The study of Antibacterial Activity of Common Spices

A
Dissertation Submitted to the
Central Department of Microbiology
Tribhuvan University

In Partial Fulfillment of the Requirements for the Award of the
Degree of Master of Science in
Microbiology
(Medical Microbiology)

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2008

RECOMMENDATION

This is to certify that **Mr. Dinesh Maharjan** has completed this dissertation work entitled **THE STUDY OF ANTIBACTERIAL ACTIVITY OF COMMON SPICES** as a partial fulfillment of M. Sc. degree in Microbiology under our supervision. To our knowledge this work has not been submitted for any other degree.

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ACKNOWLWEDGEMENT

At first, I would like to express my deep sense of indebtedness and immense gratitude to my respected supervisors, Dr. Anjana Singh, Associate Professor, Ms. Shaila Basnyat, Lecturer, Central Department of Microbiology and Mr. Lekhnath Sharma Gautam, Senior Research Assistant, Department of Plant Resources for their kind encouragement, sincere efforts, continuous guidance and overall prospective throughout my research work.

I am very much indebted to Department of Plant Resources (DPR), Thapathali and Central Department of Microbiology for providing me laboratory facilities and moral supports. I would like to acknowledge the staff of DPR and Central Department of Microbiology for their help and support. I would like to thank Mr. Ram Kishun Giri, Assistant Research Assistant, DPR for valuable suggestion and guidance during the extraction part of the study.

I would like to acknowledge Mr. Binod Lekhak for the arrangement that I could begin my research in DPR and also to Dr. Surya Kant Kalauni for his valuable support. I would like to express respect and gratitude to Dr. Dwij Raj Bhatta, Associate Professor, Dr. Shreekant Adhikari, Associate Professor and other teachers of Central Department of Microbiology.

I would like to express my gratitude to Mr. Naresh Maharjan for his ever willing help and encouragement through out my study. I am also thankful to my classmates and friends who helped me directly or indirectly.

I am obliged to Department of Microbiology, TUTH and Central Public Health Laboratory, Teku for providing required cultures of bacteria.

Lastly, I would like to express my heartfelt gratitude to my parents for their whole-hearted support, blessing and inspiration to conquer all difficulties that I could complete M. Sc.

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ABSTRACT

Spices are necessary food stuff used to impart good taste and aroma to food. It is also used as household medicines as well as preservatives. In this study, antibacterial activity of essential oils, acetone and methanol extracts of six different spices viz. *Syzygium aromaticum* Linn., *Piper nigrum* Linn., *Curcuma longa* Linn., *Trachyspermum ammi* Linn., *Coriandrum sativum* Linn. and *Cinnamomum zeylanicum* Blume. against ten bacteria viz. *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 200603), *K. oxytoca*, *Salmonella* Typhi, *S*. Paratyphi A, *Pseudomonas aeruginosa* (ATCC 27853), *Proteus mirabilis* (ATCC 49132), *P. vulgaris* and *Shigella dysenteriae* by agar well diffusion method. Among six spices, clove, ajowan and cinnamon were found to have relatively higher antibacterial activity.

Essential oil of cinnamon was found to show better antibacterial activity inhibiting all tested bacteria. Essential oil of clove showed antibacterial activity against *E. coli, S. aureus, K. pneumoniae, K. oxytoca, S.* Typhi *and P. aeruginosa.* Essential oils of ajowan showed antibacterial activity against all test bacteria except *P. aeruginosa*.

Acetone extract of clove showed antibacterial activity against *E. coli, S. aureus, K. pneumoniae, S.* Paratyphi A, *P. aeruginosa Proteus* spp. and *S. dysenteriae*. Acetone extract of cinnamon inhibited all test bacteria except *P. aeruginosa*.

Methanol extract of clove showed better antibacterial activity against *S. aureus, S*. Paratyphi A, *P. aeruginosa Proteus* spp. and *S. dysenteriae*.

MBC was determined for those extracts that possess inhibitory activity by two fold serial dilution method. The MBC value ranged from 0.39 to 25mg/ml. The lowest MBC value was given by essential oil of cinnamon against *E.coli, S. aureus* and *S. Typhi.*

Gram positive bacteria were found to be more sensitive to spices than Gram negative bacteria. *S. aureus* was inhibited by all spices, 13 out of 18 test suspensions and *E. coli* was also inhibited by all spices but 8 out of 18 test suspensions. Other Gram negative bacteria were inhibited by one or two spices.

Key words: Spices, Essential oil, Acetone extract, Methanol extract, Antibacterial activity, MBC

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
DCA-	Deoxycholate Citrate Agar
DMSO-	Dimethyl Sulfo oxide
EMB-	Eosin Methylene Blue
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
MA	MacConkey Agar
MBC	Minimum Bactericidal Concentration
mcg	microgram
MHA	Mueller-Hinton Agar
MIC	Minimum Inhibitory Concentration
MR	Methyl Red
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MSA	Mannitol Salt Agar
NA	Nutrient Agar
NB	Nutrient Broth
SAARC	South Asian Association Regional Cooperation
TUTH	Tribhuvan University Teaching Hospital
VP	Vages-Proskauer
XLD	Xylose Lysine Deoxycholate Agar
ZOI	Zone of Inhibition

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CHAPTER I

1. INTRODUCTION

Spice is defined as "one or other of various strongly flavored or aromatic substances of vegetable origin, obtained from tropical plants, commonly used as condiments or employment for other purposes on account of their fragrance and preservatives qualities" (Oxford dictionary). In other words, a spice is dried parts of aromatic plants that enriches or alters the taste of food, especially in a small degree. They consists of rhizomes, barks, leaves, fruits, seeds and other parts of plants. They play an important role in human nutrition and medicine. It is proved that spices not only alter the taste of the food but also do have medicinal value because of its antimicrobial activity (Arora and Kaur, 1999).

