CHAPTER I

INTRODUCTION AND OBJECTIVES

1.1 Background

Plants are the major source of food, fiber, fodder, medicines and many other useful products for mankind. Different plant parts such as roots, stem, leaves, fruits, flowers/inflorescence and seeds are utilized for day-to-day requirements of the human beings. Various insects, bacteria, viruses, fungi and other pests attack the plants at the various stages of their development. It reduces their productivity and leads to a huge loss to mankind (Tapwal et al 2011).

Plant pathogens such as fungi, bacteria, nematodes and viruses cause various diseases in plants or may damages the plants (Montesinos 2003). Plants also suffer competition from weeds and are often damaged by attacks of insects. It is conservatively estimated that diseases, insects and weed together annually interfere with the production of, or destroy, half of all crops produced worldwide. Out of 36.5% average of total losses, 14% are caused by diseases, 10.2% by insects, and 12.2% by weeds. Oerke and Dehne (2004) reported that the actual losses were estimated at 26-30% for sugar beet, barley, soybean, wheat and cotton, and 35% to 40% for maize, potatoes and rice, for the periods 1996-1998. The total annual worldwide loss from plant disease is about $220 billion (2002 prices) (Agoris 2005).Plant pathogens produce an array of enzymes capable of degrading plant cell wall components (Baer and Gudmestad, 1995).

Continual and indiscriminate use of synthetic antibiotics to control bacterial disease of crop plants has caused health hazard in animals and humans due to their residual toxicity (Raghavendra et al 2006). A bioactive principle isolated from plant appears to be one of an alternative for control of plant and human pathogens developed resistant to antibiotics. Plant originated- antibacterial compounds can be one approach to plant disease management because of their eco- friendly nature (Bolkan and Renert, 1994). *Xanthomonas* pathovars known to cause disease on vegetables and plantation crops has developed resistance to ampicillin, kanamycin, Penicillin, streptomycin and copper compounds (Cooksey 1987; Verma et al 1989; Rodriguez et al 1997).

More importantly, fungi are the main pathogens that harm the plants. Fungal diseases cause a considerable loss of crop yields in agricultural industries worldwide. For example, fungi such as *Fusarium* spp., growing on plants, are able to produce mycotoxins that can seriously harm consumers (Mahlo and Eloff, 2014). To colonize plants and cause disease, pathogenic fungi use diverse strategies. Some fungi kill their hosts and feed on dead material (necrotrophs), while others colonize the living tissue (biotrophs) (Doehlemann et al 2011). Antimycotics play an important two major roles in agriculture; firstly, they are used to control fungal growth on plants and fruits. Secondly, they can be used to prevent or to ease the problem of post-harvest spoilage of plants and fruits (Hof 2001). *Alternaria solani, Alternaria zinniae, Fusarium oxysporum, Rhizoctonia solani* and *Curvularia lunata* are some of the fungal pathogens causing disease in plants. The presence and growth of this fungus in food and animal feed threatens human and animal health, respectively.

Plants have been classified as an essential source of medicinal agents for centuries and a huge number of novel drug components have been isolated from natural plant sources. Many of these plants and their extracts were used in traditional medicine. Medicinal plants play a key role in health care with 80% of plant population relying on the use of traditional medicine which is predominantly based on plants (Owolabi et al 2007). Plant derived medicines have made large contribution to human health (El-Astal et al 2005). This is due to the significant healing power of the traditional medicinal systems (Adebolu and Oladimeji, 2005). Medicinal plants are distributed worldwide but they are most abundant in tropical countries (Elvin-Lewis, 2001; Naovi et al 1991). Although there is a growing interest in the use of medicinal plants to control the plant diseases, only about 2,400 plant species among more than 250,000 higher plants have been screened for the phytoactivity (Oluwalana and Adekunle, 1998; Oluwalana et al 1999; Khafagi and Dewedar, 2000).

Botanical or bio rational pesticides are the pesticides other than conventional which are derived from plants that interfere with the lifecycle of pest. Commonly used botanicals include plant extract such as neem (*Azadirchta indica*) and garlic (*Allium sativum*), Chilli pepper (*Capsicum* spp) and essential oils such as neetle (*Utrica* spp), rue (*Ruta graveolens*, Linn), thyme

(*Thymus vulgaris*, Linn) and tea tree (*Melaleuca alternifolia*) (Gurjar et al 2012). Plants are rich in a wide variety of secondary metabolites with antimicrobial properties such as tannins, terpenoids, alkaloids and flavonoids having mode of action to kill insects and inhibit phytopathogens (Al-Momani et al 2007; Bisignano et al 2000; Bouzada et al 2009; Chakraborty and Branter 1999; Cowman 1999; Setzer et al 2000; Sohail et al 2011).

Neem has been proven to have immunomodulatory, anti-inflammatory, anti-hyperglycaemic, antiulcer, antimalarial, antifungal, antibacterial, antioxidant as well as anti-carcinogenic properties (Packia et al 2012). Garlic has been used for centuries to combat various diseases. It has been used to prevent wound infection and food spoilage (Arora and Kaur, 2007). Chili peppers are used worldwide in foods for their pungent flavor, aroma, and to prolong food spoilage. With capsaicin contents ranging from zero to millions of Scoville heat units, the different varieties offer a wide range of options for people all over the world. In addition to their use in cuisines, chili peppers have been explored for their antimicrobial and antifungal properties (Omolo et al 2014).

Cow urine contains 95% water, 2.5%urea, and the remaining 2.5% a mixture of salts, hormones, enzymes, and minerals (Bhadauria 2011). It has been considered that cow urine is very useful in agricultural operations as a biofertilizer and biopesticide as it can kill number of pesticide and herbicide resistant bacteria, viruses, fungi (Dharma et al 2005). Cow urine in combination with plant extract is used to prepare disinfectant which is biodegradable and ecofriendly with good antibacterial action (Mandavgane et al 2005).

The use of pesticides has increased many folds over the past few decades. According to an estimate, about 5.2 billion pounds of pesticides are used worldwide per year. The use of pesticides for pest mitigation has become a common practice all around the world. Their use is not only restricted to agricultural fields, but they are also employed in homes in the form of sprays, poisons and powders for control-ling cockroaches, mosquitoes, rats, fleas, ticks and other harmful bugs. Due to this reason, pesticides are frequently found in our food commodities in addition to their presence in the air (Mahmood et al 2016). The majority of farmers are unaware of the potential

toxicities of pesticides. They have no information about types of pesticides, their level of poisoning, hazards and safety measures to be taken before use of those pesticides. These compounds have long term effects on human health. Awareness should be arranged for these farmers to reduce the uses of toxic pesticides (Sharma et al 2012).

The plant based pesticides are cheap, locally available, non-toxic, and easily biodegradable. There are evidences from earlier works that several plant species possess antifungal and antibacterial properties (Manoharachary and Gourinath, 1988; Bandara et al 1989; Srivastava and Lal, 1997; Maji et al 2005; Nduagu et al 2008; Yasmin et al 2008; Harlapur et al 2007 and Akinbode and Ikotun, 2008). The presence of antifungal and antibacterial compounds in higher plants has long been recognized as an important factor in disease resistance (Mahadevan 1982). Such compounds, being biodegradable and selective in their toxicity, are considered valuable for controlling some plant diseases (Singh and Dwivedi, 1987). Natural products from plants may offer new agents for antimicrobial use. A special feature of higher plants is their capacity to produce a large number of organic chemicals of high structural diversity the so called secondary metabolites (Novi et al 1991). Natural products seem to be a viable solution to the environmental problems caused by the synthetic pesticides and many researchers are trying to identify the effective natural products to replace the synthetic pesticides (Kim et al 2005). Hence, the main objective of this work is to evaluate the effect of plant extract as natural pesticide for the control of different plant disease causing pathogens reducing the indiscriminate conventional pesticide application among farmers.

1.2 Objectives

1.2.1 General Objective

To perform anti-phytopathogenic evaluation of synergistically formulated aqueous extract and cow urine extract of selected plants

1.2.2 Specific Objectives

i. To formulate cow urine extract and aqueous extract of selected plants

ii. To evaluate antifungal and antibacterial activities of crude aqueous extract and cow urine extract against selected fungi and bacteria.

iii. To determine the minimum inhibitory concentration of crude aqueous extract and cow urine extract.

CHAPTER II

LITERATURE RIVEW

2.1 Natural antimicrobials from plants

According to Cowan (1999), antimicrobials are substances that kill or inhibit the growth of microorganisms that could be in the form of antibiotics, which are products of microorganisms or synthesized derivatives), antimicrobial peptides produced by complex organisms as well as some microbes (Jenssen et al 2006) and medicinal plants, which appear to be the focus of mainstream medicine today (Cowan 1999). In an attempt to combat the various forms of disease that have continued to plague humans from time immemorial to this day, different types of antimicrobials have been developed to fight the pathogens responsible for these diseases. Antimicrobials can exist in different forms such as antibiotics, anti-viral, anti-fungal, anti-protozoan etc. Antibiotics are used in the treatment of bacterial infections and can be obtained from either natural (chloramphenicol, streptomycin, erythromycin, tetracycline etc.) or synthetic sources (sulphonamides, quinolones and oxazolidinones). Most antibiotics exert their action either by inhibition of the bacterial cell wall or protein synthesis. Exceptions are the quinolones that inhibit DNA synthesis, and the sulphonamides that inhibit the synthesis of metabolites used for the synthesis of deoxyribonucleic acid (DNA) (Singh and Barrett 2006).

Investigation of other sources of antimicrobials, such as medicinal plants, for their antimicrobial properties is gaining ground, because of the re-occurring resistance of pathogenic microorganisms to antibiotics, as well as the side effects presented by these antibiotics. Plants produce secondary metabolites (phytochemicals), which have demonstrated their potential as antibacterial when used alone and as synergists or potentiators of other antibacterial agents. Phytochemicals frequently act through different mechanisms than conventional antibiotics and could therefore be of use in the treatment of resistant bacteria (Abreu et al 2012).

