wrapped with parafilm to prevent evaporation and contamination. They were kept for incubation at 30°C for 16-20 h. The readings were taken in a microtitre plate reader (BIORAD 680) at 655 nm, 60s prior shaking was given to the microtitre plates. The activity index and proportion index was calculated for different extracts (appendix A).

Pure compounds:

Curcumin (HiMedia), quercetin (HiMedia), and lycopene (HiMedia) were dissolved in DMSO while gallic acid (HiMedia) was dissolved in distilled water and then filter it with the disposable syringae filters. In case of gallic acid there was no need to put turbidity control because gallic acid did not contain any color. For curcumin, quercetin and lycopene modified lactose minimal media (appendix B) was used because of precipitation of compounds (Atlas, 2010). Microbroth dilution assay was performed same as plant extracts.

3.4.3.2. Microbroth dilution method against fungi

Microbroth dilution method against fungi was carried out according Manual of clinical microbiology (Turnidge and Jorgensen, 2003). RPMI 1640 broth (without bicarbonate) (HiMedia, Mumbai) was used for testing against phytopathogenic fungi. In RPMI media, 10.3 ml of L-glutamine (200mM) was added per liter of media In case of *T. indica* (MeOH) and *P. sylvestris* (EtOH) extract, fungi minimal media (appendix B) was used because of precipitation of extract (http://www.fgsc.net/methods/anidmed.html). Extracts were reconstituted in DMSO and further dilutions were prepared. The volume of broth, extract, antifungal (Amphoterecin B, HiMedia, Mumbai), normal saline, solvent and inoculum was adjusted in such a way so that the extract gets diluted up to 100 times in each well and hence the volume reaches to 200 µL. Also, the DMSO concentration was adjusted 1% v/v of the total well concentration. For this, the total volume per well was kept 200 µL. Wells of growth control (150 µL broth + 50 µL inoculum), negative control (14 µL broth + 2 µL DMSO + 50

µL inoculum) as well as positive control (148 µL broth + 2 µL antifungal + 50µL inoculum) were also kept along with the experimental controls (148 µL broth + 2 µL extract + 50 µL normal saline). The inoculum used (prepared from 48 h old cultures), was already adjusted at 530 nm (0.09-0.11 OD) (Ingroff and Pfaller, 2003). The microtitre plates were wrapped with parafilm to prevent evaporation and contamination. They were kept in the incubator at 30°C for 48 h. The readings were taken in a microtitre plate reader (BIORAD, 680) at 655 nm, 900s prior shaking was given to the microtitre plates before taking the reading.

3.5. Minimum bactericidal concentration (MBC)

An aliquot of 20 µL from tubes/wells showing visually complete inhibition of growth were transferred to nutrient agar plate and were spread evenly on it with the help of sterilized glass spreader. Another nutrient agar plate was inoculated from the tube/well which is having negative control; this plate is kept as control. The plates were kept for incubation at 30°C up to 72 hours for cidal/static/post extract effect. If the experimental plate shows presence of one or two colonies indicating 99.9% inhibition and that concentration is referred to as MBC. Presence of few colonies compared to the control indicates that the extract is having bactericidal effect. While the presence of colonies equivalent to control indicates that the extract is bacteriostatic and Ramadan et al., (1995), reported persistent suppression of bacterial growth after exposure to plant extracts has been defined as the post-extract effect (PEE) and PAE (Post Extract Effect) of standard antibiotics azithromycin and erythromycin against *Streptococcus* spp. (2-4 h).

3.6. Time required to kill

In order to determine the time required for a bactericidal extract to kill the susceptible test organism, the test bacteria was challenged with the extract at its MBC in a test tube, from which an aliquot of 20 µL was transferred to a nutrient agar plate (devoid of extract) at intervals of 0, 2, 4, 6, 8, 10, and 24 h and was spread evenly. The plates were kept for incubation at 30°C for 72 h. Time corresponding to the plate with no growth was taken as the time required by the extract to kill the bacteria.

3.7. Disc diffusion assay of antimicrobial agents

For disk diffusion assay of antibiotics discs (HiMedia, Mumbai) and for antifungal assay hexadiscs (HiMedia, Mumbai) (hexa antimyco- 01) were taken (Table 4.17 b and 4.20). Sterile petriplates having diameter 150 mm was poured with 60 ml of sterile Mueller Hinton agar (MHA) and for antifungal sterile potato dextrose agar to give a mean depth of 4.00 ± 0.5 mm. the inoculum was prepared in sterile normal saline (0.85% NaCl solution) from 24h old bacterial and fungal culture and the turbidity of the inoculum was visibly adjusted using sterile normal saline solution to approximate that of 0.5 McFarland turbidity standard which is equivalent to 10^6 - 10^8 CFU/mL. Within 15 min of adjusting the inoculum turbidity to 0.5 McFarland turbidity standards, 500 µL of the suspension was taken and spread over the plate with the help of sterile glass spreader in order to get a uniform microbial growth. The inoculum was allowed to be absorbed for at least 3 min under aseptic condition discs were put on to the agar surface by applying gentle pressure with the help of sterile forcep to ensure complete contact of disc with agar. Plates were incubated at 30ºC for 18-20 h and in case of fungi 48 h required. After incubation diameter of zone of inhibition was measured.

3.8. Synergistic effect of extract

Two extracts were mixed in equal proportion and were evaluated for their synergistic potential (Williamson, 2004) by broth dilution assay. Ethanol (50 and 100 µg/mL) and methanol (50 and 100 µg/mL) extract of *S. cumini* seeds were mixed in 1:1 ratio and tested against *X. campestris.* Similarly, ethanolic extract of *P. sylvestris* (50 and 100 µg/mL) and *S. cumini* (50 and 100 µg/mL) were mixed and tested against *X. campestris.*

While against *A. tumefaciens,* ethanolic extract of *P. sylvestris* (300, 500, 600 µg/mL) and *T. indica* (MeOH) extract (300, 500, 600 µg/mL) were combined in 1:1 ratio.

3.9. Separation of crude extract by thin-layer chromatography

3.9.1. Analytical TLC

TLC is the one of the most widely used and easiest method for purifying a small number of components. Readymade plates (Merck) of 20 × 20 cm, precoated with sorbent (silica gel 60 f_{254} and non fluorescent) of 0.25 mm thickness were taken. The compound mixture was loaded at 1.5 cm from the bottom edge of the plate as a spot. Twelve spots of 2 µl were

loaded on one TLC plate. The distance between two spots and also from margins was 1.5 cm. The plates were then lowered into Merck chamber (25 cm×14 cm×25 cm) which was presaturated (minimum 2 h) with 250 ml solvent (chloroform: acetone, 90:10) to obtain a layer of 2cm from the bottom of the chamber [Harborne, 1998]. Similarly, n-butanol: water (1:1) and MeOH: conc.ammoniumhydroxide (200:3) was used for separating different components. The chamber was then covered with glass lid which was close tightly by applying grease at the edges to prevent evaporation. The solvent front was marked after 75 min of the run. Translucent spot was visible in UV lamp (Uvitec 06 12812, UK) at 365 nm. The plates were also kept in iodine chambers for development of spots but no colored spots were obtained.

3.9.2. Preparative TLC

TLC plates were also carried out on readymade preparative plates (Merck) of 20 × 20 cm, precoated with sorbent (silica gel 60 f_{254} and non fluorescent) of 0.25 mm thickness. The extract was applied as a band (5 µl was loaded). The distance of band from both margins was 1.5 cm. The plates were then lowered into Merk chamber (25 cm×14 cm×25 cm) which was presaturated (minimum 2 h) with 250 ml solvent (chloroform: acetone, 90:10) to obtain a layer of 2cm from the bottom of the chamber. Similarly, n-butanol: water (1:1) and MeOH: conc. ammoniumhydroxide (200:3) were used for separating different components. The separated compound was visible in UV lamp (Uvitec 06 12812, UK) at 254 nm and 365 nm.

3.9.3. Recovery preparative

These spots were marked and then later on scraped off and collecting in vials (Merck, 15 ml) along with silica. They were reconstituted in same solvent and centrifuged at 12,000 rpm for 15 min the supernatant was centrifuged again at the same speed and time to remove traces of silica, so as to ensure that all of the silica has been removed and that vial contain only the active isolated constituent and they were subjected to refrigeration.

3.10. Activity of isolated compounds

The antibacterial activity of this isolated active compound was further confirmed by repeating disc diffusion assay. As the isolated compounds were reconstituted in methanol, its

negative control (1%v/v) was kept and also crude extract was kept along with other discs. ZOI was measured after 18-20 h.

3.11. Experiments with host plants

Ability of ethanolic extract of *P. sylvestris* to inhibit growth of *X. campestris* on cabbage leaf was also investigated. Equal sized pieces of fresh cabbage leaf were treated with 1 % $HgCl_2$ for 60 s, followed by 60 s treatment with 95% ethyl alcohol (Satyanarayana and Vaeghese, 2007). Then wash with sterile distilled water was given to remove any traces of previously used chemicals. 20 µl of ethanolic extract of *P. sylvestris* (300 µg/mL) was evenly applied on surface of the disinfected cabbage leaf, followed by application of 20 µl of *X. campestris* suspension. Extract was replaced with DMSO to prepare negative control. Cabbage leaf inoculated with test organism (with no extract applied) served as growth control. One disinfected leaf piece (with neither extract nor organism applied on it) was also kept as control. Incubation was carried out at 30ºC for 5 days, with visual observation at regular intervals.

3.12 Effect of extracts on mycelia growth and aflatoxin production by *A. parasiticus*

This assay was carried out in 25 ml of flasks (Borosil). The system of 7 mL was taken in flask. The flasks were labeled as negative control, growth control, turbidity control and experimental (table 3.3). Assay was carried out in triplicates. Czepek dox medium was used as growth medium of *A. parasiticus* for aflatoxin production. The inoculum was prepared from 48 h old culture and the turbidity was adjusted at 530 nm (0.09-0.11 nm). The test agent as plant extract of 500 µg/mL concentration was added in flask. The flasks were incubated at 30ºc for 5 days. Shaking was done at regular interval of time.

Table 3.3: Estimation of aflatoxin

Controls	Media (µL)	Extract (µL)	DMSO (µL)	Inoculum (µL)	N. saline (µL)
Growth	5250	-	-	1750	-
Negative	5180	-	70	1750	-
Experiment	5180	70	-	1750	-
Turbidity	5180	70	-	-	1750

After 5 days incubation, the content was centrifuged at 14,000 rpm for 15 minutes. From this 4 ml of supernatant was added into 15 ml flat bottom vials (Merck). In this vials toluene: acetonitrile (9:1) solvent was mixed in 1:1 proportion [Nesheim and Stack, 2001]. This mixture was kept on shaker for overnight at room temperature. Next day upper layer was separated from the vials and readings were taken in UV spectrophotometer (ELICO SL160) at 350 nm. Aflatoxin concentration was found from given formula. For calculation the values of ε and molecular weight were different on basis of type of aflatoxin (appendix C).

$$(\mu g/mL) = A \times mw \times 1000/\varepsilon.$$

Where, A: absorbance at 350 nm

mw: molecular weight of aflatoxin

ε: Molar absorptivity

Mycelial growth of fungi was kept in hot air oven at 70ºC. Next day dry weight was calculated by this formula: (weight of flask + mycelia weight) – (empty weight of flask).