Traditional medicine particularly based on medicinal plants or herbal medicines have been used to treat various human diseases for centuries and its interest has been increasing worldwide during last decade. More than 80% of the population use herbal medicine to treat disease (WHO, 2002). Herbal medicines were used by Vaidyas, Hakims and in Siddha ways of treating patients, which were based mostly on Ayurvedic, Unani and Siddha systems (Baral and Kurmi, 2005).

Though the exact origin of the herbalism is unknown, plants have been used as a drug from the beginning of human civilization probably as far as 4500 BC to 1000 BC when the Rig Veda, perhaps the oldest repository of human knowledge, was written. It describes the healing properties of some 67 plants. Later on special faculty was developed known as Ayurveda, which deals with human philosophy of health including utilization of medicinal plants so as to restore normal physical fitness and has also mentioned 290 herbal drugs (Malla and Shakya, 1984/85; Sharma, 1981). Susruta samhita and Charaka samhita were followed after Ayurveda which contain the art of surgery and the therapeutic and medicine in detail (Joshi, 2000; HMG, 1970).

It is expected that Nepal is the home for more than 7000 higher plants and 10% of the total higher plants do have medicinal value (Jha, 2005). 701 species of medicinal plants have been reported by Department of Plant Resources (DPR, 2007).

There is an increasing interest in medicinal plants as a natural alternative to synthetic drugs, particularly against microbial agents (Fabio *et al*., 2007) so as to overcome the problem of resistant bacteria (Farah, 1998; Freeman, 1997).

Primarily, spices are taken as the condiments but not common food stuffs. Spice helps to preserve food, to make it digestible, season or flavor food. They supply much nutritional prophylactic substance. They are of great economic value as they are incorporated in perfumes, cosmetic and some dietary products and also in medicine due to their sweet scent (El-Gammal, 1993; Loewenfeld and Back, 1979).

From time immemorial, certain wild herbs were known for their healing power (Loewenfeld & Back, 1979). Spices have been shown to posses medicinal value. Several spices particularly garlic, ajowan, black pepper, clove, ginger, cumin and caraway are used mostly in the Indian diet and in Indian medicine (Arora and Kaur, 1999).

In recent time the growing concern about food safety has led to the development of natural antimicrobials to control food borne pathogens and spices are some of the most commonly used natural antimicrobials in food (Nanasombat and Lohasupthawee, 2005).

Spices are aromatic and pungent product of plants whose properties are often based on essential oils which are only benzene or terpene derivatives. Spices include leaves, flower, bulbs, fruits, stem, rhizomes and other plant parts (Shelif, 1989). They have been used for thousand of years to increase the flavour, color and aroma of food. In addition spices are also known for their preservative and medicinal value (deSouza, 2005). The antioxidant and antimicrobial property of added material is very important to preserve the quality of food material and at the same time provide safety to

consumer (Singh *et al*., 2007). Antimicrobial activity of spices and herbs has been known and described for several centuries (Bagamboula *et al*., 2003). So common culinary herbs, spices and aromatic plants the exhibit antimicrobial activity could provide source of acceptable, natural alternatives (Deloquis *et al*., 2002). Medicinal value of spices is due to antimicrobial activity exhibited by different bioactive compound like alkaloids, flavonoids, isoflavonoids, tannins, cumarins, glycosides, terpens and phenolic compounds.

Spices are used as flavourings or as seasoning both in their fresh and dried forms which is obviously the original use of spices in food (Oiye and Muroki, 2002). A part from the seasoning of the food, spices are found to be beneficial in cases of flatulence and colic, alleviating the cough and pharynx complaints, as expectorants in chest trouble, as stimulant to excite languid stomachs and as emetics. They are also helpful in cases of gastritis and dyspepsia. Externally, some spices are of value in treating rheumatism, lumbago, neuralgia, bronchitis and similar obdurate complaints. The antiseptic properties of clove and thyme have a place in tooth paste, mouth refresher and throat sprays (Parry, 1969). Essential oils are widely used in food production due to their antimicrobial, antioxidative, colouring and flavouring properties.

Besides flavouring agents, spices, as effective preservatives (i.e. as antioxidants and antimicrobials) have been demonstrated but application of those findings in food processing has been minimal. Spices can be the alternative to chemical food additive (Chandarana *et al*., 2005). So research on *in vitro* as well as *in vivo* effects of spices should be carried out.

Herbal medicine is the primary health care system available in the remote area because modern medicine hasn't reached those areas and is expensive too. To be safe from the side effect of synthetic drug, use of herbal medicine should be encouraged and exploration of the flora to establish their medicinal value should be carried out extensively.

In Nepal, spices also take as important part in every day meal. Some of the spices are imported from India and other SAARC countries and some of them are cultivated. Some of the spices are used as home herbal remedies for common ailments like cough, cold, stomach disorder, headache, nausea and vomiting, headache, dysentery etc.

In Nepal, little work has been done in the study of antimicrobial activity of medicinal plants and essential oil. This study mainly focused on the antibacterial properties of the some common spices used in Nepal like clove, coriander, turmeric, black pepper, ajowan and cinnamon. The antibacterial activity of essential oils, acetone fraction and methanol fraction of these spices is studied.