2.2 Botanical Plants

2.2.1 History of botanical plants

According to fossil records human use of plants as medicines could be dated back to the Middle Paleolithic Age, which is about 60000 years ago, (Fabricant and Farnsworth, 2001). Herbals especially medicinal herbs have constantly acted as an overall indicator of ecosystem health (Sing 2002). Medicinal plants have been transformed into one of the oldest sciences in countries such as China, Greece, Egypt and India. In ancient Persia, plants were commonly used as a drug and disinfect and aromatic agent (Hamilton 2004). In fact, the use of medicinal plants for the treatment of diseases dates back to the history of human life, that is, since human beings have sought a tool in their environment to recover from a disease, the use of plants was their only choice of treatment (Halberstain 2005). The earliest written evidences have been found on a Sumerian clay slab for the use of medicinal plants for the preparation of drugs (Qui 2007).

Commonly used plants product were oils of *Cedrus* species (cedar) and *Cupressus sempervirens* (cypress), *Glycyrrhiza glabra* (licorice), *Commiphora* species (myrrh) and *Papaver somniferum* (poppy juice) most of which are still in use today for treating ailments ranging from coughs and colds to parasitic infections and inflammation (Gurib-Fakim 2006). The plant parts includes different type of seeds, root, leaf, fruit, skin, flowers or whole plant having direct or indirect therapeutic effects and are used as medicine (Phillipson 2001). In the form of crude drugs such as tinctures, teas, poultices, powders, and other herbal formulations these medicines are being used today (Balick and Cox, 1996; Samuelsson 2004). Through oral history and information regarding medicinal plants eventually recorded in herbals, knowledge of the specific plants to be used and methods for application were passed down (Balunasa and Kinghorn, 2005).

2.2.2 General uses of botanicals.

In the maintenance of human health since ancient times, plants as a source of medicinal compounds have continued to play a dominant role. According to the World Health Organization plant extracts or their active constituents are

used as folk medicine in traditional therapies of 80% of the world's population. Over 50% of all modern clinical drugs are of natural product origin (Kirbag et al 2009).

In order to obtain the extract from the whole plant or parts of it, which could be leaves, roots, flowers or fruit most of the plants material are used fresh. Mostly the bark, roots and other parts are used as woody form. Plants such as ginger, cloves and coriander are also usually added as fresh or dried materials (Rao and Arora, 2004). For example: *Citrus limon* and *Rosmarinus officinali* essential oils possess antioxidant properties (Chang et al 2002, Noguera et al 2011). *Citrus aurantium* has immunological effects in humans. *Eucalyptus globules* oil has good antimicrobial activities (Cimanga et al 2002; Takarado et al 2004). *Thymus pannonicus* essential oil has an excellent effect against *E. coli* (Babu et al 2007). Light thyme essential oil inhibits the growth of *E. coli* in foods (Bruts 2004). *Brillantaisia lamium* extract exhibits antibacterial and antifungal effects against *Staphylococcus aureus, Enterococcus faecalis, Candida tropicalis,* and *Cryptococcus neoformans* (Tamookou et al 2011).

Many plants have been used because of their antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant (Nascimento et al 2000). Plant in the form of extracts are used to control of the phytopathogens have been obtained mainly from tree species such as eucalyptus and neem (24% of the studies with extracts) and herbaceous species like garlic, citronella, mint, rue, yarrow, ginger, basil, camphor, turmeric and ocimum (54%) (Stangarlin 2008). *Cinnamomum zeylanicum* essential oil has antibacterial and antifungal activities as well as anti-diabetic properties (Hamann 2003).

2.3Active components of plant extract

Plants have evolved secondary biochemical pathways that allow them to synthesize a raft of chemicals, often in response to specific environmental stimuli, such as herbivore-induced damage, pathogen attacks, or nutrient depravation (Raymond et al 2000; Hermsmeier et al 2001). These secondary metabolites can be unique to specific species or genera and increase their overall ability to survive and overcome local challenges by allowing them to

interact with their environment. Some of the roles of secondary metabolites are relatively straight forward; for instance, they play a host of general, protective roles (e.g. as antioxidant, free radical-scavenging, UV light-absorbing, and antiproliferative agents) and defend the plant against microorganisms such as bacteria, fungi, and viruses (Harborne 1993). The active compounds of plants are mostly secondary metabolites such as alkaloids, steroids, tannins, and phenol compounds, which are synthesized and deposited in specific parts or in all parts of the plant (Joseph and Raj, 2010).

2.3.1 Alkaloids

Alkaloids are a structurally diverse group of over 12,000 cyclic nitrogen-containing compounds that are found in over 20% of plant species (Goldman 2001). Alkaloids have been found to have antimicrobial properties with microbicide effects against *Giardia* and *Entamoeba* species as well as antidiarrheal effects, which are probably due to their effects on transit time in the small intestine (Cowan 1999).

2.3.2 Flavonoids

Flavonoids are a subclass of phenolic synthesized by plants in response to microbial infection and they have been found in vitro to be effective antimicrobial substances against a 12 wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls (Cowan 1999).

2.3.3Terpenoids

Terpenoids are condensation products of C5 isoprene units which are important constituents of essential oils (Pichersky and Gershenzon, 2002). They are active against bacteria, fungi, viruses, and protozoa. The mechanism of action of terpenes is not fully understood but is speculated to involve membrane disruption by the lipophilic compounds (Cowan 1999).

2.3.4 Tannins

Tannin is a general descriptive name for a group of polymeric phenolic substances capable of tanning leather or precipitating gelatin from solution, a property known as astringency (Haslam 1996). In every plant part: bark,

wood, leaves, fruits, and roots etc. tannins can be found (Scalbert 1991). Tannins can be toxic to filamentous fungi, yeasts, and bacteria (Scalbert 1991). The growth and protease activity of ruminal bacteria is prevented by binding of the cell wall of bacteria by tannins (Jonas et al 1994). Low tannin concentrations modify the morphology of germ tubes of *Crinipellis perniciosa* (Brownlee et al 1990).

2.4 Significance of antimicrobial susceptibility testing

Evaluation of biological activity is essential for the assessment of susceptibility of pathogens to the antimicrobial agent while screening new antimicrobials or antibiotics. To determine the resistance of certain microbial strains to different antimicrobials and to determine the efficacy of novel antimicrobials from biological extracts against different microorganisms in pharmacological research antimicrobial susceptibility testing is used (Das et al. 2010). There are number of ways such as viable counts, direct microscopic counts, turbidity measurement, bioluminescence and fluorimetry for the measurement of microbial growth and its inhibition (Grare et al 2008). The agar well diffusion method and the broth microdilution method are commonly used antimicrobial susceptibility method employed to evaluate the effect of the plant extracts or any other antimicrobial on disease-causing pathogens. Similarly, poison food technique is one of the ways for determining antifungal activity of the antimicrobials or plant extracts.

Generally antifungal activity is determined by poisoned food technique (Grover and Moore, 1962; Mishra and Tiwari, 1992; Nene and Thapliyal, 2000). Five- seven day old fungal culture is punched aseptically with a sterile cork borer of generally 6-7mm diameter. The fungal discs are then put on the gelled agar plates prepared by impregnating desired concentration of plant extract at a temperature of 45-50°C. The plates are then incubated at temperature 26 ± 1°C for fungi. Colony diameter is recorded by measuring the two opposite circumference of the colony growth. Percentage inhibition of mycelial growth is evaluated by comparing the colony diameter of poisoned plate (with plant extract) and non-poisoned plate (with distilled water) and calculated using the formula given below (Verma and Kharwar, 2008).

$$\%Mycelial\ inhibition = \frac{Mycelial\ growth\ (control) - Mycelial\ growth\ (treatment)}{Mycelial\ growth\ (control)} X100\%$$

The zones of inhibition exhibited by the plant extracts is determined by agar well diffusion method while, the broth microdilution method, which has been recommended by the Clinical and Laboratory Standards Institute (2003), is used in determining the minimum inhibitory concentration (MIC) of plant extracts. The use of microplates allows large amounts of data to be generated quickly. Bacterial growth could be assessed either visually by grading turbidity or better spectrophotometrically by measuring optical density (Grare et al 2008). Microdilution method may lacks objectivity and precision during the visual assessment of bacterial growth. The accuracy of spectrophotometric readings may be hampered by additives or antibacterial compounds that affect the spectral characteristics of growth media, the aggregation of bacteria, or bacterial pigments (Eloff 1998). Colorimetric methods using tetrazolium salts as indicators could be an alternative method, since bacteria convert them to coloured formazan derivatives that can be quantified (Grare et al 2008).

2.5 Extraction technique of plant extract

Extraction is the crucial first step in the analysis of medicinal plants because it is necessary to extract the desired chemical components from the plant materials for further separation and characterization (Sasidharan et al 2011). Among different techniques, the conventional techniques are the most commonly used for the plant extraction. In conventional extraction, the release of the desired compounds traditionally requires soaking and maceration in mild solvents (Chan et al 2012). In traditional Chinese medicinal practices, decotation in water was broadly employed and was an effective method that can be considered in cases where the presence of a chemical solvent is undesirable (Das et al 2010). Acetone, petroleum ether and hexane are some of the other solvents that can be used for plant extraction. Liquid nitrogen has also been used as a form of extraction in some research work (Karuna et al 2000). Cow urine based preparations are able to counter viral, microbial, and fungal ailments. These potions promote powerful antimicrobial, antiviral, antiallergic, and antioxidant activity. So the current research is mainly centered on the exploration of the antimicrobial powers of cow urine and also

its phytochemical properties (Shivkumar et al 2011). This increased action may be due to the hydrolytic state of cow urine and the presence of amino acids in urinary peptides, by increasing the bacterial cell surface hydrophobicity. Further increase in the antimicrobial activity of cow urine may be due to the formation of reactive compounds like formaldehyde, sulffinol, ketones, and amines during long term storage, heating, and photoactivation (Minocheherhom and Vyas, 2014). Other than conventional techniques, techniques such as lyophilization (Chen et al 2003; Grover et al 2000) and sonification (Chukwujekwu et al 2009; Yang et al 2009) are further methods that can be employed.