3.13. Estimation of alkaloid

Methanolic extract of *T. indica* seeds (100 µL) was taken on a clean slide and 200 (µL) of dragondroff's reagent (Merck) was added on extract. Brown precipitation indicates the presence of alkaloids in extract (Eloff, 2004).

Results and discussion

4. RESULTS AND DISCUSSION

4.1 Extraction

The extraction and reconstitution efficiencies of five seed extracts in different solvents is reported in table 4.1. Highest extraction yield was achieved in ethanolic extract of *S. cumini* seeds. Ethanolic extract of *S. cumini* seeds showed 29% of extraction yield [Kothari et al., 2011] so MAE method is better for *S. cumini* ethanolic extraction from seeds. Lowest yield was achieved in hydroalcoholic extract of *A. squamosa* seeds (table 4.1). Reconstitution efficiency is highest for methanolic extract of *T. indica* seeds and *S. cumini* seeds.

Table 4.1: Extraction efficiency and reconstitution efficiency

Seed	Solvent	Extraction efficiency (%)	Reconstitution efficiency (%)
A. squamosa	Ethanol (50 %)	6.04	73.84
	Acetone	12.11	40.97
M. zapota	Acetone	7.47	49.94
	Methanol	7.02	59.85
P. sylvestris	Ethanol (50 %)	6.65	79.51
T. indica	Methanol	**14.43**	**94.43**
S. cumini	Ethanol (50 %)	**24.33**	79.68
	Methanol	**17.34**	**93.88**

4.2 Antibacterial Assay

4.2.1. Result of Microbroth dilution assay

The percent inhibition by *A. squamosa* ethanolic and acetone seed extract against all phytopathogens is reported in table 4.2 and table 4.3 respectively. From these tables it was clearly showed that % inhibition was not concentration dependent. For example, in case of ethanolic extract at 100 µg/mL *X. campestris* showed 49.23% inhibition but at higher concentration % inhibition was not increased.

Table 4.2: Percent inhibition at various concentrations of ethanol extract of *A. squamosa* seeds

Conc. (µg/mL)	% Inhibition				
	X. campestris	***A. tumefaciens***	***P. marginalis***	***P. syringae***	***P. caratovorum***
50	NI	30.95	31.39	NI	Not done
100	49.23	Not done	32.55	NI	Not done
150	NI	Not done	26.74	7.1	Not done
200	30.70	38.09	33.72	NI	Not done
257.2	21.53	36.85	29.06	10	13.11
300	7.40	Not done	Not done	29.26	Not done
400	22.22	33.33	23	31.70	Not done
514	NI	31.19	28.31	29.26	9.40
600	NI	16	26.26	NI	Not done
771.6	NI	Not done	17.18	NI	9.60

Table 4.3: Percent inhibition at various concentrations of acetone extract of *A. squamosa* seeds

Conc. (µg/mL)	% Inhibition			
	X. campestris	***A. tumefaceins***	***P. marginalis***	***P. syringae***
50.00	15.02	30.30	15.18	NI
95.08	35.08	37.78	18.98	7.40
100.00	33.39	25.00	NI	NI
150.00	NI	34.20	8.18	5.71
190.04	24.61	16.66	13.63	NI
200.00	43.00	NI	25.90	NI
250.00	38.49	NI	3.99	NI
285.09	30.95	34.20	14.54	14.28
380.31	45.83	27.10	19.40	NI
475.40	Not done	NI	14.57	NI

The antibacterial activity as percent inhibition of acetone and methanol extracts of *M. zapota* seeds is shown in table 4.4 and 4.5. There was no significant antibacterial activity in *M. zapota* seed extracts (acetone and methanol) against phytopathogens but only methanolic extract able to inhibit *A. tumefaceins* (71.42%) at 900 µg/mL.

Table 4.4: Percent inhibition at various concentrations of acetone extract of *M. zapota* seeds

Conc. (µg/mL)	% Inhibition				
	X. campestris	***A. tumefaciens***	***P. marginalis***	***P. syringae***	***P. caratovorum***
50	14.82	34.54	9.85	3.20	Not done
100	6.86	30	12.67	16.04	Not done
200	5.86	27.27	NI	13.95	Not done
289.5	NI	23.63	NI	NI	Not done
400	NI	26.56	NI	14.33	Not done
579	NI	23.04	2.66	13.33	4.9
723	NI	22.17	Not done	27.55	Not done
868.5	Not done	28.26	Not done	22.18	Not done

Table 4.5: Percent inhibition at various concentrations of methanol extract of *M. zapota* seeds

Conc. (µg/mL)	% Inhibition				
	X. campestris	***A. tumefaciens***	***P. marginalis***	***P. syringae***	***P. caratovorum***
50	51.42	28	8.33	NI	7.69
100	22.85	32	14.28	NI	6.41
200	NI	19.85	6.66	NI	NI
300	NI	29.21	20	NI	2.56
400	37.14	44.63	7.61	NI	NI
500	35.51	70.14	17.14	NI	16.66
600	21.42	53.44	Not done	Not done	Not done
700	31.03	Not done	Not done	Not done	Not done
800	25	56.89	9.67	4.76	13.82
900	29.62	**71.42**	18.18	16.07	NI
1050	34.48	69.6	14.56	NI	26.68

Ethanolic extract of *P. sylvestris* showed antibacterial activity against *X. campestris, P. caratovorum* and *A. tumefaceins* (table 4.6). It can be seen that slight differences in concentration of the extracts can cause a significant increase or decrease in % inhibition. For example, in case of *P. sylvestris* (EtOH) seed extract (table 4.6) against *X. campestris*, 50 µg/mL increase in concentration (from 100 to150 µg/mL) caused 80% increase in % inhibition but in case *P. sylvestris* (EtOH) against *P. marginalis*, 100 µg/mL increase in concentration cause no significant increase in % inhibition (from 42.36 µg/mL to 43.75 µg/mL. In case of *A. tumefaciens* and *P. caratovorum* showed MIC at 858.1 µg/mL and 700

µg/mL respectively. There was no significant inhibition against *P. marginalis* and *P. syringae* so concentration > 922.7 µg/mL is required to achieve MIC value.

Table 4.6: Percent inhibitions at various concentrations of ethanol extract of *P. sylvestris* seeds

Conc. (µg/mL)	% Inhibition				
	X. campestris	***A. tumefaciens***	***P. marginalis***	***P. syringae***	***P. caratovorum***
50	NI	37.07	NI	NI	12.29
100	NI	40.44	NI	26.98	1.53
150	**80**	Not done	Not done	Not done	Not done
200	89.43	58.42	54.57	33.34	9.83
300	**94.23**	65.16	54.57	22.23	7.37
400	100	56.17	58.33	22.23	29.50
500	100	54.21	42.36	30.15	58.68
600	92.82	53.47	43.75	Not done	49.99
700	82.91	68.75	40.65	Not done	**91.66**
750	Not done	50	Not done	Not done	Not done
800	79.16	68.75	Not done	Not done	71.42
858.1	Not done	**83.33**	50	Not done	64.28
922.7	90.56	Not done	57.91	44.45	Not done

As evident from the table 4.7 (a) and table 4.7 (b), with an increase in concentration of the extract, there was a visible decrease in turbidity (growth of organism), which was later confirmed from OD at 655 nm. So, this was a concentration dependent response. It can be seen from the table 4.7 (a) and table 4.7 (b) the growth of *P. syringae*, *X. campestris* and *A. tumefaceins* was 100% inhibited at 400 µg/mL, 500 µg/mL and 625 µg/mL respectively, so these organisms are more sensitive to *T. indica* (MeOH) seeds extract.

Table 4.7 (a): Percent inhibition at various concentrations of methanol extract of *T. indica* seeds

P. syringae		*X. campestris*		*P. marginalis*	
Conc. (µg/mL)	**%Inhibition**	**Conc. (µg/mL)**	**%Inhibition**	**Conc. (µg/mL)**	**%Inhibition**
100	47.5	400	77.78	100	41.66
150	**53.03**	**425**	**82.35**	**200**	**50.00**
300	67.84	450	88.23	300	58.33
350	**85.35**	**500**	**100**	400	66.67
400	**100**			500	70.83
				600	**82.35**

Table 4.7 (b): Percent inhibition at various concentrations of methanol extract of *T. indica* seeds

A. tumefaceins		*P. caratovorum*	
Conc. (µg/mL)	**%Inhibition**	**Conc. (µg/mL)**	**%Inhibition**
550	NI	700	55.54
600	72.73	**750**	**81.18**
625	**100**	800	95.45

Table 4.8: Percent inhibition at various concentrations of ethanolic extract of *S. cumini* seeds

Conc. (µg/mL)	**% Inhibition**				
	X. campestris	***A. tumefaciens***	***P. marginalis***	***P. syringae***	***P. caratovorum***
100	**54.75**	**52.17**	NI	**50.49**	21.69
150	**84.39**	Not done	Not done	Not done	Not done
200	90.85	67.15	NI	77.27	29.24
300	89.28	67.05	NI	**81.34**	36.79
400	96.42	**80.79**	NI	81.68	38.67
500	89.28	87.92	NI	85.44	34.83
1000	Not done	94.44	**57.21**	**91.89**	28.04
1100	Not done	Not done	59.20	88.37	Not done
1300	Not done	Not done	70.67	100	Not done
1633.1	Not done	Not done	60.63	100	Not done

X. campestris and *P. syringae* were susceptible to methanolic extract of *S. cumini* seeds at 225 µg/mL and 525 µg/mL respectively**.** This extract inhibits 50% growth of *P. marginalis* but *A. tumefaciens* and *P. caratovorum* were not susceptible to this extract (table 4.9).

Table 4.9: Percent inhibition at various concentrations of methanol extract of *S. cumini* seeds

Conc. (µg/mL)	% Inhibition				
	X. campestris	***A.tumefaciens***	***P. marginalis***	***P. syringae***	***P. caratovorum***
50	17.5	NI	1.44	NI	NI
100	37.5	NI	8.65	29.03	NI
200	75	NI	28.84	45.16	NI
225	**92.39**	Not done	Not done	Not done	Not done
300	87.96	34.78	12.59	51.61	8.9
400	96.80	43.47	25	70.96	Not done
500	100	39.13	35.57	64.51	6.7
525	Not done	Not done	Not done	**87.05**	Not done
1000	Not done	17.64	50.47	83.78	17.80

Table 4.10: Relation between extraction efficiency and average total activity

Extract	Extraction efficiency %	Total activity (ml/g)
P. sylvestris (EtOH)	6.65	210.21
T. indica (MeOH)	14.43	289.74
S. cumini (EtOH)	24.33	946.16
S. cumini (MeOH)	17.34	550.52

These extracts were selected on the basis of their MIC values. There is a strong positive linear correlation between extraction efficiency and average total activity (fig 1, p 44). A positive linear correlation (r = 0.960) between these two quantities for the same extracts against human pathogenic bacteria was reported from our lab previously [Kothari, 2011]. Thus it is important to develop an effective method of extraction while screening crude extracts for bioactivity. Suitability of MAE for extracting antimicrobial compounds (simultaneously holding heat-denaturation of the active components at a minimum) from plant seeds has also been indicated earlier [Kothari & Seshadri, 2010; Kothari, 2011]. The hydroalcoholic extract

of *P. sylvestris* have lesser extraction efficiency but showed effective antibacterial activity against phytopathogens (table 4.6 and 4.1) and the hydroalcoholic extract of *S. cumini* have highest extraction efficiency and effective in their antibacterial activity against phytopathogens (table 4.8 and 4.1).