CHAPTER II

2. OBJECTIVES

2.1. General objectives

- To study the antibacterial activity of some common spices (clove, coriander, turmeric, black pepper, ajowan and cinnamon).

2.2. Specific objectives

- To extract an essential oils, acetone and methanol extract of spices.
- To assay the antibacterial activity of an essential oils, acetone and methanol extracts.
- To determine minimum bactericidal concentration (MBC).
- To compare antibacterial activity of essential oils with antibiotic sensitivity pattern of test bacteria.

CHAPTER III

3. LITERATURE REVIEW

3.1. Medicinal plant

Reviewing the history of the development of medicines, we came to know that most of herbal medicines were originally derived from the food. A single medicinal plant may be defined as a food, a functional food, a dietary supplement as an herbal medicines in different countries, depending on the regulations applying to foods and medicines in each country (WHO, 2005). The plants that posses therapeutic properties or exert beneficial pharmacological effects on the human body are termed as “Medicinal plants”. In Nepal especially, Ayurvedic doctors, Traditional healer, Jhakri, Amchi etc deals with disease in their own way.

3.2. Spices

Spices may be defined as "any dried, fragrant, aromatic or pungent vegetables or plant substances in whole, broken or ground forms that contribute flavour, whose primary function in food is seasoning rather than nutrition and that may contribute piquancy of foods and beverages." Spices enrich or alter the quality of a thing, altering the taste of the food. Spices contain essential oil and other chemical substances which give them fragrant, aromatic, pungent, acrid, bitter or other properties of aroma and taste. In other word it may be defined as plant substances from indigenous or exotic origin, aromatic or with strong taste, used to enhance the taste of food. Spices constitute essential items used daily in food. Spices impart good taste and odour to food and nutritional products and also used as preservatives (Chandarana *et al*., 2005; El-Gammal, 1993).

Spices include leaves (bay, mint, rosemary, coriander, laurel and oregano), flower (clove), bulbs (garlic, onion), fruits (cumin, red chilli and black pepper), stem (coriander, cinnamon), rhizomes (ginger) and other plant parts (Shelif, 1989). These

plant parts contain essential oil and other chemical substance that gives them frangrant, aromatic, pungent, acrid, bitter or other properties of aroma and taste.

3.3. Essential oil of spices

Essential oil is a volatile oil, obtained by distillation, and marked by the characteristic odour of the plant or substance from which it is obtained, as the oil of laurel, oil of turpentine etc. now often as a synonym of 'volatile oil' (oxford dictionary). Essential oils (also called volatile or ethereal oils) are aromatic oily liquids obtained from plant material (flower, buds, seeds, leaves, twings, bark, herbs, wood, fruits, and roots). It is obtained mostly by steam distillation, however hydrodistillation can also be used. The term 'essential oil' is thought to derive from word *Quinta essentia*. An estimated 3000 essential oils are known, of which 300 commercially important are predetermined chiefly for the flavours and fragrances market along with medicinal value. Besides antibacterial properties, essential oils or their components have shown antiviral, antimyotic, antioxigenic, antiparasitic and insecticidal properties.

Currently, the greatest use of essential oils and their individual components in the European is in food as flavourings, perfumes for their fragrances and aftershaves and pharmaceuticals for their functional properties (Burt, 2004).

Essential oils have a long history of use as natural microbial agents. Essential oils have recently been used in number of pharmaceutical, food and cosmetic products because these oils effectively inhibit the growth of a wide range of microorganisms and they cause fewer side effects than synthetic drugs in human (Park *et al*., 2007). In Traditional popular medicine, essential oils and other plants product have been used traditionally to treat respiratory tract infections. Inhalation of essential oils has been used to treat pharyngitis, bronchitis and sinusitis (Fabio *et al*., 2007).

3.4. History of spices and its tradition

Spices and herbs have played a dramatic role in the development of western civilization. Now a day there are lots of different kinds of spices, mostly used as

flavouring, but in ancient times, they were rare and precious products, used as medicine, perfume, incense and flavouring. They were valued as highly as gold.

The use and cultivation of spices, however, go back to the beginning of history. They have played a major part in all civilization of antiquity, in ancient china and India, in Babylon and Egypt and in Greece and Rome. The popularity of Indian apices is older than the recorded history, more than 7000 years old, centuries before Greece and Rome had been discovered. The Assyrian myth claims that the gods drank sesame wine the night before they created the earth. However, the first authentic, if fragmentary, records of the use of spices and herbs may date from the Pyramid Age in Egypt, approximately 2600 to 2100 BC. Onions and garlic were fed to the one hundred thousand laborers who toiled in the construction of the Great Pyramid of Cheops, as medicinal herbs to preserve their health. Most of the king and queens of ancient Egypt were deeply interested in perfumes and spices.

In Genesis, Joseph's older brothers sold him to a passing caravan of spice merchants traveling from Gilead to Egypt. Later, in the book, the Queen of Sheba made a tribute to King Solomon in the form of spices, gold and precious stones. Around 400 BC Hippocrates, the Greek physician, listed more then 400 medicine made with spices and herbs, about half of which we still use today.