Non-conventional methods that can be used are the supercritical fluid extraction and microwave-assisted techniques. In research carried out by Taiwanese research teams, supercritical fluid extraction was used to investigate the antioxidant activity of the extract of lotus gem (Li et al 2009). In the investigation of antioxidant activity of the extract of lotus gem carried out by Taiwanese research team, superficial fluid extraction was used (Li et al 2009). Microwave-assisted extraction has also been used to investigate the bioactivity of tea flower polysaccharides (Wei et al 2010). The advantages presented by these two non-conventional techniques are short extraction time and solvent-free active compounds.

2.6 Medicinal plants under the study

2.6.1 *Azadirachta indica*

Neem tree belongs to the family Meliaceae which is found in abundance in tropical and semitropical regions like India, Bangladesh, Pakistan, and Nepal. It is a fast-growing tree with 20–23 m tall and trunk is straight and has a diameter around 4-5 ft. The leaves are compound, imparipinnate, with each comprising 5–15 leaflets. Its fruits are green drupes which turn golden yellow on ripening in the months of June–August. Taxonomic position of *Azadirachta indica* (neem) is classified as in **Table 2.6.1**(Girish et al 2008).

Classification of *Azadirachta indica* (Neem)

Kingdom	Plantae
Order	Rutales
Suborder	Rutinae
Family	Maliaceae
Sub-family	Melioideae
Tribe	Melieae
Genus	*Azadirachta*
Species	*indica*

Source: Interntional Research Journal of Pharmacy

2.6.1.1 Active components of Neem

Azadirachta indica (neem) shows therapeutics role in health management due to rich source of various types of ingredients. The most important active constituent is azadirachtin and the others are nimbolinin, nimbin, nimbidin, nimbidol, sodium nimbinate, gedunin, salannin, and quercetin. Leaves contain ingredients such as nimbin, nimbanene, 6-desacetylnimbinene, nimbandiol, nimbolide, ascorbic acid, n-hexacosanol and amino acid, 7-desacetyl-7-benzoylazadiradione, 7-desacetyl-7-benzoylgedunin, 17-hydroxyazadiradione, and nimbiol (Ali 1993; Kokate et al 2010; Hossain 2011). Quercetin and ß-sitosterol, polyphenolic flavonoids, were purified from neem fresh leaves and were known to have antibacterial and antifungal properties (Govindachari 1998) and seeds hold valuable constituents including gedunin and azadirachtin.

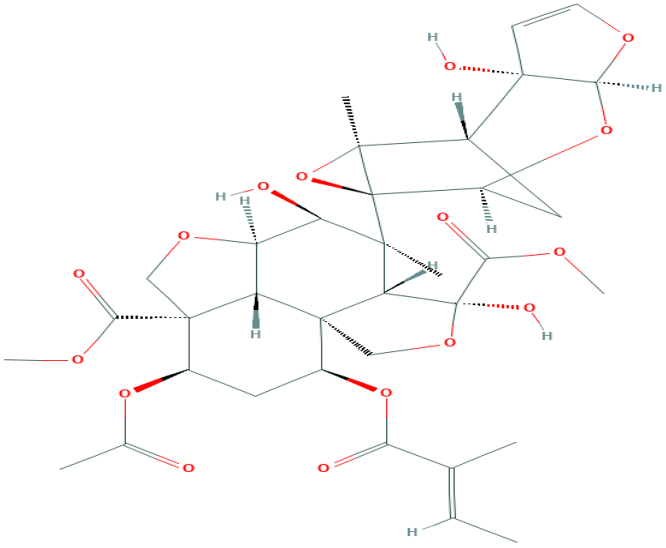


Fig 1: Azadirachtin*:* (Source: Pubchem)

2.6.1.2 General uses of Neem

Neems possess antimicrobial, anticancerous, antioxidant, properties as well as shows hepatoprotective, wound healing, and antidiabetic activity. Neem and its ingredients play role in the inhibition of growth of numerous microbes such as viruses, bacteria, and pathogenic fungi. Neem (*Azadirachta indica*) plants parts shows antimicrobial role through inhibitory effect on microbial growth/potentiality of cell wall breakdown. Azadirachtin, a complex tetranortriterpenoidlimonoid present in seeds, is the key constituent responsible for both antifeedant and toxic effects in insects (Mordue et al 2009). The aqueous extracts of neem cake have shown the antimicrobial role in the inhibition of spore germination against three sporulating fungi such as *Cocgliobolusm lunata*, *Helminthosporium pennisetti* and *Colletotrichum. Gloeosporioides* f. sp. *mangiferae* (Anjali 2013) and results of the study revealed that methanol and ethanol extract of *Azadirachta indica* showed growth inhibition against *Aspergillus flavus*, *Alternaria solani*, and *Cladosporium* (Shrivastava 2014).

2.6.2 *Allium sativum*

Allium sativum, commonly known as garlic, is a species of the onion family Alliaceae (Saravanan et al 2010). *Allium sativum* is classified as shown in **Table 2.6.2.**

Classification of *Allium sativum*

Kingdom	Plantae
Phylum	Magnoliophyta
Class	Lilopsida
Order	Liliales
Family	Liliaceae
Genus	*Allium*
Species	*sativum*

Source: Bioweb

2.6.2.1 Active Components of *Allium sativum* (Garlic)

Allin is known to be main active component of garlic (Benkeblia and Lanzotti, 2007). Allin converts into allicin when crushed which an antibiotic. Sulphur-

containing compounds such as ajoene, diallylsulfide, dithiin, S-allylcysteine, and enzymes, B vitamins, proteins, minerals, saponins, flavonoids are also present as active constituent in garlic. Furthermore, a phytoalexin (allixin) has been found (Pandya et al 2011). Phytoalexin a non-sulphur compound with a γ-pyrone skeleton structure that has antioxidant effects, antimicrobial effects, antitumor promoting effects, inhibits aflatoxin B2 DNA binding, and neurotrophic effects (Yamasaki et al 1991). The compounds such as g-glutamyl-S-allyl-L-cysteines and S-allyl-L-cysteine sulfoxides (alliin) contains non-volatile sulphur which are abundant in intact garlic. These sulfoxides are then converted into thiosulphinate (such as allicin) through enzymatic reactions (Amagase 2006). The antibacterial, antifungal, antiviral and antiprotozoal effect of garlic has been ascribed to the above mentioned constituents of the plant.

2.6.2.2 General uses of *Allium sativum* (Garlic)

Allium sativum is a natural plant being used as a food as well as medicine for centuries in all over the world. Reuter et al 1996 described garlic as a plant with various biological properties like antimicrobial, anti-cancer, antioxidant. Garlic also possesses different properties such as antiviral, antifungal, expectorant, anti-septic; anti-histamine (Hanna et al 2011). It has also been used as a treatment for cold, cough and asthma and is reported to strengthen the immune system. It has many medicinal effects such as lowering of blood cholesterol level, antiplatelet aggregation, anti-inflammatory activity and inhibition of cholesterol synthesis (Shobana 2009).

Different garlic extracts demonstrated activity against Gram negative and Gram positive bacteria including species of *Escherichia, Salmonella, Staphylococcus, Streptococcus, Klebsiella, Proteus, Bacillus, Clostridium, Helicobacter pylori* and even acid-fast bacilli (AFB) such as *Mycobacterium tuberculosis*. Allicin is thiosulfinate compound of garlic reported for its antibacterial activity. Allicin is proved to be anti-bacterial as it inhibits RNA synthesis (Hannan et al 2011).

2.6.3 *Capsicum annum* (Chili pepper)

Capsicum annum is one of the domesticated species belonging to genus *Capsicum* which is a member of the Solanaceae family (Moscon et al 2007). *Capsicum annum* is classified as shown in **Table 2.6.3.**

Classification of *C. annum* (Chili pepper)

Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Solanales
Family	Solanaceae
Genus	*Capsicum L*
Species	*annum*

Source: Research Gate

2.6.3.1 Active components of *Capsicum annum*

The pungency of chili peppers is due to the accumulation of capsaicinoids (also known as capsinoids), a group of naturally produced compounds that are unique to the *Capsicum* genus (Mortensen 2009). Capsaicin and dihydrocapsaicin are alkaloids which are responsible for 90 % of the intense organoleptic sensation of heat (Govindarajan 1987; Campos 2013). Hot taste is due to the presence of Capsaicinoids, particularly Capsaicin (N-(4-hydroxy-3-methoxy-phenyl) methyl) 8-methyl-non-6-enamide) and dihydrocapsaicin (N-(4-hydroxy-3-methoxy-phenyl) methyl) 8-methyl-nonamide) which are responsible for 80-90 % of the spiciness (Kosuge 1970). The active principles found in plants are the bioactive compound having pharmaceutical and therapeutic applications (Manjuet al 2002). These compounds are vitamins and other secondary metabolites such as phenolic compounds, terpenoid, steroids and alkaloids (Eloff 2004).

2.6.3.2 General uses of *Capsicum annum*

Capsaicin is considered to be an active principle in arthritis pain reliever and anti-inflammation (Chad et al 1999). The plant flavonoids are potentially important dietary factory in cancer as chemo-protective agents and they show anti-allergic (Kurian et al 2002), anti-inflammatory (Vatsalya et al 2012), anti-microbial, anti-mutagenicity effect, anticancer and high antioxidant activities

(Lee 2000). It is also used as traditional medicine for the treatment of ulcers, diabetes and Rheumatism (Tolan 2004). Rose et al (2010) investigated the effect of methanol and aqueous extract of *Capsicum annum* on selected bacteria (*Staphylococcus aureus, Vibrio cholera* and *Salmonella typhimurium.* The methanolic and aqueous capsicum extracts was found to be effective with the minimum inhibitory concentration of 0.20 mg/ml and 0.25mg/ml respectively. Consequently, natural antimicrobials such as chili peppers are receiving a good deal of attention for a number of microorganism-control issues (Brito et al 2009).