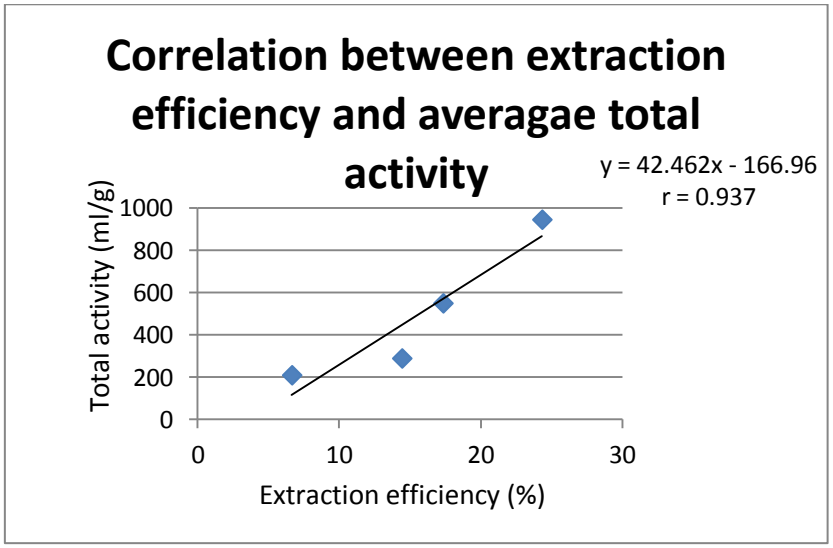


Fig 1: Correlation between extraction efficiency and average total activity

The summary of antibacterial susceptibility testing by Microbroth dilution assay is reported in table 4.10. Ethanol and acetone extract of *A. squamosa* seeds did not give significant inhibition against phytopathogens (table 4.2) but crude ethanolic extract of *A. squamosa* seeds showed antibacterial activity against *Bacillus subtilis, Pseudomonas aeruginosa* and *Salmonella typhi* [Ahmad and Sultana, 2003]. Also reported, there is poor antioxidant activity in ethanol and acetone extract of *A. squamosa* seeds [Kothari and Seshadri, 2010 (a)]. Methanolic extract *A. squamosa* seeds showed strong antifungal (*Botrytis cinerea, Blumeria graminis, Puccinia recondite*) and nematicidal (*Meloidogyne incognita* and *Bursaphelenchus xylophilus*) activity at 250 µg/mL [Dang et al., 2011].

Acetone extract of *M. zapota* seeds was not effective against strains of same genus i.e. *P. marginalis* and *P. syringae* even at concentration 868.5 µg/mL (table 4.4) but same extract was found to be effective against human pathogenic *Pseudomonas oleovorans* at 323 µg/mL and *Vibrio cholerae* at 216.5 µg/mL [Kothari and Seshadri, 2010 (b)], result in different mode of action. Methanolic extract of *M. zapota* seeds had not significant inhibition against phytopathogens except *A. tumefaciens* (table 4.5) but same extract showed

antibacterial activity against *Vibrio cholerae* and *Shigella flexneri* [Kothari and Seshadri, 2010 (b)]. So this extract is active against human pathogens.

Ethanolic extract of *P. sylvestris* was effective against three phytopathogens (table 4.6) also human pathogenic *Salmonella paratyphi A* was notable susceptible to *P. sylvestris* [EtOH] extract [Kothari, 2011].

Methanolic extract of *T. indica* (table 4.7a and 4.7b) showed effective activity against all five phytopathogens so this might be due to polar phytochemicals compounds are largely contributing to total antibacterial activity also methanolic extract of *T. indica* seeds was potent against *Salmonella paratyphi A* and *Staphylococcus epidermidis* [Kothari and Seshadri, 2010(b)]. Phytochemical tests indicated the presence of alkaloids in methanolic extract of *T. indica*. Ethanolic and acetone extract of *T. indica* showed positive results for phenols and flavones [Kothari and Seshadri, 2010 (b)]. Ara and Islam, (2009), reported the presence of alkaloids in ethanolic extract of *T. indica.*

Methanolic extract of *S. cumini* against was effective against *X. campestris* and *P. syringae* but this extract was effective against human pathogens *E. coli* and *V. cholerae* at 1100 µg/mL (Kothari et al., 2011). Methanolic extract *S. cumini* of also showed anti-inflammatory activity [Kumar et al., 2008]. *S. cumini* (EtOH) seed extract was found to be effective against three phytopathogens i.e. *A. tumefaceins, X. campestris,* and *P. syringae*. Most susceptible organism against this extract was *X. campestris.* The antibacterial activity of this extract was not significant against *P. caratovorum* (table 4.8). Ethanolic extract of *S. cumini* seeds showed effective antibacterial activity against human pathogens in range of 150-450 µg/mL [Kothari et al., 2011]. *S. cumini* leaves [Kaneria et al., 2009] and barks [Sharma et al., 2009] also showed antibacterial activity. Methanolic extract of *Rauvolfia tetraphylla* callus and *Physais minima* leaves inhibit *Xanthomonas axonopodis* and *Xanthomonas vesicatoria* at >2000 µg/mL [Shariff et al., 2006].

T. indica (MeOH) seed extract showed bactericidal activity against two phytopathogens (table 4.11) i.e. *A. tumefaciens* (fig 2, p 56) and *P. syringae* (fig 3, p 57). Methanolic extract of *T. indica* showed bactericidal against human pathogens *S. paratyphi A* and *S. epidermidis* at 242.5 and 53 µg/mL [Kothari and Seshadri, 2010(b)]. Ethanolic extract of *P. sylvestris* and *S. cumini* seed showed post extract effect against *X. campestris* i.e. growth of organism was inhibited up to 36 h. (table 4.11). The ratio of MBC/MIC less than 4 [Forlenza et al., 1981; Choia et al., 2010] and less than 2 [Brown et al., 2008] is consider for bactericidal agents.

Table 4.11: Results of broth dilution assay of various seed extracts against different organisms

Extract	Organism	IC_{50} (µg/mL)	MIC (µg/mL)	MBC (µg/mL)	MBC/MIC
S. cumini (MeOH)	*X. campestris*	150	225	-	NA
	P. syringae	300	535	-	
	A. tumefaciens	-	>1000	-	
	P. marginalis	1000	>1000	-	
	P. caratovorum	-	>1000	-	
T. indica (MeOH)	*X. campestris*	-	425	-	
	P. syringae	150	350	400	1.14
	A. tumefaceins	-	625	625	1.0
	P. marignalis	200	600	-	NA
	P. caratovorum	700	800	-	
P. sylvestris (EtOH)	*X. campestris*	-	150	300*	
	A. tumefaciens	200	850	-	
	P. caratovorum	500	700	-	
	P. syringae	>500	>922.7	-	
	P. marginalis	>600	>858.8	-	
S. cumini (EtOH)	*P. syringae*	100	400	-	
	X. campestris	100	150	150*	
	A. tumefaciens	100	400	-	
	P. marginalis	1000	>1633.1	-	
	P. caratovorum	>1000	>1633.1	-	
M. zapota (Acetone)	*P. syringae*	-	>868.5	-	
	X. campestris	-	>723	-	
	A. tumefaciens	-	>868.5	-	
	P. marginalis	-	>579	-	
	P. caratovorum	-	>579	-	
M. zapota (MeOH)	*P. syringae*	-	>1050	-	
	X. campestris	>500	>1050	-	
	A. tumefaciens	>500	>1050	-	
	P. marginalis	-	>1050	-	
	P. caratovorum	-	>1050	-	
A. squamosa (EtOH)	*P. syringae*	>400	>771.6	-	
	X. campestris	-	>771.6	-	
	A. tumefaciens	>257.2	>771.6	-	
	P. marginalis	>200	>771.6	-	
	P. caratovorum	-	>771.6	-	
A. squamosa (Acetone)	*P. syringae*	-	>475.4	-	
	X. campestris	>285.09	>475.4	-	
	A. tumefaceins	>285.09	>475.4	-	
	P. marginalis	-	>475.4	-	

- static effect was confirmed; NA- not applicaple; *post exract effect (PEE)

4.2.2. Total activity of seed extracts

Total activity is a measure of the amount of material extracted from a plant in relation to the MIC of the extract, fraction or isolated compound. It indicates the degree to which the active extracts, fractions or compounds present in 1g can be diluted and still inhibit the growth of the test organism. Total activity of different extracts was calculated as reported in table 4.12. The total activity values of the extracts are very high which shows that the extract can be diluted up to many folds and still it will retain its antibacterial potency [Eloff, 2004].

As reported in the table 4.12, highest total activity obtained in ethanolic extract *S. cumini* seed was 946.16 mL/g which shows that the extract can be diluted up to 946.16 mL and still it will retain its antibacterial potency. Same extract showed highest total activity against human pathogens [Kothari et al., 2011]. Lowest activity index obtained in case of *S. cumini* (EtOH) and *P. sylvestris* (EtOH) against *X. campestris* (table 4.12). So these extracts showed effective antimicrobial potency against *X. campestris*. Difference in activity index of methanolic and ethanolic extract of *S. cumini* may be due to difference secondary metabolites. Activity index of ethanolic and water extract of *Myxopyrum serratulum* leaves is reported by Gopalkrishnan et al., (2010) and both extracts were effective against *Streptococcus faecalis, Baccillus subtilis, Baccillus cereus, Escherichia coli, Proteas vulgaris, Pseudomonas aeruginosa*, *Klebsiella* aerogens and *Asparagillus flavans*. In case of *T. indica* (MeOH) we obtained 100% proportion index i.e., all phytopathogens selected for antibacterial assay were susceptible to this extract [Borgio et al., 2008]. Gopalkrishnan and Vadelivel, (2011) have reported proportion index of ethanolic and water extract of *Bauhinia tomentosa* (bark).

It is difficult to find out a promising source of antibacterial against gram negative organism because they contain an outer membrane of lipopolysacharides. The extracts used for experimental purpose showed activity against phytopathogenic gram-negative organisms.