The majority of spices originated in the Asiatic tropics and was among the first objects of commerce between the East and West. Long before Christian era, the Greek merchants hosted the markets of South India, buying many expensive items amongst which spices were one. From 1000 BC trading in spices between East and West was dominated by Arabia. The Romans broke the Arabian spice trade monopoly by discovering their long-held secret of the monsoon winds, and increased the demands for the various herbs and spices. Then, comes Europeans, who dealt directly with these Eastern cultures for spices and explorers, sought new worlds in their quest for exclusive trade routes. Later other countries took over the spice trade. For many years, Venice was the leader. In the 16th century, the Portuguese assumed control and held a

virtual monopoly for two centuries. They were supplemented by the Dutch, who were supreme for many years. Later on British Empire shared with Holland most of the spice trade of the world. Americans began their entry into the world spice race in 1672 AD. As their influence grew, Americans made many new contributions to the spice world. From the beginning of history the strongest nations have controlled the spice trade. The same true today, the United States is now the world's major spice buyer, followed by Germany, Japan and France.

The ancient Chinese knew a lot about spices and their uses, preserved in their famous historical book the Classic Indian history also has similar information about the use of spices in food and medicine (Loewenfeld and Back 1979; El-Gammal, 1993; Sharma, 1996)

3.5. Herbs and spices for health, cooking and cosmetics

In the passage of time, the uses of the spices have steadily increased. Herbs and spices have become the important parts of the human diet. It is important that the food we eat should contain the necessary nutritive and bactericidal properties. Spice helps to preserve food, to male it digestible and at the same time provided the basis of their medicines. Even today herbs and spices not only flavour and improve the taste of our food, but supply us with many nutritional prophylactic substances. Most of spices contain varying degree of alkaloids, essential oils, minerals, trace element, sugar, fatty oils, protein and starch which are important to maintain sound health. Some alkaloids produce a stimulating effect on the nervous system, while others stimulate the appetite and it is though they enable the body to build up protein. Spices activate the lower digestive glands without which food is not digested properly. Essential oils increase the production of white blood corpuscles and improve the circulation of the blood to the skin, also increase production of mucus and bile. They promote perspiration, sooth inflammation and possess bactericidal and disinfectant properties. Tannin has an astringent effect on mucous membranes and is antibiotic by nature.

They have become indispensable in the culinary art, adding savor to insipid dishes, tang to beverages and zest to appetizers. In the food manufacturing industry, the spices have a most important place. The essential oils of the spices are used as flavouring extracts in different variety of foods. Most dishes, including processed and frozen foods, are improved by addition of the appropriate herbs or spices, singly or in mixtures.

Herbs and spices were once the main source and foundation of all cosmetics, before methods were discovered of synthesizing their properties. Most of the spices acts directly on the skin, clearing spots, pimples or refining it, depending on the herbs used. There is also no doubt that herbs and spices with their lovely natural perfumes and volatile oils, produce a sense of relaxation (Loewenfeld and Back, 1979)

3.6. General description of spices under study

3.6.1. *Syzygium aromaticum* Linn.

(Family: Myrtaceae)

Nepali name: Lwang English name: Clove

The clove tree is a native of some islands of the Malay Archipelago, especially Moluccas. It is cultivated in Zanzibar and Pemba (Tanzania), Indonesia, Penang, Malagasy and to a lesser extent in the Seychelles, Mauritius, India and Srilanka. In India, It is grown in Tamil Nadu and Kerala.

Clove tree is a pyramidal or conical evergreen tree, 9-12m high, sometimes taller. Main stem is erect with 100cm in girth, often forking at a height of 1.5-1.8m. Bark is smooth and grey. Leaves are elliptical, dark green, entire, lanceolate, in pair, acute at both ends, gland dotted, fragrant. Flower small rosy and borne in cluster of three at the end of branches. Flower buds borne at the end of branches, greenish, turning pink at the time of maturity, aromatic, drupes (mother of clove) develops which is about 1inch long and half inch broad, fleshy, dark pink. Seed is oblong, soft, grooved on one

side 1.5cm long. The trees begin to bear fruit when they are six to seven years old (The Wealth of India, 1976).

Use: The clove is highly esteemed as a flavouring material and is extensively used, whole or in ground form, as culinary spice. The oil exerts bactericidal action against most of bacteria. Clove also exerts preservative action against oxidative rancidity.

The Cloves are aromatic, stimulant and carminative. They are used in various forms of gastric irritation and dyspepsia to relieve nausea and vomiting, to cure flatulence and to excite languid digestion. The oil is used as a local analgesic for hypersensitive dentine and carious cavities, a mixture of oil and zinc oxide is used a temporary filling for tooth cavities. Externally the oil is rubefacient and counter irritant, and internally it is carminative and antispasmodic.

Because of its antiseptic and antibacterial properties, many pharmaceutical industries use clove oil in the product such as perfumes, toilet waters, soaps, toothpaste etc (The Wealth of India, 1976).

Clove is used in preparation such as Raktalavangadi, Churna, Lavangadi vati, Avipattikara churna, S.V Tulasi, Herbal tea (Jha, 2005).

3.6.2. *Piper nigrum* Linn.

(Family: Piperaceae)

Nepali name: Marich English name: Black pepper, Pepper

Black pepper is considered as the 'King of Spices' because of its highest volume of international trade among all the spices known. Black pepper is the dried mature but unripe fruit of *Piper nigrum* L. It is one of the most ancient crops cultivated in India. It is also cultivated in Indonesia, Malaysia, Sri Lanka, Brazil, Thailand and other tropical countries (The wealth of India, 1969). This plant is also cultivated in Nepal and dried fruits are imported (DPR, 2007).