2.7 Phytopathogens under study

2.7.1 Plant Pathogenic Bacteria

2.7.1.1 *Xathomonas oryzea* pv *oryzea*

Xanthomonas oryzae pv. *oryzae* is bacterial plant pathogens that cause bacterial blight (BB) of rice which is one of the most important diseases of rice in most of the rice growing countries (Hopkins et al 1992). *Xanthomonas oryzea* pv *oryzea* is gram negative bacteria which appear as circular, convex, and yellow to creamy yellow colour with smooth surface on the nutrient agar medium (Patil and Devanna, 2009). The casual organism is catalase +ve, citrate +ve, starch hydrolysis +ve as well as positive to 3% KOH test and gelatin liquefaction test and negative to oxidase test and H_2S test (Abhang et al 2015). *Xanthomonas oryzae* pv *oryzea* affect the rice plant through rice seed, stem and roots that are left behind at harvest. Through natural openings (water pores and growth cracks on roots) and/or leaf and root wounds, the bacterium infiltrates the plant upon introduction to host plant. *X. oryzae* grows in the plant and infects the plants leaf veins as well as the xylem causing blockage and plant wilting. Bacteria oozes from leaf lesions and is spread by wind or rain (Ou 1985). The grain maturation and quality is affected at the maturity stage where leaves turns grayish white and die (Masao Goto 2012).

2.7.1.*2 Xanthomonas axonopodis* pv *citri*

Xanthomonas axonopodis pv *citri* is causative agent of citrus cancker of citrus. *Xanthomonas axonopodis* pv *citri* is a gram-negative, motile by means of single polar flagellum. Colonies of casual organism is creamy yellow with

copious slime. The yellow color of the colony is due the pigment xanthomonadin. Organism is catalase +ve, citrate +ve, but Kovacs' oxidase is negative or weak, nitrate reduction is negative. Various carbohydrates and organic acids are used as a sole source of carbon by *X. citri*. The organism is capable of starch hydrolysis, casein hydrolysis and gelatin hydrolysis Goto (1992). Citrus canker is common and prevalent in Asia and South America and has also been reported in Arab countries (Ibrahim and Bayaa, 1989). Cancker lesions begin as light yellow, raised, spongy eruptions on the surface of the leaves. As the lesion enlarges, the spongy eruptions begin to collapse and the brown depressions appear in their central portion, forming a crater-like appearance. Cancker lesion quickly enlarges and turns to flat lesions with water-soaked appearance with frequent rain. Cancker lesions vary in size from 5 to 10 mm depending upon the susceptibility of host plant Goto (1992).

2.7.2 Plant Pathogenic Fungi

2.7.2.1 *Fusarium oxysporum* f.sp *cubense*

Fusarium oxysporum is a pathogenic fungus common in soils around the world, cause of fusarium wilt of several agricultural and horticultural crops. The fungus is known to produce sparse to abundant aerial mycelium, and white, pink, salmon and purple pigmentation on the reverse side of the colony in culture (Gerlach and Nirenberg, 1982; Nelson et al 1983). *Fusarium oxysporum* appears to rely solely on asexual reproduction and produces three types of asexual spores: microconidia, macroconidia and chlamydospores (Kistler and Miao, 1992). Forms of *Fusarium oxysporum* are divided into forma speciales based on the specific hosts (O'Donnell et al 1998). For instance, isolates of the pathogen that attack bananas are called *F. oxysporum* f. sp. *cubense*, those attacking carnation are named *F. oxysporum* f. sp. *dianthi*, and *F. oxysporum* f. sp. *lini* and *F. oxysporum* f. sp. *lycopersici* are pathogenic to flax and tomato, respectively (Booth 1971; Armstrong and Armstrong, 1981). *Fusarium* wilt of bananas, also known as Panama disease is caused by *F. oxysporum* f. sp. *cubense.* It only infects banana and relatives but it may survive as a parasite of non-host weed species. Infection due to *F. oxysporum* f. sp. *cubense* triggers the self-defense mechanisms of the host plant causing the secretion of a gel occurs followed by formation of tylose in

the vascular vessels which blocks the flow of water to the upper part of the host plant (Stover 1972). Yellow leaf syndrome is the classic symptom of *Fusarium* wilt on banana, the oldest leaves turned to a faint off-green to pale-yellow beginning with patches or streaks at the base of the petiole, close to the midrib and hang down, forming a skirt of death leaves around the pseudostem (Stover 1962; Pérez-Vicente, 2004).

2.7.2.2 *Bipolaris oryzea*

*Bipolaris oryzae*is the causal agent of rice brown spot disease and is responsible for significant economic losses. *Bipolaris oryzae* is classified in the subdivision Deuteromycotina (imperfect fungi), class Deuteromycetes, order Moniliales, and family Dematiaceae (Aldesuquy and Baka, 1992). *Bipolaris oryzea* have black with fluffy growth or grey with fluffy growth and white spots or it may have grey with suppressed growth (Valarmathi and Ladhalakshmi, 2018). Conidia are 5-10 septate with the oldest conidium towards base. Typically conidia are slightly curved and widest at the middle. The optimum temperature for growth and conidial germination has been found to be 27-30°C and 25-30°C respectively (Ou 1985). Conidia are formed between 5-38°C, optimum being 25°C (Ou 1985; Vinay Kumari et al 1997). The pathogen attacks the crop from seedling to milky stage. The symptoms appear as minute spots on the coleoptile, leaf blade, leaf sheath and glume, being most prominent on leaf blades and glumes. On leaves, typical spots are brown in colour with grey or whitish center resembling sesame seed with typical yellow halo over the spot (Sunder et al 2005). As foliar pathogens, *B. oryzae* affects the photosynthetically active tissues of rice. It may reduce the rate of the photosynthesis in the infected leaves by affecting either the chloroplasts or chlorophyll content directly, or through the enzymes concerned with photosynthesis (El Wahsh 1997).

CHAPTER III

MATERIALS AND METHODOLOGY

3.1 Materials

A list of materials, equipment's, chemicals, reagent, antibiotic and media used for the research study are listed in Appendix A.

3.2 Methods

3.2.1 Study site

The study was carried out in central campus of Technology, Dharan, Nepal from November 2018 to April 2019.

3.2.2 Sampling sites

The plants materials were collected from the different parts of Dharan. The selection of plant material was done the basis of their uses and as described by literature and identified at Central Campus of Technology, Tribhuvan University, Dharan, Nepal. The list of medicinal plant materials with their corresponding parts used for evaluating antiphytopathogenic properties, location and date of sample collection are given in Appendix B.

3.2.3 Processing of samples

The selected plant materials were washed with water to remove soil and unwanted particles and were chopped into small pieces to reduce time for drying and to grind easily. The plant materials were kept under shade at room temperature for 2 weeks to dry and grinded with grinder to obtain fine powder of plant material**.**

3.2.4 Preparation of plant extract

Plant extract were prepared by following the method described by (Ndip et al 2007; Ganguly et al 2007; Rajpandiyan et al 2011). Sterile distilled water and cow urine were used as a solvent to prepare the crude plant extract as to mimic the traditional style and they are easily available. These plant parts were administered as either infusions or decoctions. Hundred grams of each powdered plant material were macerated in 1000ml of each solvent in

extraction pots such that the level of the solvent was above that of the plant material. The macerated mixtures were then left on the waterbath shaker for 72 hours at room temperature for continuous shaking. The mixtures were then allowed to settle for 24 hour and solvent containing water was decanted. The decanted extracts were then filtered by two fold muslin cloth followed by Whattman filter paper No.1 (pore size 11µm). After filtration solvent was evaporated in water bath at 40°C to dryness to obtain solid mass of the extract and were stored at 4°C until use.

3.2.5 Calculation of percentage yield of extract

After the complete dryness of plant material, the percentage yield of plant extract was calculated. To calculate plant extract yield, the weight of the weight of the pre-weighed beaker was subtracted from the weight of the beaker with dry extract. The crude plant extract was then transferred in a sterile bottle with the help of spatula and was labeled and stored in refrigerator for further use. The percentage yield of the plant extract was calculated as below:

$$\text{Percentage yield (\%)} = \frac{\text{Dry wt.of Extract}}{\text{Dry wt.of plant material}} \times 100$$

3.2.6 Phytochemical screening of plant extract

The crude extracts (water and cow urine) were subjected to qualitative phytochemical screening to detect the major phytochemicals present in them. The Phytochemical screening were carried out to identify the constituents (tannins, phlobatannins, saponins, alkaloids, flavonoids, terpenoids) using standard procedures as described by Sofowora (1993); Trease and Evans (1989) and Harborne (1973); Prashant et al (2011).

3.2.6.1. Test for tannins

About 2ml of the aqueous extract were stirred with 2ml of distilled water and few drops of $FeCl_3$ solution were added. The formation of a green precipitate was an indication for the presence of tannins.

3.2.6.2. Test for saponins

5 ml of aqueous extract were shaken vigorously with 5 ml of distilled water in a test tube and warmed. The formation of stable foam was taken as an indication for the presence of saponins.

3.2.6.3. Test for phlobatannins

About 2ml of aqueous extract were added to 2ml of 1% HCl and the mixture was boiled. Deposition of a red precipitate was taken as an evidence for the presence of phlobatannins.

3.2.6.4. Test for flavonoids

To 1ml of aqueous extract was added 1ml of 10% lead acetate solution. The formation of a yellow precipitate was taken as a positive test for flavonoids.

3.2.6.5. Test for terpenoids

2ml of the organic extract were dissolved in 2ml of chloroform and evaporated to dryness. 2ml of concentrated sulphuric acid will be then added and heated for about 2minutes. A greyish color will indicate the presence of terpenoids

3.2.6.6. Test for alkaloids

3ml of aqueous extract were stirred with 3ml of 1% HCl on a steam bath. Mayer's and Wagner's reagents were then added to the mixture. Turbidity of the resulting precipitate was taken as evidence for the presence of alkaloids.

3.2.6.7. Tests for carbohydrates

(a) Molisch's test: 3ml of the aqueous extract was added to 2ml of Molisch's reagent and the resulting mixture shaken properly. 2ml of concentrated H_2SO_4 was then poured carefully down the side of the test tube. A violet ring at the interphase indicates the presence of carbohydrate.