Table 4.12: Results of total activity of various seed extracts against different organisms

Plant extract	Organism	Cidal/static/ PEE	Total activity (mL/g)	Average total activity (mL/g)	Activity index[1]	Proportion index (%)
T. indica (MeOH)	*A. tumefaciens*	**Cidal**	380	289.74	62.5	**100**
	P. syringae	**Cidal**	249.12		35	
	P. marginalis	Static	259.58		60	
	P. caratovorum	Static	194.68		-	
	*X. campestris**	Static	366.35		42.5	
P. sylvestris (EtOH)	*X. campestris**	PEE	466	210.21	**15**	60
	A. tumefaceins	Static	74.35		85	
	P. caratovorum	Static	90.28		-	
S. cumini (EtOH)	*P. syringae*	Static	608.25	**946.16**	40	60
	*X. campestris**	PEE	1622		**15**	
	A. tumefaceins	Static	608.25		40	
S. cumini (MeOH)	*X. campestris**	Static	770.76	550.52	22.5	40
	P. syringae	Static	330.28		53.5	

[1]Streptomycin was taken as standard antibiotic for all organisms, *ampicillin was taken, '-' resistant to antibiotic

4.2.3. Time required to kill

T. indica (MeOH) seed extract showed bactericidal activity against *A. tumefaciens* (fig 7, p 61) and *P. syringae* (fig 8, p 61) (table 4.13). Ethanolic extract of *Piper betel* leaves 25 µg/mL required more than 6 h to kill *Pseudomonas aeruginosa* (Datta et al., 2011). Also, ethanolic extract of *P. sylvestris* and *S. cumini* showed post extract effect against *X. campestris* up to 36 h of incubation. Time kill study is useful in determining tolerance to the lethal activity of antibacterial agents (Pfaller et al., 2004).

Table 4.13: Time required to kill

Plant extract	Organism	Concentration (µg/mL)	Time required to kill (h)
T. indica (MeOH)	*A. tumefaceins*	625	8
	P. syringae	400	8
P. sylvestris (EtOH)	*X. campestris*[1]	300*	-
S. cumini (EtOH)	*X. campestris*[1]	150*	-

[1]Duration of PEE was determined to be 36 h; *post extract effect

4.2.4. Results of broth dilution assay of pure plant components

From table 4.14 it showed that curcumin was able to inhibit 100 % growth of *X. campestris, P. marginalis* and *P. caratovorum. P. marginalis* was more sensitive instead of *P. syringae.*

Table 4.14: Percent inhibition at various concentrations of curcumin

Conc. (µg/mL)	% Inhibition				
	X. campestris	***P. syringae***	***P. marginalis***	***A. tumefaceins***	***P. caratovorum***
20	66.66	8.33	NI	NI	57.14
30	**80**	33.33	NI	5	28.57
40	100	8.33	77.78	10	28.57
50	**100**	NI	77.78	10	100
60	100	50	55.56	20	**100**
70	100	57.5	**77.78**	25	100
80	100	58.31	**100**	5	NI
100	100	**51.66**	100	10	100

Quercetin was able to inhibit 100% growth of all phytopathogens at different concentrations. In table 4.15 bold values indicate 100% inhibition and concentration (µg/mL). *P. syringae* and *P. marginalis* both were sensitive to quercetin at different concentration.

Table 4.15: Percent inhibition at various concentrations of quercetin

Conc. (µg/mL)	% Inhibition				
	X. campestris	***P. syringae***	***P. marginalis***	***A. tumefaceins***	***P. caratovorum***
10	71.42	NI	21.42	NI	-
20	62.36	NI	28.57	NI	NI
30	62.36	NI	28.57	NI	NI
40	**77.47**	NI	21.42	52	62.5
50	71.42	NI	14.28	52	50
60	71.42	NI	14.28	68	12.5
70	100	NI	12.66	56	100
80	57.14	30.76	36.36	48	37.5
90	100	46.15	15.38	48	-
100	**100**	63.48	**100**	**100**	**100**
125	-	62.5	-	-	-
150	-	**100**	-	100	-
200	-	-	-	100	-

-: not done

From table 4.16, in case of lycopene no significant inhibition was obtained against phytopathogens. From table 4.17, all phytopathogens were not susceptible to gallic acid except *A. tumefaciens* but MIC value was not confirmed because gallic acid was not able to dissolved at >100 µg/mL [The Merck Index, 2006]. Gallic acid did not show any activity against *Aeromonas hydrophila* [Kothari et al., 2011].

Table 4.16: Percent inhibition at various concentrations of lycopene

Conc. (µg/mL)	% Inhibition				
	X. campestris	***P. syringae***	***P. marginalis***	***A. tumefaciens***	***P. caratovorum***
5	11.76	9.19	17.27	NI	NI
10	17.64	22.90	6.1	NI	NI
20	8.8	21.83	14.54	NI	6.66
40	6.4	17.24	14.54	13.79	NI
60	NI	NI	9.61	NI	14.43

Table 4.17: Percent inhibition at various concentrations of gallic acid

Conc. (µg/mL)	% Inhibition			
	X. campestris	***A. tumefaciens***	***P. marginalis***	***P. caratovorum***
5	19.56	15.83	NI	4.95
10	NI	41.66	10.28	4.13
20	NI	60.00	1.86	9.09
30	2.17	39.16	4.67	6.61.
40	NI	58.33	4.67	6.61
50	4.34	48.33	5.60	4.95
60	6.52	57.50	NI	5.78
70	8.69	61.66	3.73	4.95
80	8.69	47.50	NI	5.78
90	4.34	70.00	NI	2.47
100	4.34	45.83	1.86	NI

-There was no significant inhibition against *P. syringae*

It was clear that curcumin had antibacterial activity against plant pathogens (fig 6, p 60). Curcumin has also been reported for its anti-protozoa [Cui et al., 2007] and antifungal activity [Martin et al., 2009]. At 30 µg/mL curcumin was able to inhibit 80% of growth (spectrophotmetrically) of *X. campestris* but 100% inhibition was obtained after spreading on nutrient agar plate in 6 h (fig 9, p 61).

MIC values of quercetin against *P. syringae* and *P. marginalis* obtained at 150 µg/mL and 100 µg/mL respectively. Akroum et al., (2009), had reported that quercetin 3-O-glycoside from ethanolic extract of *Mentha longifolia* inhibited *P. aeruginosa* at 60 µg/mL. The lowest activity index was obtained in curcumin and quercetin against *X. campestris* (table 4.18). Kothari et al., (2011), reported the presence of quercetin and gallic acid in methanolic extract of *S. cumini* seeds. This extract showed activity against *X. campestris* and *P. syringae* and quercetin also effective against all five phytopathogens. So might be quercetin was responsible for antibacterial activity in this extract.

Table 4.18: MIC and MBC of pure compounds

Pure compound	Organisms	MIC (µg/mL)	Activity index[1]	MBC (µg/mL)
Curcumin	*X. campestris**	30	3	30
	P. marginalis	70	7	static effect
	P. caratovorum	50	-	
Quercetin	*X. campestris**	40	4	
	P. marginalis	100	10	
	P. caratovorum	100	-	
	A.tumefaciens	100	10	
	P. syringae	150	15	

[1]Streptomycin was taken as standard antibiotic for all organisms; *ampicillin was taken; '-' *P. caratovorum* is resistant to antibiotics

4.2.5. Results of broth dilution assay and disc diffusion assay of antibiotics

Streptomycin was used worldwide in control of plant diseases [McManus et al., 2000]. *P. caratovorum* was resistant to Penicillin, Streptomycin, ampicillin, cefuroxine, co-trimoxazole, cefaclor, cefafroxii, and amikacin. But *P. sylvestris* (ethanol) and *T. indica* (MeOH) extract was effective against *P. caratovorum* at 700 and 800 µg/mL respectively. *P. marginalis* was resistant to ampicillin at 10 µg/mL of table 4.19(a).

Table 4.19 (a): Percent inhibition at various concentrations of antibiotics by Broth dilution assay

Antibiotics	Conc. (µg/mL)	% Inhibition			
		X. campestris	*A. tumefaceins*	*P. marginalis*	*P. syringae*
Penicillin	10	15.78	86.36	15.38	33.34
	20	10.52	90.90	15.38	40
Streptomycin	10	68.42	86.36	76.92	80
	20	68.42	86.36	76.92	80
Ampicillin	10	78.26	66.67	NI	70

- *P. caratovorum* was resistant to antibiotics

Table 4.19 (b): Disc diffusion assay of antibiotics against phytopathogens

Antibiotics	Conc. (µg/disc)	ZOI (mm)			
		X. campestris	*P. caratovorum*	*A. tumefaciens*	*P. syringae*
Norfloxacin	10	14	16	15	S
Gentamicin	10	14	11	18	S
Chloramphenicol	30	30	13	16	24
Cefuroxine	30	S	R	15	21
Ampicillin	10	15	R	22	S
Ciprofloxacin	5	S	22	13	30
Cefoperazone	75	18	14	16	15
Ceftazidine	30	16	16	7	S
Roxithromycine	30	30	7	20	S
Clarithromycin	15	19	S*	17	S
Co-trimoxazole	20	10	R	24	S
Netillin	30	15	9	16	S
Cefaclor	30	R	R	19	R
Cefotaxime	30	22	17	20	20
Cefafroxii	30	7	R	18	R
Azithromycin	15	28	7	17	19
Penicillin	10 units	14	12	11	7
Amikacin	30	11	R	18	20
Sparfloxacin	5	20	15	19	S

S- Sensitive, R- resistant, *moderately sensitive

4.3. Antifungal assay

4.3.1. Results of broth dilution assay

Antifungal activity of eight extracts against *Aspergillus parasiticus* is reported in table 4.20. *A. squamosa* ethanol and methanol seed extract was able to inhibit the growth of fungi but visibly growth was not reduced. 40 % growth was inhibited by *M. zapota* (MeOH) seed extract. No significant inhibition was obtained in case of *M. zapota* acetone, *T. indica* (MeOH), *P. sylvestris* (EtOH), *S. cumini* (MeOH) and *S. cumini* (EtOH) seed extracts. The required concentration to reach MIC values of different extracts is shown in table 4.20. Methanolic extract of plant *Grewia arborea* showed antifungal activity against *Aspergillus niger* at lower concentration [Bobbarala, 2009].

Table 4.20: Percent Inhibition at various concentrations of different extracts against *Aspergillus parasiticus*

Sr. no	Extract	Conc. (µg/mL)	% Inhibition	MIC (µg/mL)
1	*A. squamosa* (EtOH)	257.2	NI	> 514.4
		514.4	89.56*	
2	*A. squamosa* (MeOH)	300.5	NI	> 601
		601	70.37*	
3	*M. zapota* (MeOH)	1000	5.26	> 2000
		2000	40.53	
4	*M. zapota* (Acetone)	238.7	NI	> 716.1
		477.4	78.17**	
		716.1	84.44**	
5	*P. sylvestris* (EtOH)	858	NI	> 1716
		1716		
6	*T. indica* (MeOH)	1085.4		> 2170.8
		2170.8		
7	*S. cumini* (MeOH)	2769.8		> 5539.6
		5539.6		
8	*S. cumini* (EtOH)	1633.1		> 1633.1

*Visible growth was observed

** Visibly growth reduced but not completely inhibited

4.3.2. Results of broth dilution assay of pure plant components against *A. parasiticus*

Antifungal activity of pure plant components i.e. gallic acid, quercetin and lycopene showed no significant inhibition against *A. parasiticus*, except curcumin able to inhibit the growth of fungi by 53.26%. Gallic acid was able to inhibit 82.83% of growth but visible growth was observed (table 4.21). The required concentration to reach MIC values of these plant pure components is showed in table 4.21.