Black pepper is a branching, climbing perennial shrub. Branch's stout, trailing and rooting at the nodes. Leaves are entire 12.5-17.5 by 5.0-12.5cm, very variable in breadth, sometimes glaucous beneath, base acute rounded on cordate, equal or unequal. Flower is minute in spikes, usually dioecious. Fruit is ovoid or globose, bright red when ripe. Seed is usually globose, testa thin, albumin hard, bisexual, simple, pendulous slender spikes (The Wealth of India, 1969).

Use: The ancient Aryans considered it as a powerful remedy for various disorders of the anatomical systems and prescribed to as an effective cure for dyspepsia, malaria, deliriumtremens, haemorrhoids etc. It is also used for embalming,

In modern European medicine, black pepper is rarely prescribed, except indirectly, as an ingredient of some combined preparations. In modern Indian medicine, it is much employed an aromatic stimulant in cholera, weakness following fevers, vertigo, coma etc., as a stomachic in dyspepsia and arthritic diseases. Externally, it is valued for its rubefacient propertied and as a local application for relaxed sore throat, piles and some skin diseases. Pepper, particularly its oleoresin, has bacteriostatic and fungistatic properties.

The whole fruits are added to pickles, certain typed of sausage, etc but the bulk of the product is generally ground before use. Black pepper is mostly used for its characteristic delicate penetrating aroma and pungent, biting taste.

The characteristic aromatic odour of pepper is due to the presence of volatile oil in the cells of the pericarp. Oil of pepper is an almost of phellendrene. Oil of pepper is a valuable adjacent in the flavouring of sausages, canned meats, soups, table sauces and certain beverages and liquors. It is used in perfumery, particularly in bouquets of the oriental type of which it imparts spicy notes difficult to identify. The oil is also used in carnation compound for soaps. It finds use in medicine (The wealth of India, 1969).

Black pepper is used in the preparation such as Marichyadi taila, Trikuta churna, Mrityunjaya rasa, Pratishyayahara vati (Jha, 2005).

3.6.3. *Curcuma longa* Linn. or *Curcuma domastica* Linn.

(Family: Zingiberaceae)

Nepali name: Haledo (Beshar) English name: Turmeric

The plant is a native of Southern Asia (probably India) and is cultivated extensively throughout the warmer parts of the world. It is grown on a large scale in India, China and East Indies. It is widely cultivated in Nepal for spices (DPR, 2007).

Turmeric is erect perennial herb, 2-3ft high with a short stem; Leaves in group of 6-10 on psuedostems, about 40 cm long, broadly lanceolate, acuminate, entire, bright green with distinct midrib, base sheathening; Flowers yellowish in pairs in the exiles of the bracts, opening before the others; Rhizome short and thick (The wealth of India, 1950).

Use: Turmeric (rhizomes or powder) is an auspicious article in all religious observances in Hindu household. It is a normal constituent of condiments, curry powder and prepared mustard. It is used also for dyeing wool, silk and unmordanted cotton to which it imparts a yellow shade in an acid bath. In Indian systems of medicine, turmeric is used to some extend as a stomachic, tonic and blood purifier. It is also prescribed as an antiperiodic alternative. Mixed with warm milk it is said to be beneficial in common cold. The juice is the fresh rhizome is used as an antiparasitic for many skin infections. Externally, it is applied to indolent ulcers and a paste made from the powdered rhizome along with wine forms a remedy for inflamed joints. A decoction of the rhizome is said to relieve the pain of purulent ophthalmia. Oil of turmeric, distilled from the dried rhizomes, has feeble antiseptic properties. It is an antacid and in small doses, acts as a carminative, stomachic, appetizer and tonic. In large doses, however, it appears to act as antispasmodic inhibiting excessive peristaltic movements of the intestines.

The antioxidative properties of curcuma powder are probably due to the phenolic character of curcumin. The choleretic action of the essential oil is attributed to p-

tolylmethyl carbinol. The dye stuff acts as a cholagogue causing the contraction of the gall blader (The wealth of India, 1950).

Turmeric is used in the preparation such as Haridra khanda, Kushthadi manjan, Naram tel, Chandraprabha vati (Jha, 2005).

3.6.4. *Trachyspermum ammi* Linn.

(Family: Umbelliferae)
Nepali name: Jwano English name: Ajowan
The herbs is said to be a native of Egypt. Although it is cultivated in the Mediterranean region and in South-west Asian countries such as Iraq, Iran, Afghanistan and Pakistan, ajowan is chiefly produced in India.

Ajowan is an erect, glabrous or minutely pubescent, branched annual herb, up to 90cm tall. It is cultivated almost throughout India. Stem is striate; Leaves rather distant, 2-3 pinnately divided, segments linear, ultimate segments, 1.0-2.5cm long; Flower in terminal or seemingly, lateral pedunculate, compound umbels, white, small; Fruit ovoid, grayish brown, about 2mm long and compressed, with distinct ridges and tubercular surface, 1-seeded (The Wealth of India, 1976).

Use: Ajowan, with its characteristic aromatic smell and pungent taste, is widely used as a spice in curries. It is employed either alone or in mixture with other spices and condiments. It is also used in pickles, certain types of biscuits, confectionery and beverages. Another use of ajowan is as an ingredient of *pan*-mixtures. The more important use of ajowan in medical and it is much valued for its antispasmodic, stimulant, tonic and carminative properties. It is used in flatulence, atonic dyspepsia, diarrhea, cholera. It is also effective in relaxed sore throat and in bronchitis, and often constitutes an ingredient of cough-mixture. Externally, a paste of the crushed fruit is applied for relieving colic pains and a hot and dry fomentation of the fruits on the chest is common remedy for asthma.