(b) To 3ml of the aqueous extract was added about 1 ml of iodine solution. A purple colouration at the interphase indicates the presence of carbohydrates.

3.2.6.8. Detection of phenols

Ferric Chloride Test: Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicated the presence of phenols.

3.2.6.9. Detection of proteins and amino acids

Xanthoproteic Test: The extracts were treated with few drops of conc. Nitric acid. Formation of yellow colour indicates the presence of proteins.

Ninhydrin Test: To the extract, 0.25% w/v ninhydrin reagent was added and boiled for few minutes. Formation of blue colour indicates the presence of amino acid.

3.2.7 Antiphytopathogenic assay

3.2.7.1 Microbial pathogens used

3.2.7.1.1 Bacterial pathogens

The anti-phytopathogenic evaluation of crude plant extract was carried out on the active culture of plant pathogenic bacteria. The plant pathogenic bacteria include *Xanthomonas oryzea* pv *oryzea* and *Xanthomonas axonopodis* pv *citri. Xanthomonas oryzea* pv *oryzea* is a causative agent of bacterial leaf blight in rice whereas *Xanthomonas axonopodis* pv *citri* is the causative agent of citrus cancker in citrus. The plant samples for the isolation of these bacteria were obtained from RARS and farmer field of Tarahara, Sunsari. *Xanthomonas oryzea* pv *oryzea* was isolated as described by (Kala et al 2015), characterized and pathogenicity test was performed as described by (Patil and Devanna 2016). Similarly, isolation of *Xanthomonas axonopodis pv citri* was isolated, characterized and pathogenicity test was performed as described by (Abhang et al 2015).

3.2.7.1.2 Plant pathogenic fungi

The anti-phytopathogenic evaluation of crude plant extract was carried out on the active culture of plant pathogenic fungi. The plant pathogenic bacteria include *Fusarium oxysporum* f.sp *cubense* and *Bipolaris oryzea*. *Fusarium oxysporum* f. sp *cunbense* is the fungal pathogens that causes Panama disease of banana, also known as Fusarium wilts of banana whereas *Bipolaris oryzea* is a fungal pathogen of rice that causes brown spot disease in rice. The plant

samples for the isolation of these fungi were obtained from RARS and farmer field of Tarahara, Sunsari. *Fusarium oxysporum* f. sp *cubense* was isolated, identified and pathogenicity test was performed as described by (Udompongsuk and Soytong, 2016). Similarly, *Bipolaris oryzea* was isolated, identified and pathogenicity test was performed as described by (Sobanbabu et al 2018).

3.2.7.2 Preparation of extract

400mg of crude extract was dissolved in 10ml DMSO to make concentration of 40000µg/ml stock solution. After making stock solution the test tubes were capped and stored in at 4°C until use**.** The combined extracts were prepared by mixing the stock solution in the ratio of 50:50.

3.2.7.3 Preparation of standard culture inoculums

A single colony of the organism on NA plate was transferred to a tube containing 5ml of NB sterilized by autoclaving. The NB broth containing culture was then incubated at 37°C for 4 hours to maintain the standard turbidity of 0.5 McFarland to obtain the desired cell density of $1.5x10^8$ (cells/ml) as recommended by WHO (1991).

The 0.5 McFarland turbidity standard was prepared by adding 0.05ml of 1.175% of barium chloride dehydrate ($BaCl_2 \bullet 2H_2O$), with 9.95ml of 1% sulphuric acid (H_2SO_4).

3.2.7.4 Antimicrobial screening via Agar Well Diffusion technique and Poison food technique

The antimicrobial activity of plant extract was evaluated against the phytopathogens by agar well diffusion method for bacteria as described by Irshad et al (2012) and Poison food technique for fungal plant pathogens as described by (Rao and Srivastava, 1994). These methods were employed to evaluate the antimicrobial properties of plant extract. For antibacterial bioassay, the Agar Well Diffusion medium was prepared by pouring molten Mueller-Hinton agar on petri dishes and allowing it to solidify. The fresh bacterial inoculum with standard turbidity i.e. 0.5 McFarland was prepared as mentioned in 3.3.7.3 and the bacterial inoculum was seeded over the MHA

plates using sterile cotton swab. The inoculated plates were left for 20 minutes at room temperature. Then with the help of cork borer no 6, wells of 6mm in diameter were made in the inoculated plates and labeled. With the help of micropipette, 100µl of the stock extract prepared for diffusion method i.e., 25mg/ml and 50mg/ml was pipette onto the holes to give a concentration of 25mg and 50mg per hole and DMSO itself was tested as a control in a separate well. The plates were then incubated at 37°C for 24 hours. After the incubation, the plates were observed for the halozone around the well and the zone of inhibition was measured and recorded.

For antifungal bioassay volume of 0.5ml of each concentration was aseptically poured into the petriplate followed by the addition of 9.5ml of melted PDA and was swirled gently to achieve thorough mixing of the contents. In the control set, no extract was used. After the solidification of the media, one inoculum disc of the test fungus was aseptically inoculated upside down at the center of the petriplate and incubated at 25 +20°C. The average diameters of the fungal colonies were measured on the 7th day of incubation and percentage of mycelial growth inhibition was calculated (Shrestha and Tiwari, 2009).

Mycelial growth inhibition (%) = $\frac{gc-gt}{gc} X\ 100$

Where,

gc = growth of mycelial colony in control set after incubation period subtracting the diameter of inoculum disc.

gt = growth of mycelial colony in treatment set after incubation period subtracting the diameter of inoculum disc.

3.2.7.5 Broth microdilution assay for minimum inhibitory concentration (MIC) of bacteria.

MIC values of the plant extracts against bacterial strains were determined based on a micro well dilution method (Swanson et al 1992). The 96-well plates were prepared by dispensing 45µL of nutrient broth and 5µL of the inoculum in each well. A 50µL of plant crude extracts initially prepared at the concentration of 40mg/ml in DMSO (2.5%) was added into the first wells.

Then, 50µL from their serial dilutions was transferred into different consecutive wells to achieve concentrations from 40000µg/ml to 0.625µg/ml. The last well containing 95µL of nutrient broth without bacterial inoculum was used as a negative control. The final volume in each well was 50µL. The microtitre plates were covered with sterile lid and incubated at 37°C for 24 hrs. The lowest concentration of the test samples, which did not show any growth of tested organism after macroscopic evaluation, was determined as MICs, which were expressed in mg/ml.

3.2.7.6 Determination of minimum inhibitory concentration (MIC) of fungus

The in-vitro fungicidal activity of plant extracts were performed according to Dellavalle et al (2011) and Espinel-Ingroff et al (2002). The Crude extracts of plants were dissolved in 2.5% DMSO solution to get the initial concentration of 40mg/ml. The growth assay was performed in microtitre wells incorporated with 45µL of PDB and 5µL of fungus inoculums. Serial dilution was performed to get concentration ranging from 40000µg/ml to 0.625µg/ml. The plates were incubated at 27°C for 48 hours. The lowest concentration of the test samples, which did not show any growth of tested organism after macroscopic evaluation, was determined as MICs, which were expressed in mg/ml.

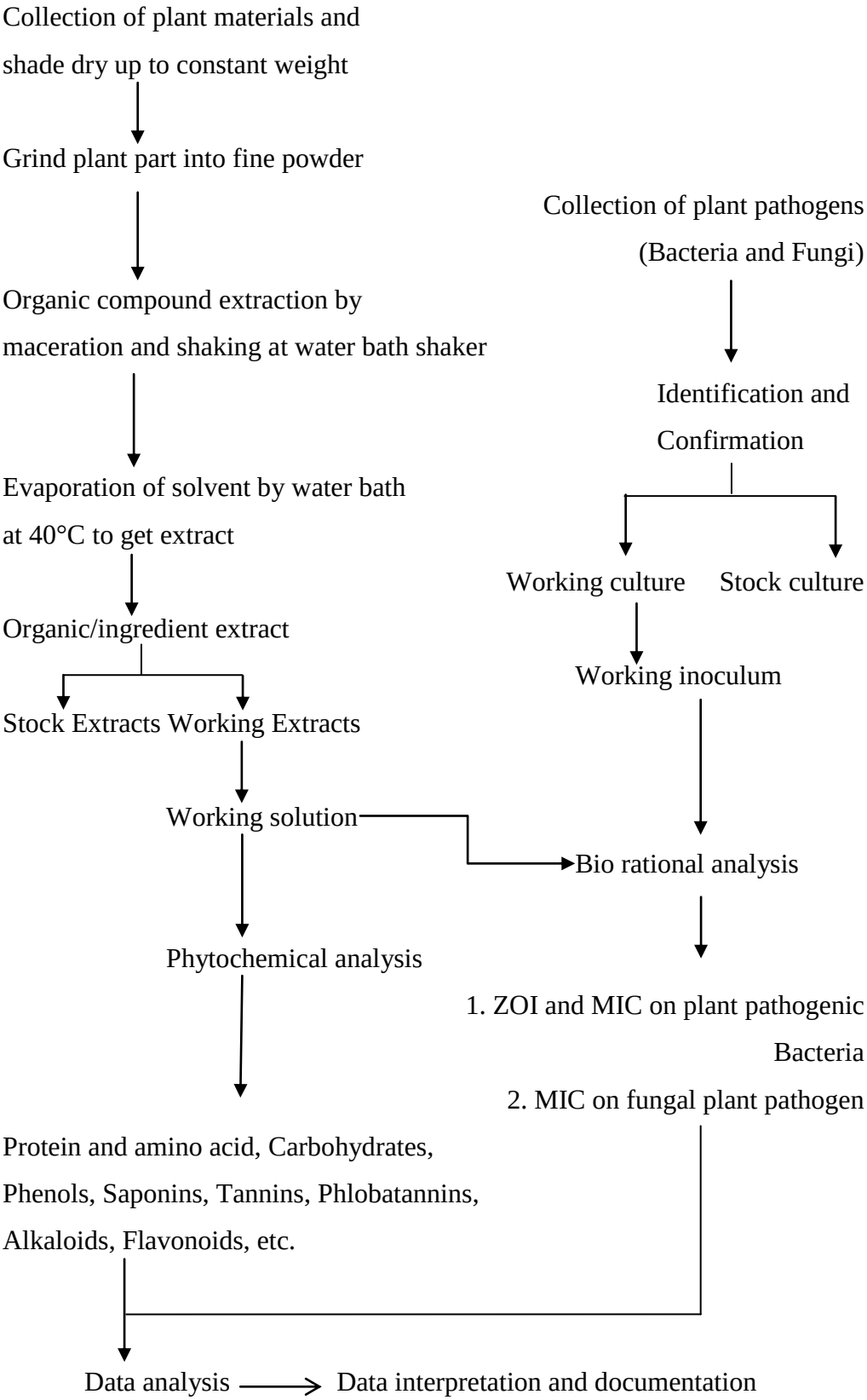
3.3 Quality control for tests

In this study, quality and accuracy of all test was maintained by following standard procedures of collection, isolation and identification. For quality control, media, antibiotics and reagents were prepared, stored and utilized as recommended by the manufacturing company. Antifungal discs were stored at refrigerator temperature.