Table 4.21: Percent inhibition at various concentrations of pure compounds

Organism	Pure compound	Conc. (µg/mL)	% Inhibition	MIC (µg/mL)
A. parasiticus	Gallic acid	100	NI	> 200
		200	82.83*	
	Quercetin	100	65.68*	>200
		200	NI	
	Curcumin	100	46.46	>200
		200	**53.26**	
	lycopene	60	52.08*	> 60

*Visibly growth was observed

4.3.3. Results of disc diffusion assay of against fungi

The zone of clearance around the disc is indication of potency of antifungal against *A. parasiticus* such clear zone was observed in case of clotrimazole, itraconazole and ketaconazole. Also there was no significant inhibition of growth in presence of amphotericin B, flucanazole, and nystatin (table 4.22, fig 13, p 64).

Table 4.22: Disc diffusion assay of antifungal against *A. parasiticus*

Sr. no	Antifungal	Conc.(µg/mL)	ZOI (mm)
1	Amphotericin B	100 units	NI
2	Clotrimazole	10	12
3	Fluconazole	25	NI
4	Itraconazole	10	8
5	Ketaconazole	10	10
6	Nystatin	100 units	Sensitive

4.3.4. Result of effect of extracts on mycelia growth and aflatoxin production by *A. parasiticus*

In table 4.23 the bold indicates the p value <0.05. Acetone extract of *M. zapota* was able to reduce aflatoxin production and mycelia growth i.e 57.04% (fig3, p 57) and 19.11 % (fig 2, p 56) respectively. Interestingly methanolic extract of *T. indica* showed decrease in mycelia growth but increase in aflatoxin production. So, there was no correlation between mycelia growth and aflatoxin production. *M. zapota* (acetone) seed extract was inhibiting 57.11 % total aflatoxin production by *A. parasiticus*, decrease in aflatoxin production was observed under 365 nm (fig 14, p 64). Reddy et al. (2009), reported that no relation between mycelia growth and aflatoxin production by *A. flavus*. Methanolic and ethanolic extract of both *M. zapota* and *T. indica* leaves have been reported to possess antifungal activity against *A. flavus* [Satish et al., 2007]. But ethanolic extract of *P. sylvestris* showed parallel increase of aflatoxin production and mycelia growth. Al-Rahmah et al., (2011), reported that a direct correlation between fungal growth and aflatoxin production. In case of leaf extracts of *A. squamosa, Azadirachta indica, Acalypha indica* and *Allium cepa* inhibit the aflatoxin production by *A. flavus* at 100 and 300 mg/ml [Reddy et al. 2009].

Acetone extract of *A. squamosa* showed activity against both *A. flavus* and *A. parasiticus*. So this extract was might be genus specific. But in case of methanolic extract of *T. indica* showed decrease in aflatoxin production against *A. flavus* but aflatoxin production was increase in *A. parasiticus*, so this response was might be species specific (appendix E).

Table 4.23 Effect of extracts on mycelia growth and aflatoxin production by *A. parasiticus*

Plant extract	Control myceilial weight (g)	Exp. Myceilial weight (g)	% Difference	Aflatoxin B_1 in control (µg/mL)	Aflatoxin B_1 in exp. (µg/mL)	% Difference	Total Aflatoxin in control (µg/mL)	Total Aflatoxin in exp. (µg/mL)	% Difference
A. squamosa (MeOH)	0.091±0.001	0.106±0.08	+16.48	2.00±0.77	2.89±0.00	+ 44.5	8.55±3.31	12.36±0.00	+44.56
A. squamosa (EtOH)	0.094±0.004	0.094±0.004	0.00	9.39±0.11	12.47±4.06	+32.80	40.15±0.49	46.82±7.81	+16.61
S. cumini (EtOH)	0.034±0.002	0.036±0.015	+5.88	0.51±0.11	0.80±0.16	+56.86	2.95±0.64	3.41±0.72	16.78
S. cumini (MeOH)	0.094±0.000	0.080±0.000	**-14.73 ***	3.04±0.07	2.84±0.02	**-6.57***	13.44±0.25	12.14±0.10	**-9.67***
M. zapota (MeOH)	0.040±0.021	0.038±.0210	-5.0	2.61±0.60	1.46±0.02	+44.06	11.16±2.58	6.41±0.07	+42.56
M. zapota (Acetone)	0.068±0.000	0.055±0.001	**-19.11***	7.31±1.01	3.14±1.16	**-57.04***	31.29±5.01	13.42±4.94	**-57.11***
T. indica (MeOH)	0.091±0.007	0.079±0.001	**-13.18 ***	1.87±0.19	5.75±0.04	**+207.48***	7.43±1.63	24.61±0.19	**+231.22***
P. sylvestris (EtOH)	0.059±0.001	0.068±0003	+15.25	1.13±0.18	4.36±0.08	**+285.84***	4.82±0.73	18.64±0.39	**+286.72***

'-' decrease; '+' increase; * p <0.05

Fig 2: Potency of extracts on mycelia growth of *A. parasiticus*

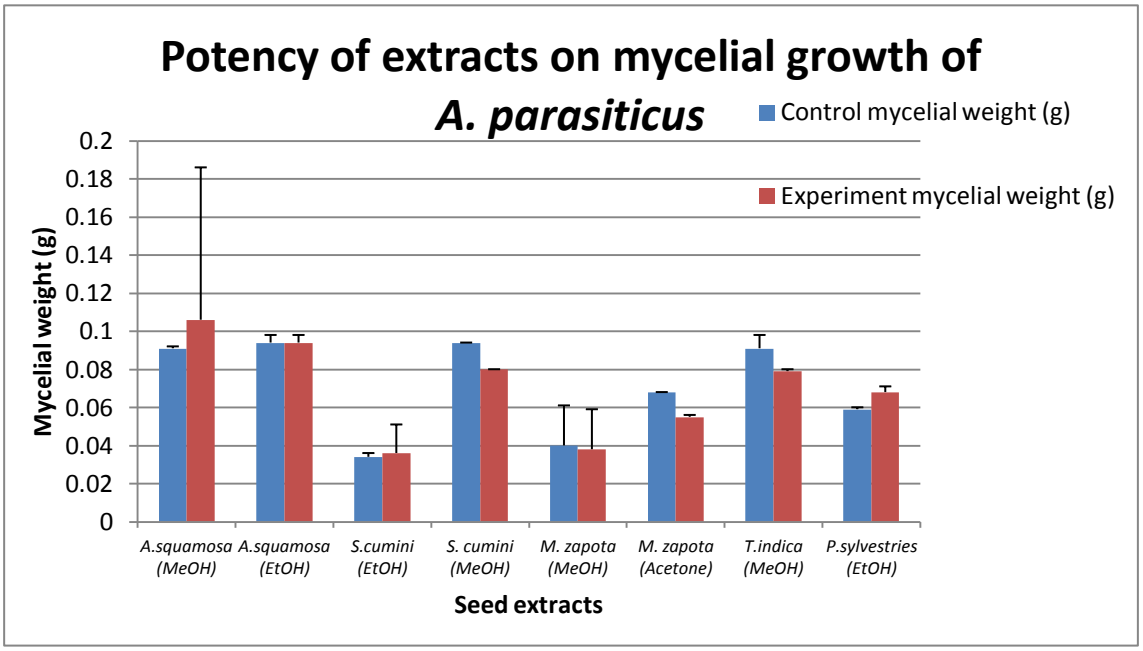
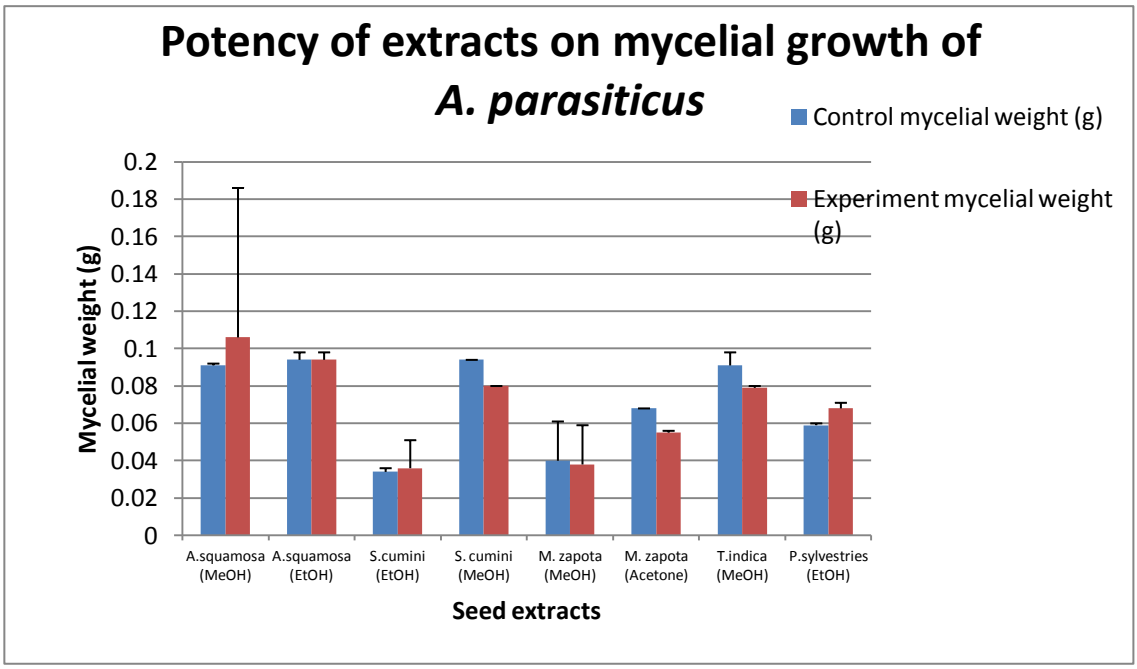


Fig 3: Potency of extracts against aflatoxin production by *A. parasiticus*



4.4 Synergistic effect of extracts

Extracts from two different solvents (MeOH and EtOH) of the same seed (*S. cumini*) when combined in 1:1 ratio did not give significant inhibition against *X. campestris*. In case of different seeds *S. cumini* and *P. sylvestris* but same solvent ethanol also did not give any significant inhibition. *T. indica* (MeOH) and *P. sylvestris* (EtOH) were not able to show effective inhibition against *A. tumefaciens* (table 4.24). Synergistic effect of plant extracts against food borne diarrheagenic bacteria has been reported by Karmegam et al., (2008).