The root of the ajowan plants are reported to possess diuretic and carminative properties and are used in febrile conditions and in stomach disorder. It is also used in the preparation of lotions and ointments applied for checking chronic discharge (The wealth of India, 1950).

Ajowan is used in the preparation such as Jeecaba bindu, Bhivaneshwara vati, Hingwashtaka churna (Jha, 2005).

3.6.5. *Coriandrum sativum* Linn.

(Family: Umbelliferae)

Nepali name: Dhaniyaa English name: Coriander

The coriander plant is native to Eastern Mediterranean region and is extensively grown in India, Russia, Central Europe, Asia Minor and Morocco (The wealth of India, 1950). Plant is also cultivated in Nepal (DPR, 2007).

Coriander plant is an annual herb, 1-3ft. high, with smack, white or pinkish purple flower borne in compound terminal umbels. The lower leaves are broad with crenately lobed margins, while the upper ones are narrow, finely cut with linear lobes. The fruit are globular and ribbed yellow brown in colour and range in size from 2.0-3.5mm in diameter. The fruit consists of two united carpel, each carpel containing a single seed. The crop matures in $3\text{-}3^{1}/_{2}$ month after sowing (The Wealth of India, 1950).

Use: The whole plants can be used. All parts of the plants have a pleasant aromatic odour. Aromatic odour and taste of the coriander is due to an essential oil. The whore plants, when young, are used in preparing chutneys and sauces and the leaves are used for flavouring curried and soups. The fruit are extensively used as condiment in the preparation of curry powder, pickling spices, sausages and seasonings. They are used for falvouring pastries, cookies, buns and cakes and tobacco products.

The volatile oil is used chiefly as a flavouring agent for spirituous liquors and cocoa and chocolate industries. It is also employed in medicine as a carminative or as a

flavouring agent to cover the taste or correct the nauseating or griping qualities of other medicines. Oils of coriander are a valuable ingredient in perfumes (The wealth of India, 1950).

The fruit are considered carminative, diuretic, tonic, stomachic, antibilious, refrigerant and aphrodisiac. They are used chiefly to conceal the odour of other medicines and to correct the groping qualities of rhubarb and senna and also the foul breath. An infusion of the seeds in combination with cardamom and caraway seeds is useful in flatulence, indigestion, vomiting and intestinal disorders (The Wealth of India, 1950).

3.6.6. *Cinnamomum zeylanicum* Blume.

(Family: Lauraceae)

Nepali name: Daalchini English name: Cinnamon

Cinnamon is native of South India and Ceylon. The plant is extensively cultivated in Ceylon, which constitute the world's principal source of supply of cinnamon (The Wealth of India, 1950). This plant is also cultivated in Nepal and dried bark is also imported (DPR, 2007).

Clove is small evergreen tree, with thick bark, outer surface rusty, brown, striated; inner surface smooth; Leaves is 10-20 cm long, glabrous, ovate or ovate-lanceolate, 3-5 nerved; Flower is 5-6mm long, often bisexual, numerous and white (Baral and Kurmi, 2005).

Use: Parts used for medicinal purpose is inner barks of the tree. Cinnamon is one of the oldest and most praised spices which are valuable for its fragrance and medicinal properties.

Cinnamon bark oil is extensively used for flavouring confectionery, liqueurs, pharmaceuticals, soaps and dental preparations. It has high germicidal activity and also fungicide. It is employed as adjuvant in stomachic and carminative medicines. As a powerful local stimulant it is sometimes prescribed in gastrodynia, flatulent colic and gastric debility. Cinnamon bark, either as small pieces or as powder, is

extensively used as spice or condiment. It is aromatic, astringent, stimulant and carminative. It possesses the property of checking nausea and vomiting (The Wealth of India, 1950). It is also externally in the treatment of rheumatism, neuralgia, headache and toothache; internally used in dyspepsia, flatulence, useful in hemorrhagia, gonorrhea, tuberculosis and enteric fever (Baral and Kurmi, 2005). Leave and stem extract is inhibitory against Ehrlich ascites tumour cells (Joshi, 2000).

Cinnamon used in the preparation such as Jeerakadyarista, Sitopaladi churna, Sutashekara rasa, Chandraprabha vati (Jha, 2005).

3.7. Screening and evaluation of Antimicrobial activity

3.7.1. Antimicrobial agent

Antimicrobial agent is the chemical substance that kills or inhibits the growth of microorganisms. Antimicrobials are generally described as bacteriostatic when, at usual dose, they prevent the active multiplication of bacteria and described as bactericidal when, at usual dose, they kill bacteria. The term 'broad spectrum' is applied to antimicrobial agents with activity against a wide range of bacteria.

3.7.2. Antimicrobial activity

Not all antimicrobials, at the concentration required to be effective are necessarily non-toxic to human cells. Antimicrobial must be safe to be used in the treatment of disease. However most antimicrobial show selective toxicity to be of value in the treatment of microbial disease

There are various mechanisms by which antimicrobials act on bacteria. They are as follows:

a. by inhibiting cell wall formation leading to cell lysis,
b. by damaging the bacterial cell membrane, leading to loss of cell contents and so to call death,
c. by inhibiting protein synthesis and therefore arresting bacterial growth, and

d. by inhibiting the production of nucleic acids and therefore preventing bacteria from reproducing and
e. by inhibiting of foliate synthesis (Cheesbrough, 1993; Denyer *et al*.,2004).