3.4 Data analysis

The information collected from questionnaire was documented and tabulated. The data were statistically analyzed at 5% level of significance by SPSS version 16.

Flow diagram of the study



CHAPTER IV
RESULTS

The plant samples were collected, shade dried and powdered into fine particles. The fine powder (100gram) of the selected plants were dissolved in 1L of water and cow urine, kept at a water bath shaker for 78 hours at 40ºC, filtered and dried to obtain the dried extract. The extracts were further processed for phytochemical screening and antimicrobial activity on plant pathogens and results were observed as follow:

4.1 Physical characteristics of samples

The crude plant extract with two different solvent i.e. aqueous and cow urine with different plant parts showed variations with the percentage yield, texture and consistency. The consistency of most of the extract was solid with sticky and oily in touch while some plant extract were semi solid in appearance. The percentage yield of plant extract varied from 12.53% to 40.28%. *A. indica* (aqueous extract) had the lowest yield in water whereas *A. sativum* (cow urine) had the highest yield in cow urine. The characteristics and percentage yield of the obtained extracts have been listed in **Table 4.1.**

Table 4.1: Physical characteristics of plants extracts

SN	Plant	Solvent	Characteristics of extract		Dry weight (gm)	Weight of extract (gm)	% Yield
			Color	Consistency			
1.	*A. indica*	Water	Dark green	Solid	100	12.53	12.53
		Cow urine	Dark green	Solid and sticky	100	14.52	14.52
2.	*A. sativum*	Water	Yellow	Solid	100	31.55	31.55
		Cow urine	Yellow	Semi-solid	100	40.28	40.28
3.	*C. annum*	Water	Dark red	Solid	100	19.87	19.87
		Cow urine	Dark-red	Semi-solid and sticky	100	25.42	25.42

4.2 Qualitative phytochemical screening of selected plants

Preliminary qualitative phytochemical screening of the aqueous and cow urine extracts showed the presence of different kinds of phytochemicals such as tannins, saponins, alkaloids, flavonoids, terpenoids, sugars, etc. as listed in **Table 4.2**. Both the aqueous extract and cow urine extract had shown the similar result. All the plant extract had shown the presence of tannins, saponins and aminoacids whereas phlobatanins and terpenoids were not detected on any plant extracts.

Table 4.2: Phytochemical Screening of Samples

Test	*A. indica*		*A. sativum*		*C. annum*	
	Aqueous extract	Cow urine extract	Aqueous extract	Cow urine extract	Aqueous extract	Cow urine extract
Tannins	+	+	+	+	+	+
Saponins	+	+	+	+	+	+
Phlobatanins	-	-	-	-	-	-
Flavonoids	+	+	+	+	-	-
Terpenoids	-	-	-	-	-	-
Alkaloids	+	+	+	+	+	+
Carbohydrates	+	+	+	+	-	-
Proteins	-	-	-	-	-	-
Phenols	-	-	-	-	-	-
Amino Acids	+	+	+	+	+	+

4.3 Microbial sensitivity test

4.3.1 Antibiotic Sensitivity pattern of *X. oryzea* pv *oryzea* and *X. axonopodis* pv *citri*

The antibiotic susceptibility assay was examined using six different antibiotics against isolated bacteria. The standard Gentamycin revealed highest antibiotic activity with 21mm and 24mm diameter of zone of inhibition for *X. oryzea* pv *oryzea*and *X. axonopodis* pv *citri* respectively, with 10µg/disc concentration. On the other hands, standard ampicillin showed lowest activity with 7mm and 6mm diameter of zone of inhibition for *X. oryzea* pv *oryzea* and *X. axonopodis* pv *citri* respectively with disc potency of 10µg/disc which is shown in **Table 4.3.1**

Table 4.3.1: AST of *X. oryzea* pv *oryzea* and *X axonopodis* pv *citri*

Name of Antibiotics	**Disc Potency (µg/disc)**	**Diameter of zone of inhibition (mm)**		**p-value**
		X. oryzea* pv *oryzea	***X. axonopodis* pv *citri***	
Gentamycin	10	21	24	0.00
Kanamycin	30	14	18	
Chloramphenicol	30	24	22	
Ampicillin	10	7	6	
Amoxicillin	10	9	9	
Tetracycline	30	20	22	

4.3.2 Antimicrobial activities of plant extracts

4.3.2.1 Zone of Inhibition (ZOI) of plant extract (aqueous) against bacterial plant pathogens

The crude aqueous extract of *A. indica* showed the best inhibitory among the bacterial plant pathogens under the study. The inhibitory effect of aqueous extract of *A. sativum and C.annum* decreased respectively as listed below in **Table4.3.2.1.** The crude aqueous extract of *A. indica* showed the highest ZOI (14mm) against *X. axonopodis* pv *citri* and *X. oryzea* pv *oryzea* 12.3 mm ZOI with 50 mg/ml concentration. Similarly, crude aqueous extract of *A. indica* showed the highest ZOI (10mm) against *X. axonopodis* pv *citri* and *X. oryzea* pv *oryzea* and 9mm ZOI with 25 mg/ml concentration.

Table 4.3.2.1: ZOI of plant extract (aqueous) against bacterial plant pathogens

SN	Plant extract	Bacteria	Zone of Inhibition (mm)			p-value
			DMSO	25mg/ml (mm)	50mg/ml (mm)	
1.	*A. indica*	*X. oryzea* pv *oryzea*	-	9	12.3	0.014
		X. axonopodis pv *citri*	-	10	14	
2.	*A. sativum*	*X. oryzea* pv *oryzea*	-	8	11	0.05
		X. axonopodis pv *citri*	-	8.5	11.8	
3.	*C. annum*	*X. oryzea* pv *oryzea*	-	7.8	10	0.14
		X. axonopodis pv *citri*	-	8	10.6	

4.3.2.2 Zone of Inhibition (ZOI) of plant extract (cow urine) against bacterial plant pathogens

The crude cow urine extract of *A. indica* showed the best inhibitory among the bacterial plant pathogens under the study. The inhibitory effect of aqueous extract of *A. sativum and C. annum* decreased respectively as listed below in **Table 4.3.2.2.** The crude aqueous extract of *A. indica* showed the highest ZOI (14.7mm) against *X. axonopodis* pv *citri* and *X. oryzea* pv *oryzea* 14mm ZOI with 50mg/ml concentration. Similarly, crude aqueous extract of *A. indica* showed the highest ZOI (12mm) against *X. axonopodis* pv *citri* and *X.oryzea* pv *oryzea* 11mm ZOI with 25mg/ml concentration.

Table 4.3.2.2: ZOI of plant extract (cow urine) against bacterial plant pathogens

SN	Plant extract	Bacteria	Zone of Inhibition (mm)			p-value
			DMSO	25mg/ml (mm)	50mg/ml (mm)	
1.	*A. indica*	*X. oryzea* pv *oryzea*	-	11	14	0.01
		X. axonopodis pv *citri*	-	12	14.7	
2.	*A. sativum*	*X. oryzea* pv *oryzea*	-	10	12	0.01
		X axonopodis pv *citri*	-	10.4	12.6	
3.	*C. annum*	*X. oryzea* pv *oryzea*	-	9.2	12.8	0.02
		X. axonopodis pv *citri*	-	10	13	

4.3.2.3 Mycelial growth inhibition (in percentage) by the crude aqueous extract of selected plants against fungal plant pathogens

The crude aqueous extract of *A. indica* showed the best mycelia growth inhibition of the fungal plant pathogens under study. The inhibitory effect of *A. sativum* and *C. annum* decreased gradually as, listed in Table 4.3.2.3. The crude aqueous extract of *A. indica* had showed the greatest mycelial growth inhibition of 78% for *F. oxysporum* f. sp. *cubense* and 42% for *B. oryzea* at 50 mg/ml concentration. Similarly, the crude aqueous extract of A. indica had showed the greatest mycelial growth inbition of 42% for *F. oxysporum* f.sp. *cubense* and 35% for *B. oryzea* at 25 mg/ml concentration.

SN	Plant extract	Fungi	Mycelial growth inhibition (%)			p-value
			DMSO	25mg/ml (mm)	50mg/ml (mm)	
1.	*A. indica*	*F.oxysporum* f. sp. cubense	-	42	78	0.05
		B. oryzea	-	35	72	
2.	*A. sativum*	*F. oxysporum* f. sp. cubense	-	36	68	
		B. oryzea	-	30	64	
3.	*C. annum*	*F.oxysporum* f. sp. cubense	-	30	58	
		B. oryzea	-	25	52	

4.3.2.4 Mycelial growth inhibition (in percentage) by the crude cow urine extract of selected plants against fungal plant pathogens

The crude cow urine extract of *A. indica* showed the best mycelia growth inhibition of the fungal plant pathogens under study. The inhibitory effect of *A. sativum* and *C. annum* decreased gradually as, listed in **Table 4.3.2.4**. The crude cow urine extract of *A. indica* had showed the greatest mycelial growth inbition of 92% for *F. oxysporum* f. sp. *cubense* and 86% for *B. oryzea* at 50mg/ml concentration. Similarly, the crude cow urine extract of *A. indica* had showed the greatest mycelial growth inhibition of 56% for *F. oxysporum* f. sp. *cubense* and 50% for *B. oryzea* at 25mg/ml concentration.