Table 4.24: Synergistic effect of extracts

Extracts	Conc. (µg/mL)	% Inhibition	
		X. campestris	*A. tumefaceins*
S. cumini (EtOH) + *S. cumini* (MeOH)	50	3.7	Not done
	100	11.11	
S. cumini (EtOH) + *P. sylvestris* (EtOH)	50	NI	Not done
	100	NI	
P. sylvestris (EtOH) + *T. indica* (MeOH)	300	Not done	NI
	500		NI
	600		33.33

4.5. Characterization of crude extract

4.5.1. Results of thin-layer Chromatography

TLC of *T. indica* (MeOH) seed extract was done by using three different solvents system $CHCl_3$: acetone (90:10), n-butanol: water (1:1) and MeOH: conc. ammoniumhydroxide (200: 3) [Harborne, 1998]. The successful isolation of components was done by using solvent system $CHCl_3$: Acetone (90:10), R_f values of fractions obtained is reported in table 4.25 (fig.10, p 62). The solvent front was 10.5 cm. The bands of different R_f values were observed differently in visible, UV (365 nm & 254 nm) light (table 4.25). Extract was not successfully isolated by other two solvent systems.

Table 4.25: Results of separation of extracts of *T. indica* seeds by TLC ($CHCl_3$: acetone)

Extract	Fraction no.	Appearance of separated spot		R_f (cm)	hR_f
		365 nm	254 nm		
Methanol extract	1	Translucent	Not visible	0.15	15
	2	Translucent	Not visible	0.96	96
	3#	Red	brown	1.0	100

In daylight only fraction 3 of methanol extract was visible as a green colored component

4.6. Antibacterial activity of isolated fraction by disc diffusion assay

All the fractions obtained through TLC of *T. indica* (MeOH) seed extract, crude methanolic extract and methanol (negative control) were tested against all five phytopathogens bys disc diffusion method. All three fractions were capable of inhibiting growth *P. caratovorum* (table 4.26, fig 11, p 63) but there was no zone of inhibition against other phytopathogens. The R_f1, R_f2, R_f3 were labeled as fraction 1, 2, 3 respectively.

Table 4.26: Disc diffusion assay for fractions separated on TLC plate

Extract	Organism*	Fraction	ZOI (mm)	Negative control (mm)	Crude extract (mm)
T. indica (MeOH)	*P. caratovorum*	1	4	No zone of inhibition	4
		2	5		
		3	5		

*No ZOI (mm) obtained against *P. marginalis, X. campestris, A. tumefaciens, P. syringae*

4.7. Experiments with host plants

After 24 h of incubation there is no disease condition visible in any of controls and experimental plates (fig 12a, p 63). *X. campestris* is able to cause disease (black rot) on cabbage leaf (fig 12 c, p 63) in absence of extract. After five days of incubation cabbage leaf was completely spoiled due to disease caused by pathogen but cabbage leaf in presence of extract was not affected by *X. campestris* (fig 12 b, p 63). There was no disease condition develop in plate no. (ii) and (v). From plate (v), there is no counter effect of DMSO in developing disease condition (fig 12 a, p 63). Also from plate (ii) the host is not affected by environmental conditions (fig 12 a, p 63).

a) Negative control: *A. tumefaciens* b) Experiment, 625 (µg/mL)

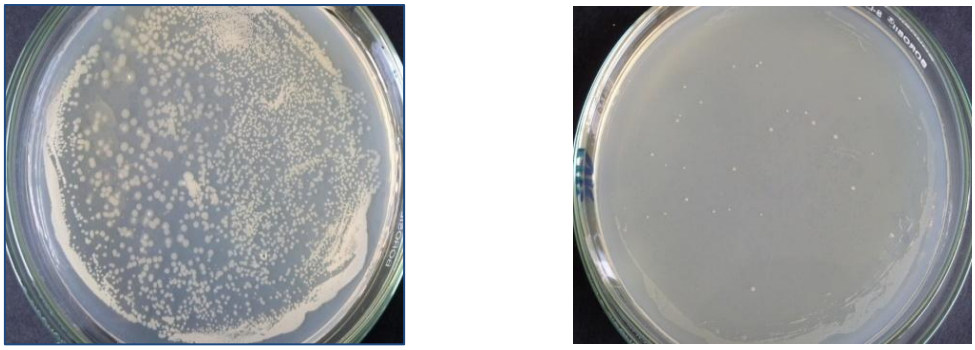


Fig 4: Cidal effect of *T. indica* (MeOH) seed extract against *A. tumefaciens*, 100 %inhibition

a) Negative control: *P. syringae* b) Experiment, 400 (µg/mL)

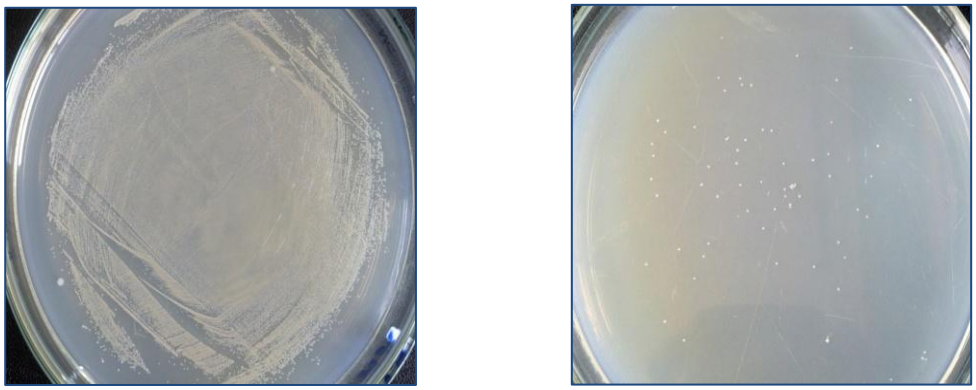


Fig 5: Cidal effect of *T. indica* (MeOH) seed extract against *P, syringe*, 100% inhibition

a) Negative control: *X. campestris* b) Experiment, 30 (µg/mL)

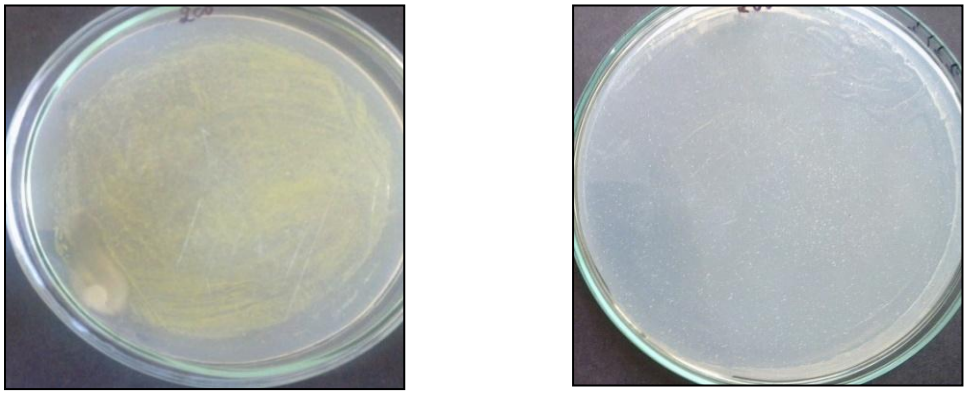


Fig 6: Cidal effect of Curcumin against *X. campestris*

a) Negative control: *A. tumefaciens* b) Experiment (8 h)

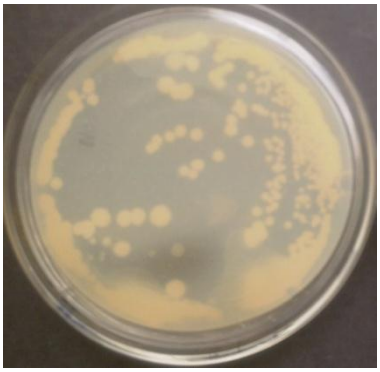
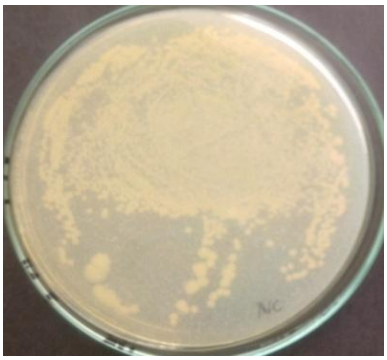


Fig 7: Time required to kill *A. tumefaciens* at concentration 625 µg/mL of *T. indica* (MeOH) extract

a) Negative control: *P. syringae* b) Experiment (8 h)



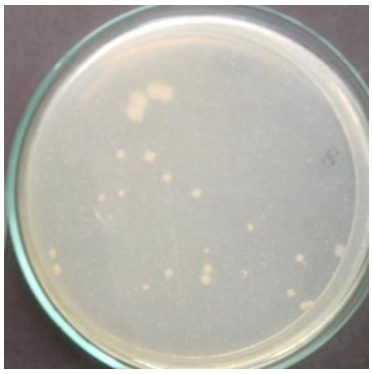
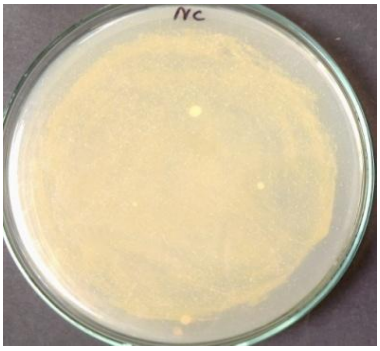


Fig 8: Time required to kill *P. syringae* at concentration 400 µg/mL of *T. indica* (MeOH) extract

a) Negative control: *X. campestris* b) Experiment (6 h)



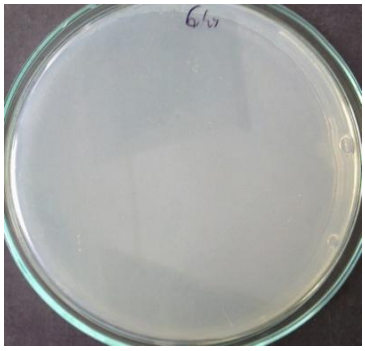


Fig 9: Time required to kill *X. campestris* at concentration 30 µg/mL of Curcumin