As evidenced from the findings of different researches, spices have been shown to possess activity against different microorganisms including bacteria, fungi and virus. Herbs and spices, possessing antimicrobial activities are used as preservatives. Antimicrobial activity of spices is mostly due to essential oils. Some of the notable works conducted from the world includes:

In the study done by Sampurna and Nigam (1980), one mixture, containing the oils of *Eucalyptus citriodora, Cinnamomum zeylanicum, Cuminum cyminum, Mentha arvensis, M. spicata*, and *M. piperita* and another mixture, containing *M. piperta, E. citricodora, C. zeylanicum, Trachypermum ammi, Carum carni* and *C. cyminum* possess potent antibacterial efficacy against *Salmonella* Typhi.

Huhtanen (1980) reported inhibition of *Clostridium botulinum* by spice extracts. They found that clove and nutmeg showed the antimicrobial activity with the MIC of 500ppm and 125ppm against it. White and black pepper had MIC of 125ppm. Venkataram *et al*. (1983) reported that water distillate and essential oils of clove and cinnamon had preservative property with out altering the physicochemical properties of Kwathas.

Jain and Kar (1992) studied the antimicrobial activity of *Piper nigrum*. They found equal zone of inhibition against *E. coli, B. subtilis, S. aureus* and *S*. Typhi i.e. equal to 8mm. where filter discs used was 6mm in diameter.

Essential oils from clove exhibited significant antimicrobial activity against a collection of 20 different genera of test bacteria and 20 different isolates of *Listeria monocytogens* (Deans *et al.* 1995). Agnihotri and Vaidya (1995) reported that clove inhibited all seven test organisms which include *Escherichia coli, Staphylococcus*

aureus, Salmonella Typhi, *Pseudomonas aeruginosa, Shigella flexneri* and *Proteus vulgaris*. Cinnamon inhibited all the organisms except *P. aeruginosa* and *S. flexneri*.

A crude methanol extract of clove exhibited better growth inhibitory activity against Gram negative periodontal oral pathogens including *Porphyromonas gingivalis* and *Prevotella intermedia* (Cai and Wu, 1996). Baratta *et al.* (1998) studied the chemical composition, antimicrobial and antioxidative activity of coriander essential oil. They reported that coriander oil demonstrated significant activity against *Acinetobacter calcoaceticu, Beneckea natriegens, Citrobacter freundii, Erwinia carotovora, Lactobacillus plantarum, Micrococcus luteus* and *Staphylococcus aureus*. Coriander showed maximum activities.

The extract from *Acrous calamus, Ocimum sanctum, Zingiber officinale, Cinnamomum zeylanicum. Moringa oleifer,* and *Piper bettle* were analyzed for antimicrobial activity against *S. aureus, E. coli, P. aeruginosa* and *Klebsiella* spp. *E. coli* exhibited higher resistant to antibiotics and plants extracts (Rajendhran *et al.,* 1998).

Pradhan *et al.* (1999) investigated antibacterial activity of *Piper nigrum* against *E. coli, S. aureus, Bacillus cereus and Salmonella* Typhimurium. They found that the active compound present, 3,4-dihydroxyphenylethanol glycosides was effective at 2.25mmol/l against *E. coli, S. aureus, B. cereus and Salmonella* Typhimurium.

Among 14 essential oils, cinnamon bark, lemon grass and thyme oil showed the lowest MIC followed by essential oil containing terpene alcohol as major constituent against respiratory tract pathogens like *Haemophilus influenzae, Streptococcus pneumoniae, S. pyogens and Staphylococcus aureus* (Shigeharu *et al.,* 2001).

Delaquis *et al.* (2001) studied antimicrobial activity of individual and mixed fraction of dill, cilantro, coriander and eucalyptus oils. They found that mixing fraction resulted in additive, synergistic or antagonistic effect against test microorganisms. Distilled fractions of coriander oils were considerably more potent than the crude oil.

Arora and Kaur (1999) analyzed the antimicrobial activity of garlic, ginger, clove, black pepper and ground green chilli and their aqueous extract on human pathogenic bacteria and found that yeast was totally killed in 5hrs by clove and it also inhibited the growth of *Shigella flexneri* to a moderate extent.

The antimicrobial activity of black pepper, clove, geranium, nutmeg, oregano and thyme against 25 different genera of bacteria was analyzed (Dorman and Dean, 2000). The volatile oils exhibited considerable inhibitory effects against all the organism under test while their major components demonstrated various degree of growth inhibition.

Black pepper extracts showed complete reduction of colonies against tested bacteria strains of *S. aureus, B. cereus* and *B. subtilis* at 5 and 10µl level (Singh *et al*., 2005). In the study, Indu *et al*. (2006) found that black pepper showed no antimicrobial activity against 20 different serotypes of *E. coli*, 8 serotypes of *Salmonella, Listeria monocytogens* and *Aeromonas hydrophila.*

In their study, Agaoglu *et al*. (2007) found that cinnamon was most effective spice against all of the test strains including *S. aureus, K. pneumoniae, P. aeruginosa, E. coli, Enterococcus faecalis, Mycobacterium smegmatis, Micrococcus lutius* and *Candida albicans.* The most susceptible bacteria to cinnamon were *S. aureus* and *C. albicans*. *P. aeruginosa* and *E. coli* were the most resistant strains to spice sample.