SN	Plant extract	Fungi	Mycelial growth inhibition (%)			p-value
			DMSO	25mg/ml (mm)	50mg/ml (mm)	
1.	*A. indica*	*F.oxysporum* f.sp. cubense	-	56	92	0.039
		B. oryzea	-	50	86	
2.	*A. sativum*	*F. oxysporum* f.sp. cubense	-	42	75	
		B. oryzea	-	35	62	
3.	*C. annum*	*F.oxysporum* f.sp. cubense	-	35	65	
		B. oryzea	-	40	60	

4.3.2.5 Minimum Inhibitory concentration of crude aqueous plant extract against *X. oryzea* pv *oryzea* and *X. axonopodis* pv *citri*

The aqueous extract of *A. indica* and in combination with *A. sativum* showed the best inhibitory effect against *X. axonopodis* pv *citri* with lowest MIC 1250 µg/ml and 625 µg/ml respectively. Similarly, combined extract of *A. indica* and *C. annum* showed the best inhibitory effect against *X. oryzea* pv *oryzea* with lowest MIC of 1250 µg/ml and the plant extract of *A.indica*, *A. indica+ A. sativum*, *A. sativum + C. annum* showed the similar MIC of 5000 µg/ml against *X. oryzea* pv *oryzea* which is shown in **Table 4.3.2.5**.

Table 4.3.2.5: **MIC of crude aqueous plant extract against *X. oryzea* pv *oryzea* and *X. axonopodis* pv *citri***

SN	Plant extract(aqueous)	MIC (µg/ml)		p-value
		X. oryzea* pv *oryzea	***X. axonopodis* pv *citri***	
1	*A. indica*	5000	1250	0.00
2	*A. sativum*	10000	2500	
3	*C. annum*	20000	5000	
4	*A. indica + A. sativum*	5000	625	
5	*A. sativum + C. annum*	5000	2500	
6	*C. annum + A. indica*	1250	2500	

4.3.2.6 Minimum Inhibitory Concentration (MIC) of crude cow urine plant extract against *X. oryzea* pv *oryzea* and *X. axonopodis* pv *citri*

The cow urine extract of *A. indica* + *A. sativum* showed the best inhibitory effect against *X. axonopodis* pv *citri* with lowest MIC 312.5µg/ml. The inhibitory effect of Similarly, combined extract of *A. indica* + *A. sativum* showed the best inhibitory effect against *X. oryzea* pv *oryzea* with lowest MIC of 1250 µg/ml which is shown in **Table 4.3.2.6**.

Table 4.3.2.6: **MIC of crude cow urine plant extract against *X. oryzea* pv *oryzea* and *X. axonopodis* pv *citri***

S.No.	Plant extract (Cow urine)	MIC (µg/ml)		p-value
		X. oryzea* pv *oryzea	***X. axonopodis* pv *citri***	
1	*A. indica*	2500	625	0.04
2	*A. sativum*	5000	2500	
3	*C. annum*	10000	1250	
4	*A. indica* + *A. sativum*	1250	312.5	
5	*A. sativum* + *C. annum*	5000	1250	
6	*C. annum* + *A. indica*	5000	625	

4.3.2.7 Minimum inhibitory concentration (MIC) of crude aqueous plant extract against plant pathogenic fungi *Fusarium orysporum* f. sp *cubense* and *Bipolaris oryzea*

The combined aqueous extract of *A. indica + A. sativum* showed the best inhibitory effect against *B. orzea* with lowest MIC of 2500µg/ml. The inhibitory effect of *A. indica*, *A. indica+ A. sativum, C. annum + A. indica* shows the best inhibitory effect with similar MIC 1250 µg/ml as shown in **Table4.3.2.7**.

Table 4.3.2.7: MIC of crude aqueous plant extract against plant pathogenic fungi *Fusarium orysporum* f.sp *cubense* and *Bipolaris oryzea*

SN	Plant extract (aqueous)	MIC (µg/ml)		p-value
		F. oxysporum f.sp *cubense*	*B. oryzea*	
1	*A. indica*	1250	5000	
2	*A. sativum*	5000	10000	
3	*C. annum*	5000	20000	0.127
4	*A. indica + A. sativum*	1250	2500	
5	*A. sativum + C. annum*	5000	10000	
6	*C. annum + A. indica*	1250	5000	

4.3.2.8 Minimum inhibitory concentration (MIC) of crude cow urine plant extract against plant pathogenic fungi *Fusarium orysporum* f.sp *cubense* and *Bipolaris oryzea*

The cow urine extract of *A. indica*, *A. indica+ A. sativum*, *C. annum + A. indica* showed the best inhibitory effect against with similar MIC of 2500 µg/ml. Similarly, *A. indica+ A. sativu* and *C. annum + A. indica* showed best inhibitory effect against *F. orysporum* f.sp *cubense* with lowest MIC value of 312.5µg/ml, which is shown in **Table 4.3.2.8**.

Table 4.3.2.8: MIC of crude cow urine plant extract against plant pathogenic fungi *Fusarium orysporum* f.sp *cubense* and *Bipolaris oryzea*

S.N.	Plant extract (cow urine)	MIC (µg/ml)		p-value
		F. oxysporum f.sp *cubense*	*B. oryzea*	
1	*A. indica*	625	2500	0.047
2	*A. sativum*	2500	5000	
3	*C. annum*	5000	10000	
4	*A. indica + A. sativum*	312.5	2500	
5	*A. sativum + C. annum*	1250	5000	
6	*C. annum + A. indica*	312.5	2500	

CHAPTER V

DISCUSSIONS

Plants have been classified as an essential source of medicinal agents for centuries and a huge number of novel drug components have been isolated from natural plant sources. Many of these plants and their extracts were used in traditional medicine. Several species of plants has been proven to have antifungal, antibacterial, antioxidant as well as anti-carcinogenic properties (Packia et al 2012). Due to the indiscriminative use of chemical pesticide for the control of plant disease their increase the risk for human health as well as plant health too. Therefore, the researchers are giving more importance for screening the different properties of plants. This research work also attempts in the study of some botanicals for their antimicrobial and antifungal properties. For this work, three botanicals were selected and their phytochemicals have been evaluated that can help to justify the antimicrobial properties of selected plants. The plants were collected, washed and dried on a shade to preserve the bioactive components for extraction.

In this work, different parts of plants were selected on the basis of reported use for its antimicrobial properties. *A. indica, A. sativum* and *C. annum* have been used by local farmers as they are easily available and posse's antimicrobial properties for the control of disease causing plant pathogens. Water and cow urine had been used as a solvent to obtain the plant extract as they are easily available and cheap for the farmers or local people to use. Water is a universal solvent and dissolves non polar components and has low toxicity. Similarly, cow urine contain contains 95% water, 2.5% urea, and the remaining 2.5% a mixture of salts, hormones, enzymes, and minerals (Bhadauria 2011) and cow urine based preparations are able to counter viral, microbial, and fungal ailments. Cow urine based preparations are biodegradable and ecofriendly too. Maceration and infusion method had been employed as it helps to dissolve the soluble particles of plants. The variations in extraction method usually depends upon the length of the extraction period, solvent used , $_{P}{}^{H}$ of the solvent, temperature, particle size of the plant tissue and the solvent to sample ratio (Tiwari et al 2011).

There was a difference in the percentage yield of the solvent extracts from different plant samples. The differences ranges from 12.53% to 31.55% with water extract whereas 14.52% to 40.28% with cow urine extract. The differences in yield might be due different type and part plant materials, particle size of the plant sample, maturity of plant during sampling and extent of dryness etc.

In our study the phytochemical screening of aqueous neem extract had shown the presence of phytochemicals such as saponins, alkaloid, flavonoids, and tannins. Similar result was observed by Ramadas and Subramanan (2018), where the aqueous neem extract had shown the presence of all these phytochemicals. Similarly, aqueous chili extract shows the presence of tannins, saponins and alkaloids whereas the study performed by Hemalatha (2013) had shown the presence of terpenoids along with these phytochemicals. This vary in result might be due the ratio of solvent to plant sample while extraction, reagents used etc. The aqueous garlic extract had shown the presence of tannins, saponins, flavonoids and alkaloids which is similar to the study of Huzaifa et al (2014) where the phytochemical screening of aqueous garlic extract had shown the presence of similar phytochemicals.

In this study, the phytochemical screening of cow urine neem extract had shown the presence of tannins, saponins, flavonoids and alkaloids. This result have shown similarity with the study of Rajapandiyan et al (2011), where cow urine neem extract had also shown the presence of similar phytochemicals. Similarly, the cow urine chili extract had shown the presence of tannins, saponins and alkaloid as that of aqueous extract. The cow urine chili extract had shown the presence of tannins, saponins, alkaloid and flavonoid in our study. Phytochemical analysis of incubated cow urine extract of *A. indica* showed the presence of flavonoids, quinines, alkaloids, coumarin, tannin, saponin and phenol (Shanti et al 2015). In our study the Phytochemical screening of Cow urine extract of *A. indica* reported presence of Flavonoids, Tannins, Saponins, Alkaloids, Carbohydrates and amino acids. The difference in antimicrobial properties of different plant extract might be due to the difference in the type and amount of phytochemicals present in them.