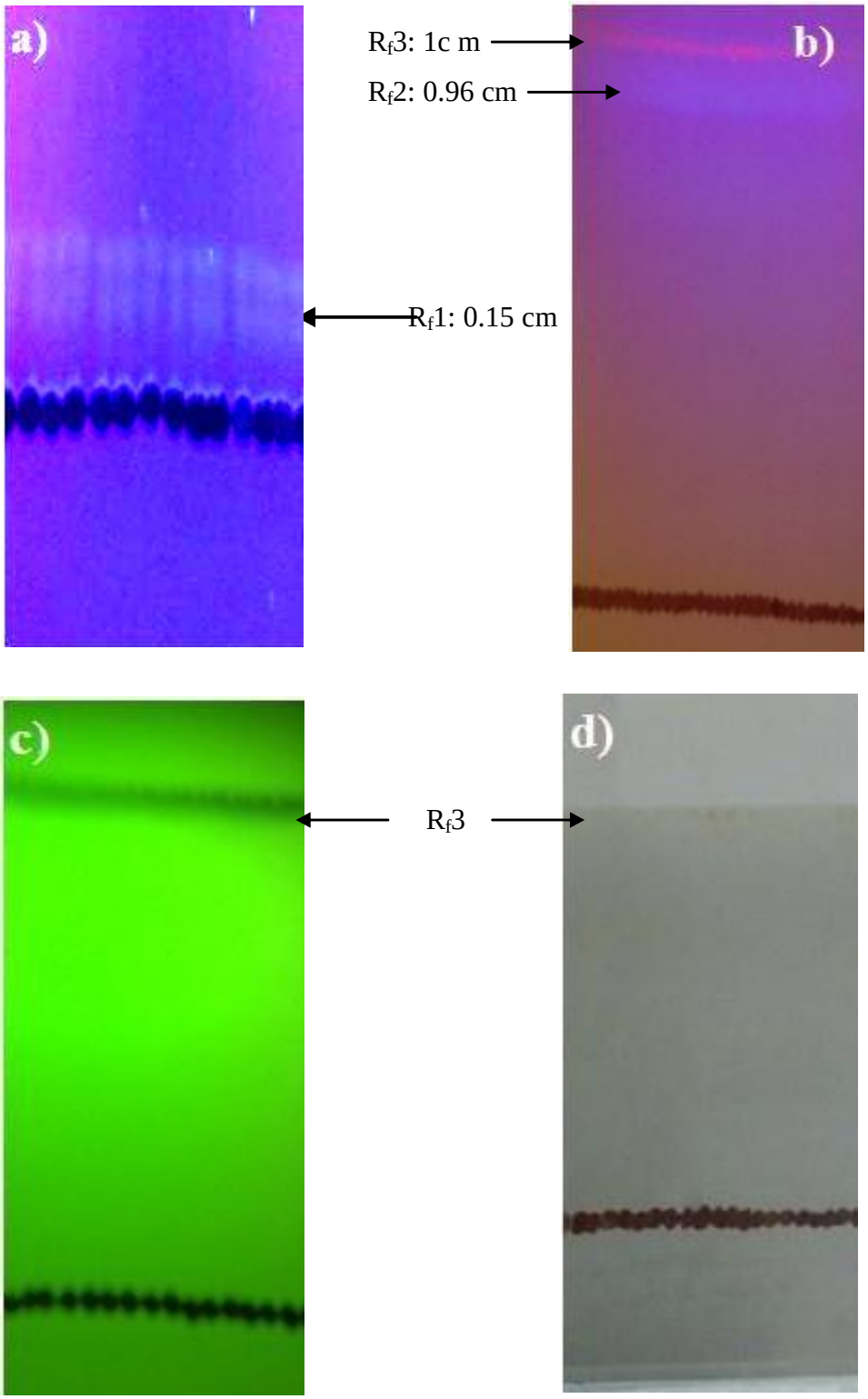


Fig 10: TLC of *T.indica* (MeOH) extract in $CHCl_3$: acetone (90:10)

a) and b) under 365 nm, c) under 265 nm d) Day light

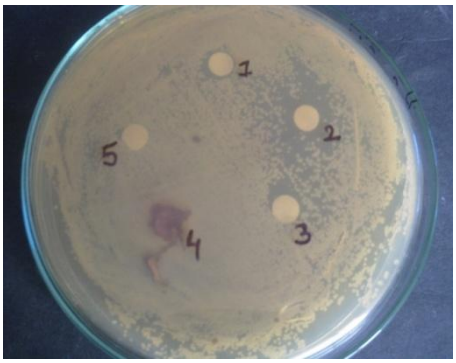


Fig 11: Disc diffusion assay of isolated fractions from TLC of *T. indica* (MeOH) extract against *P. caratovorum*

(1) R_f1, (2) R_f2, (3) R_f3, (4) crude extract, (5) Negative control (MeOH)

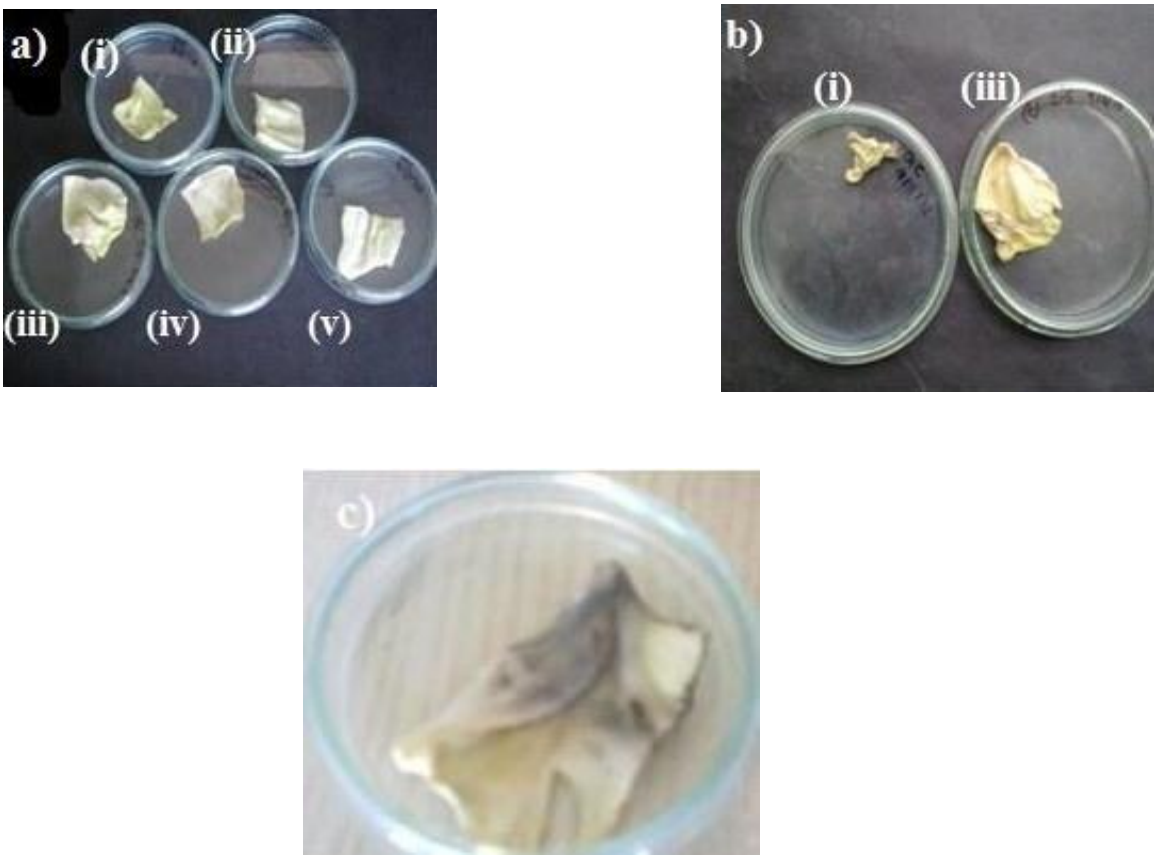


Fig 12: Study of disease caused by *X. campestris* on host cabbage (leaf)

a) After 24 h: (i) Growth control, (ii) control, (iii) and (iv) Experiment, (v) negative control

b) After 5 days: (i) Growth control, (iii) experimental (treated with extract)

c) Black rot disease of cabbage

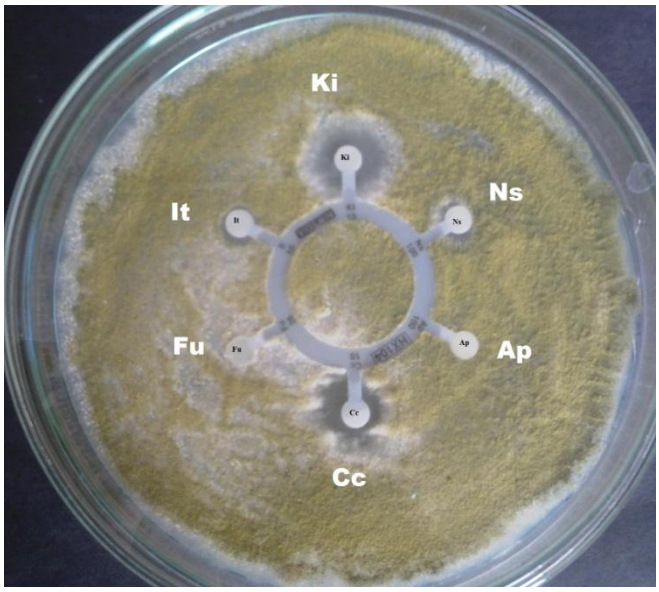


Fig 13: Antifungal testing by disc diffusion

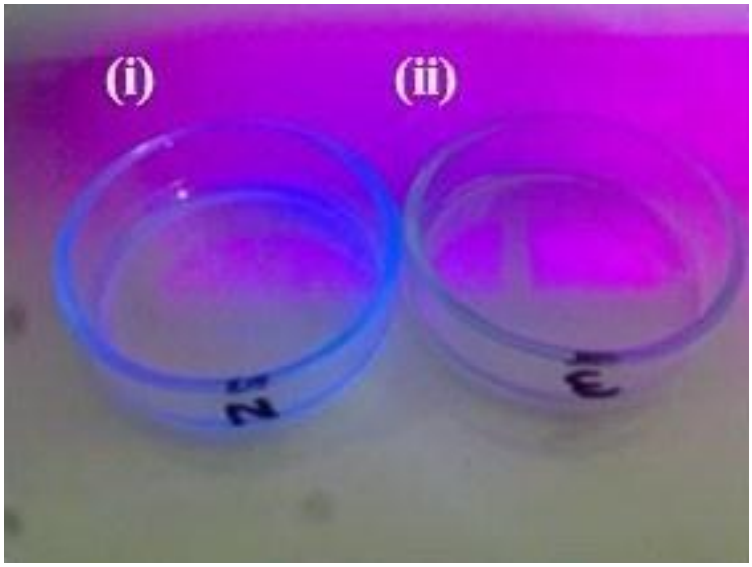


Fig 14: Decrease in aflatoxin production by *M. zapota* (acetone) seed extract at 500 µg/mL (365 nm)

i) Negative control

ii) Experiment

Conclusion

5. Conclusion

Various extracts of *T. indica, S. cumini, M. zapota, P. sylvestris* and *A. squamosa* seeds were tested for their antimicrobial activity. Out of the five seeds, *T. indica, S. cumini and P. sylvestris* showed antibacterial activity against phytopathogens. Maximum extraction efficiency (24.33%) was obtained in case of ethanolic extract of *S. cumini*. Among these seeds most potent is *T. indica*, it inhibits all five phytopathogens. *X. campestris* is the most susceptible organism as it showed susceptibility against more than one extract. Also, there was a strong positive linear correlation (r = 0.937) between extraction efficiency and total activity, indicating the importance of development of efficient extraction methods.

MIC values of ethanol extract of *S. cumini* seeds against *P. syringae, X. campestris* and *A. tumefaceins* were 400, 150, 400 µg/mL respectively. Methanolic extract of *S. cumini* was effective against *P. syringae* and *X. campestris. P. caratovorum* was resistant to few antibiotics but *P. sylvestris* (EtOH) *and T. indica* (MeOH) was effective. Methanolic extract of *T. indica* showed bactericidal activity against *P. syringae* and *A. tumefaceins* and PEE effect was found in *P. sylvestris* (EtOH) and *S. cumini* (EtOH).

M. zapota acetone extract showed decrease in aflatoxin production against *A. parasiticus* i.e 57.11%. Curcumin and quercetin showed antibacterial activity against phytopathogens but no significant inhibition was obtained with gallic acid and lycopene as the test agent.

TLC of methanol extract of *T. indica* was done in solvent system butanol: water (1:1) by which three fractions were isolated. Fractions having R_f1(0.15 cm), R_f2 (0.96 cm) and R_f3 (1.00 cm) were observed to possess antibacterial activity against *P. caratovorum*. This was determined by performing disc diffusion assay of the separated components. No synergistic activity was found between extracts.

The separated fractions of the potent extracts can be further investigated by IR, NMR and HPLC analysis to identify which secondary metabolite is responsible for antibacterial activity. Further *in vivo* studies and clinical trials can be conducted to further evaluate the potential of these extracts as an antibacterial tool.

Appendices

6. APPENDICES

Appendix A: Definitions and formula

Appendix B: Media and chemicals

Appendix C: Values of ε and mw of aflatoxins

Appendix D: McFarland Standards

Appendix E: Results of effect of plant extracts on mycelial weight and aflatoxin production by *A. flavus* and *A. parasiticus*

Appendix A: Definitions and formula

1. **Extraction efficiency:**

$$\text{Extraction efficiency}(\%) = \frac{\text{weight extracted (mg)}}{\text{weight of initial material (mg)}} \times 100$$

Weight extracted (mg) = (Weight of petriplate after evaporation of solvent)-(weight of empty plate)

2. **Reconstitution efficienc**y:

$$\text{Reconstitution efficiency}\ (\%) = \frac{\text{weight of extract reconstituted (mg)}}{\text{total weight of dried extract (mg)}} \times 100$$

Weight of extract reconstituted (mg) = (weight of petridish)-(weight of petridish after reconstitution)

3. **MI**C: MIC is defined as the lowest concentration of the agent that inhibits visible growth (Collins, Lyne and Grange, 2001).
4. **MBC:** MBC is the lowest concentration of an agent which kills a defined proportion (usually 99.9%) of the population after incubation for 72 h. The growth reappears after 40-50 h then it is said post effect of extract.
5. **Total activity:** It is a measure of the amount of material extracted from a plant in relation to the MIC of the extract, fraction or isolated compound. It indicates the degree to which the active extracts, fractions or compounds present in 1g can be diluted and still inhibit the growth of the test organism (Eloff, 2004).
6. **Synergy:** Synergy can be defined as interactions of constituents within a total extract of a single plant part, as well as between different plant extracts in a formulation (Williamson, 2004).
7. **IC50:** The concentration of the drug which inhibits 50% growth of any organism (Khan and Ather, 2006).
8. **Post antibiotic effect (PAE)**: The persistent suppression of bacterial growth after exposure to antibiotic has been defined as the post-antibiotic effect (PAE) and similarly Post Extract effect (PEE) is found.

9. **Post extract effect (PEE):** The persistent suppression of bacterial growth after exposure to plant extracts has been defined as the post-extract effect (PEE).

10. **Proportion index** (%)**:**

$$\text{Proportion index (\%)} = \frac{\text{No of positive results}}{\text{No of total test}} \times 100$$

11. **Activity index:**

$$\text{Activity index} = \frac{\text{MIC of extract}}{\text{MIC of antibiotics}}$$

12. **% Inhibition :**

$$\text{Growth (\%)} = \frac{\text{OD of the extract} - \text{OD of the absorbance control}}{\text{OD of the negative control}} \times 100$$

$$\text{Inhibition (\%)} = 100 - \text{ Growth (\%)}$$

Appendix B: Media and chemicals

1) Modified lactose minimal media (Atlas, 2010)

Per liter

Sucrose	15.0g
K_2HPo_4	5.0g
NH_4Cl	2.0g
NaCl	1.0g
$MgSo_4$	0.1g
Yeast extract	0.1g
Distilled water	1000 ml

Autoclaving was performed at 121°C, 15 psi for 15 min for sterilization purpose of media.

2) Minimal media for fungi (http://www.fgsc.net/methods/anidmed.html)

Per liter

$NaNo_3$	6.0 g
KCl	0.52 g
$MgSo_4.7\ H_2O$	0.52 g
KH_2PO_4	1.52 g

Adjust pH to 6.5 (usually requires 1 ml of 1 NaOH)

Glucose (dextrose)	10.0 g

2 ml of Hutner's trace elements

Hutner's trace elements

H_2O	100 ml
$ZnSO_4$	2.2 g
H_3BO_3	1.1 g
$MnCl_2.4H_2O$	0.5 g
$FeSO4.7H_2O$	0.5 g
$CoCl2.6H_2O$	0.16 g
$CuSO4.5H_2O$	0.16 g
$(NH4)\ MO7024.4H_2O$	0.11 g
EDTA*	5.0 g

To prepare Hutner's trace elements: Heat to boiling, cool to 60ºC, add KOH adjusting pH to 6.5-6.8. Solution goes to deep purple after standing for several days. If in titrating pH, pH exceeds 7.0 discard and start over.

*ethylene diamine tetra acetic acid (Disodium salt is easiest)

Autoclaving was performed at 121°C, 15 psi for 15 min for sterilization purpose of media.

3) Mueller-Hinton HiVeg^{TM} broth (MV391-500G)

Per liter

HiVeg infusion	2.00 g
HiVeg acid hydrolysate	17.50 g
Starch	1.50 g
pH	7.4±0.2

Autoclaving was performed at 121°C, 15 psi for 15 min for sterilization purpose of media.

Agar powder (3%) was used as solidifying agent to prepare slant and/or plate of media.

4) Nutrient HiVeg^{TM} broth (MV002-500G)

HiVeg peptone……..5.00 g
HiVeg extract……1.50 g
Yeast extract…….1.50 g
Sodium chloride……5.00 g
pH…….7.4±0.2

Commercially available nutrient broth (Nutrient HiVeg^{TM} Broth) was used. 13.0 g dry powder in 1000 ml distilled water and sterilized by autoclaving.

5) Czepek Yeast autolysate media

Yeast extract	5 g
$NaNO_3$	3 g
Sucrose solution	100 ml

$MgSO_4.7H_2O$	0.5 g
$FeSO_4.7H_2O$	0.01 g
K_2HPO_4	1 g
KCl	0.5 g

Autoclaving was performed at 121°C, 15 psi for 15 min for sterilization purpose of media.

Filter sterilization was performed for sucrose solution using membrane based disposable 0.2 µ syringe filter (Axiva Sichem Biotech).

6) RPMI-1640 (modified for autoclaving), (AT126A-5L)

7) Agar Agar type 1: HiMedia, (RM666-500G)

8) NaCl crystal pure: 500g Merck, Mumbai, (61751905001730)

9) Acetonitrile GR: Pro analysis Merck, (60000305001730)

10) Glycerol: about 87% GR Pro analysis, (10409405001730)

11) Gallic acid: SRL, (074759)

12) Quercetin LR: (3, 3', 4', 5, 7 pentahydroxy flavones) SD fine chemicals.

13) Curcumin crystals: CDH laboratory reagents

14) Lycopene: MP biomedicals, (198684)

15) L-glutamine: 200mM solution, (TCL012-20ml)

16) Hexa antimyco-01: (HX 104-2PK)

17) ICOSA Universal-1: (IC001-1PK)

Appendix C

Table 6.1 values of ε and molecular weight of aflatoxin

Mycotoxin	Molecular weight (g)	Molar absorptivity (ε)
Aflatoxin B_1	312	19,300
Aflatoxin B_2	314	21,300
Aflatoxin G_1	328	16,400
Aflatoxin G_2	330	18,300

Appendix D

McFarland Standards

McFarland turbidity standards are used to standardize the approximate number of bacteria in a liquid suspension by visually comparing the turbidity of a test suspension with the turbidity of a McFarland standard. The most commonly used standard for inoculums is 0.5 McFarland turbidity, representing approximate 1.5×10^6 bacteria/ml.

Preparation of a 0.5 McFarland standard:

Approximately 85 ml of 1% H_2SO_2 was added to a 100 ml volumetric flask. To that flask, 0.5 mL 1.175% $BaCl_2$ was added drop wise with constant swirling to the flask. The volume was made up to 100 ml with 1% H_2SO_4 optical density of the solution was set between 0.08 to 0.1 OD at 625 nm. The solution should be stored in a dark bottle at room temperature for a month.

Appendix E

Table 6.2 Results of effect of plant extracts on mycelial weight and aflatoxin production by *A. flavus* and *A. parasiticus*

Plant extract	Microorganism	Control mycelial weight (g)	Exp. Mycelial weight (g)	% Difference	Aflatoxin B_1 in control (µg/ml)	Aflatoxin B_1 in exp. (µg/ml)	% Difference	Total Aflatoxin in control (µg/ml)	Total Aflatoxin in exp. (µg/ml)
S. cumini (MeOH)	*A .parasiticus*	0.094±0	0.080±0.000	-14.73	3.04±0.07	02.84±0.02	**-6.57**	13.44±0.25	12.14±0.10
M. zapota (Aceton	*A. parasiticus*	0.068±0	0.055±0.001	**-19.11**	7.31±1.01	03.14±1.16	**-57.04**	31.29±5.01	13.42±4.94
	A. flavus	0.099±0.007	0.082±0.002	-17.17	7.11±0.870	03.50±0.02	**-50.77**	30.42±3.73	14.91±0.06
M. zapota (MeOH)	*A .flavus*	0.047±0.000	0.065±0.002	**+38.29**	0.90±0.049	01.81±0.02	**+101**	03.86±0.20	07.74±0.10
T. indica (MeOH)	*A .parasiticus*	0.091±0.007	0.079±0.001	**-13.18**	1.87±0.19	05.75±0.04	**+207.48**	07.43±1.63	24.61±0.19
	A. flavus	0.088±0.001	0.103±0.000	**-17.04**	2.60±0.077	01.93±0.17	**-25.76**	11.14±0.33	08.27±0.77
P. sylvestris (EtOH)	*A. parasiticus*	0.059±0.001	0.068±0.003	+15.25	1.13±0.18	04.36±0.08	**+285.84**	04.82±0.73	18.64±0.39
A. squamosa (MeOH)	*A. flavus*	0.103±0.000	0.106±0.009	+2.91	7.27±0.00	10.90±1.12	**+49.93**	31.09±0.00	46.60±4.79
A. squamosa (EtOH)	*A. flavus*	0.082±0.002	0.022±0.002	**-73.17**	1.87±0.084	0.00±0.00	**0**	8.01±0.38	0.00±0.00

'-' decrease; '+' increase

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