In antibiotic assay, Gentamycin showed highest 24mm diameter of zone of inhibition and Ampicillin showed lowest 6mm diameter of zone of inhibition against *X. axonopodis* pv *citri*, Ali et al (2017) reported 21mm, 20.6mm, 15mm, 20.6mm, 9.4mm and 6mm inhibition zone by Gentamycin, Tetracycline, Kanamycin, Chloramphenicol, Amoxicillin and Ampicillin respectively. Similarly, Gentamycin and Chloramphenicol showed the highest Zone of inhibition i.e. 21mm and 24mm against *X. oryzae* pv *oryzea* respectively and Ampicillin shows the least inhibition zone i.e. 7mm against the bacterial pathogen. This result coincides with the observation of Erasmus et al (1997) which proved chloramphenicol to inhibit the growth of pathogens. These research findings might be helpful for the control of bacterial leaf blight in rice and citrus cancker in lemon. However, the antibiotic resistivity has been increased among the bacteria. So, there arises a concern for the alternative solution to control these plant pathogens.

In fact ZOI and MIC are two different attributes for the evaluation of antibacterial effect and MIC for antifungal effect of obtained plant extract. The MIC value is important to evaluate the dose response relationship of plant extract with bacteria/fungi. Jabeen (2011) reported the significant activity of extract of *Azadirachta indica* on isolates of *Xanthomonas oryzae*. Naqvi et al (2019) showed significant efficacy of Neem extract and chilly extract on *Xanthomona oryzae* pv *citri*. Similar findings were even observed on our study where Neem extract exhibited greatest antimicrobial property against selected phytopathogens. Similarly, Sundar et al (2010) observed the extracts of onion bulbs and *A. cepa* and *A. indica* are effective against the white rust and blight of mustard. An important characteristics of plant extracts and their components is their hydrophobicity which enables them to partition the lipids of the bacterial membrane and mitochondria, disturbing the cell structure and leakage from bacterial cell or the exit of critical molecules and ions will led to death (Rastogi and Mehrotra, 2002). Alane SK and Swami CS (2016) reported zone of inhibition by the extract of *Moring oleifera* (10mm), *Calotropis procera*(10mm) and *Allium sativum* in (10mm) respectively against *X. axonopodis* pv *punicae*. Similarly in our study aqueous extract of *A. sativum*

had showed zone of inhibition of 11.8mm and 11mm against *X. axonopodis* pv *citri* and *X. oryzea* pv *oryzea* respectively.

Rakesh et al (2013) reported antifungal activity being displayed by the cow urine extract of certain plants against *F. oxysporum* f.sp *zingiberi* and *Phythium aphanidermatum* which causes rizome rot disease in zinger. Our study also revealed the similar result where cow urine extracts of all the selected plants exhibited antifungal activity against *F. oxysporum* f.sp *cubense* and *B. oryzea*.

In our study, aqueous extract of *A. indica* showed the highest mycelia growth inhibition followed by *A. sativum* and *C. annum* with 92%, 75% and 65% mycelial growth inhibition at the concentration of 50 mg/ml respectively. The result was similar with the research of Shrestha AK (2009) where the extract of *A. sativum* inhibited the mycelia growth of *F. solani* at the concentration of 40% and the extracts of *C. annum* and *Phyllanthus emblica* inhibited the mycelia growth completely at the concentration of 100%.Similarly, in our study, the aqueous extracts of *A. indica* showed maximum mycelia growth inhibition followed by *A. sativum* and *C. annum* with 50%, 40%and 35% mycelial growth inhibition. The result was found to be similar with the research of Bisht and Khulbe (1995) and Ganguly (1994) where *A. indica* extract has shown best inhibitory effect against *Bipolaris oryzea*.

Rakesh et al (2013) found antifungal activity being displayed by cow urine extract of certain plants against *F. oxysporum* f.sp. *zingiberi* and *Phythium aphandermatum* . Amin et al (2013) also reported combination of tobacco leaf and cow urine was found to suppress the mycelial growth and formation of sclerotia of *Sclerotium rolfsii*, causal agent of foot and rot of beetle vine. This research was similar with our study where cow urine extract of *A. indica, A. sativum and C. annum* inhibited the mycelia growth of *F. oxysporum* f.sp *cubense* and *B. oryzea* at the concentration of 25mg/ml and 50mg/ml. Similarly, the study of Akhter et al (2003), showed the inhibitory effect of different concentrations of plant extracts in combination with cow urine against conidial germination of *B. sorokiniana*.

Siny (2008) also reported that the extracts of *Adhatoda vasica, Allium cepa, A. sativum* and *A. indica* caused inhibition of the *Curvularia penneseti* Chowdappa et al (2018) studied the effect of *C. annum* against *Xanthomonas* sp and found that the aqueous extract also possessed the good antimicrobial property which increased with increase in the concentration of aqueous formulation. Sundar et al (2008) reported the extracts of onion bulbs and garlic cloves were effective against *Drechslera oryzea.* The aqueous extract of *A. indica* and in combination with *A. sativum* showed the best inhibitory effect against *X. axonopodis* pv *citri and* with lowest MIC 1250µg/ml and 625µg/ml respectively. Similarly, combined extract of *A. indica* and *C. annum* showed the best inhibitory effect against *X. oryzea* pv *oryzea* with lowest MIC of 1250µg/ml and the plant extract of *A. indica, A. indica + A. sativum, A.sativum + C. annum* showed the similar MIC of 5000µg/ml against *X. oryzea* pv *oryzea*.

Shanti et al (2015) reported cow urine extract of *A. indica* most effective than the other extracts exhibiting maximum antibacterial activity against *X. oryzae* pv *oryzae* with MIC at 1600µg/ml concentration. Similarly, even in our study cow urine extract of *A. indica* and *A. indica + A. sativum* showed maximum antibacterial activity against *X. axonopodis* pv *citri* with lowest MIC of 625µg/ml and 312.5µg/ml respectively. Similarly, combined extract of *A. indica + A. sativum* showed the best inhibitory effect against *X. oryzea* pv *oryzea* with lowest MIC of 1250µg/ml. Shanti et al (2011) also reported that cow urine extract of leaf of *Pongamiapinnata* was effective in inhibiting *Xanthomonas oryzae*.

Swami and Mukunda (2006) reported the extract of *Polialthia longifolia, Annona squamosa, Curcuma longa* and *A. indica* were found to inhibit *Alternaria solani, Curvularia lunata* and *Fusarium oxysporum*. Moslem et al (2009) found that neem leaves and extracts were effective against all tested *Fusarium oxysporium* with a complete inhibition (100%) of growth of *Fusarium oxysporium* and *R. solani* at 40 % level of ethanolic and methanolic extracts. Similarly in our study, the inhibitory effect of aqueous extract of *A. indica* alone and in combination with *A. sativum, C. annum* showed the best inhibitory effect against *F.oxysporum* f.sp *cubense*

with similar MIC 1250µg/ml. In Our study the combined aqueous extract of *A. indica* in combination with *A. sativum* showed the best inhibitory effect against *B. orzea* with lowest MIC of 2500µg/ml. Manimegalai et al (2012) also obtained good inhibitory activity of aqueous extract of Neem against *Bipolaris oryzae.* Ganguly (1994) showed good inhibitory effect of *Azadirachta indica* against *Helmithosporium oryzae*. .Study suggests that even at 10% concentration of all types of extract could cause significant inhibition of growth (Gupta and Bansal, 2003; Amadioha 2004).

Rakesh et al (2015) found antifungal activity being displayed by cow urine extract of certain plants against *F. oxysporum* f.sp. *zingiberi* and *Pythium aphanidermatum* which cause rhizome rot disease in ginger. In our study,the cow urine extract of *A. indica, A. indica + A. sativum, C. annum + A. indica* showed the best inhibitory effect against *B. oryzea* with similar MIC of 2500 µg/ml. Similarly, *A. indica + A. sativum* and *C. annum + A. indica* showed best inhibitory effect against *F. orysporum* f.sp *cubense* with lowest MIC value of 312.5µg/ml Mehta et al (2014) also observed formulations containing crude extracts from four plants with cow urine were shown to exhibit antimycotic activity against *Sclerotium rolfsii*.

In our study all the plant extract had shown the significant effect on the control of both fungal and bacterial plant pathogens. The cow urine extract showed greater inhibition than the aqueous extract which might be due to the presence of some antimicrobial substances in cow urine. The extract of *A. indica* had shown pronounced effect than other plant extracts alone and in combination with *A. sativum* and *C. annum*. The differences in antimicrobial properties might be due to variations in phytochemicals composition (Owuor et al 1986; Toda et al 1989).

CHAPTER VI
CONCLUSIONS AND RECOMMENDATIONS

6.1: Conclusions

Our study was aimed in evaluating antimicrobial effect of neem, garlic and chili using water and cow urine as a solvent. These plant sample and solvents are easily available and ecofriendly for the farmers to use. This study revealed that selected plants contained antimicrobial properties against the plant pathogens. This study also revealed that the higher the concentration higher will be the effectiveness of the selected plant extracts against the pathogens. Among all the extract, neem extract alone and in combination with garlic and chili extract was found to be more effective in comparison to other plant extracts. In comparative study plant extract using cow urine as a solvent showed significantly better result as compared to aqueous plant extract. The reason for this variation might be due to the presence of different minerals and compounds as cow urine itself possess an antimicrobial property. From this work, it can be concluded that the bio rational or botanical extract could be a safe method for the control of plant pathogen and might be helpful to replace the harmful chemical pesticide in the field too.

6.2 Recommendations

1. Within two solvents used, the cow urine was found to be best for the antimicrobial properties. So, cow urine as a solvent should be given more focus for the formulations of the plant extracts as it is easily available, cheap and eco-friendly too.

2. Cow urine extract of *A. indica* + *A. sativum* showed significant antimicrobial activity. So, research on *A. indica* and *A. sativum* extract as a bio rational control for the plant pathogens should be focused.

3. Although antibiotics disc had revealed good action against the pathogens, the plant extract had also shown the good antimicrobial properties. So, the potent plant could be discovered by further evaluation of antimicrobial properties.

4. As, the selected plant extracts showed good effect with the increase in concentration .The proper dose of plant extract should be known for its application.

5. The work was done on the *in vitro* condition; the *in vitro* results might vary in field or *in vivo* condition. Therefore, field studies on effectiveness of plant extracts should be done which would help in the utilization of natural resources.

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