Sofia *et al*. (2007) designed to evaluate the antimicrobial activity of six Indian spices extract, namely, clove, cinnamon, mustard, garlic, ginger and mint against potent foodborne pathogens, namely, *E. coli, S. aureus* and *B. cereus*. They found that the extracts of clove and mustard had good inhibitory action at 1% concentration. Cinnamon leaf volatile oil and oleoresin showed better inhibitory result in comparison to bark oils (Singh *et al.*, 2007).

In the investigation of the antibacterial potential of aqueous decoction of black pepper, bay leaf, aniseed and coriander against 176 bacterial isolates belonging to 12

different genera of bacterial population isolated from oral cavity of 200 individuals (Chaudhry and Tariq, 2006), overall aqueous decoction of black pepper was the most bacterial toxic exhibited 75% antibacterial activity with maximum activity against *S. aureus* (23mm) at the concentration of 10µl/disc.

Nanasombat and Lohasupthawee (2005) examined for the antibacterial activity of crude ethanolic extracts and essential oils of 14 spices. Among all ethanolic extracts, clove extract had the higher inhibitory effect on the growth of all bacterial strains tested and oils of clove and kaffir lime peels exhibited greater antibacterial activity against all tested strains. Coriander also showed potent inhibitory activities. Ethanolic extract of *Piper nigrum* was found to be most active against *P. aeruginosa*, in the study done by Erturk (2006).

The examination of the cytotoxicity and the antibacterial effects of a variety of essential oils on major respiratory tract pathogens including *Streptococcus pyogens*, *S. agalactiae*, *S. pneumoniae, K. pneumoniae, Haemophillus influenzae , S. aureus* and *Stenotrophomonas maltophilia* (Fabio *et al*., 2007). Cinnamon and thyme showed the strongest action followed by clove indicating that thyme can be considered as a potential antimicrobial agent for the treatment of some respiratory tract infection in man.

In their experiment, Betoni *et al.* (2006) tried to verify the synergism between 13 antimicrobial drugs and 8 plant extract including guaco, guava, clove, garlic, lemongrass, ginger, carqueja and mint against *S. aureus*. They verified that clove, guava, and lemongrass presented the highest synergism rate with antimicrobial drugs and ginger whereas garlic showed limited synergistic capacity.

A significant antibacterial activity was shown by *Coriandrum sativum* essential oil against *E. coli*, and *Bacillus megaterium* (Lo Contore *et al*., 2004). Aliphatic (2E)-alkanals and alkanals characterized from the fresh leaves of the coriander were found to possess antibacterial activity against *Salmonella choleraesuis* ssp. *Choleraesuis*

ATCC 35640. (2E)-dodelenol (C(12)) was the most effective with MBC 6.25µg/ml followed by (2E)-undecenal (C(11)) with MBC of 12.5µg/ml (Kubo *et al*., 2004).

Burt and Reinders (2003) quantified the antibacterial properties of five essential oils on non-toxigenic strains of *E. coli* O157:H7 in the presence and absence of a stabilizer and an emulsifier and at three different temperature and clove bud oil was found to be less active to it.

In the evaluation of the antimicrobial activity of essential oils of cinnamon, clove, basil, rosemary, dill and ginger, cinnamon and clove gave the strongest (very similar) inhibition, followed by basil and rosemary, with dill and ginger giving the weakest inhibition, in the solid diffusion test. The fungi were the most sensitive microorganisms, followed by Gram positive bacterial strains (Lopez *et al*., 2005). Coriander seeds showed no inhibition against tested microorganisms (Ates and Erdogrul, 2003).

Thakare (2004) investigate the antimicrobial activity of ethanolic extracts of different plants including turmeric, ginger, black pepper, cinnamon, thyme, bay leaf and clove against common poultry pathogens *E. coli, A. typhimerium, E. faecium* and *E. feacalis* and found that cinnamon and thyme were more active against test bacteria.

Essential oils of spices and herbs including thyme, origanum, mint, cinnamon, salvia and clove were found to possess the strongest antimicrobial activity among many test organisms (Kalemba and Kunicka, 2003).

Most of the foodborne bacterial pathogens examined were sensitive to extracts from plant such as cinnamon, clove, garlic, mustard, onion and oregano. Phenols, alcohols, aldehydes, and ketones, ether and hydrocarbons have been recognized as major antimicrobial components in spices (Ceylen and Fung, 2007).

Singh *et al*., (2007) had studied on antioxidant and andibacterial potential of essential oils and acetone extracts of various spices where they found that the essential oils of ajowan, tejpat, Chinese cassia bark and coriander were active against all the Gram

positive and Gram negative bacterial strains tested and also the acetone extract were less effective as compared to volatile oils.

Study about the antimicrobial activities of medicinal plants and essential oils are limited in Nepal. Even though, regular study about antimicrobial activities of medicinal plants is carried out by Department of Forestry and Plant Research. Few studies have been done in dissertation works in Central Department of Microbiology. Some of the notable works done are as follows:

Shakya (1982) performed the preliminary studies on medicinal plants for their antimicrobial activities in which he determined MIC value of plants against *S. aureus, B. subtilis, S.* Typhi, *E. coli, S. dysenteriae, C. albicans, S. cerevisiae* and *C. neoformans.*

Shrestha and Sharma (1988) observed the antimicrobial activities of some plant products viz. *Mentha arvensis, Acorus calamus, Zanthoxylum oxyphyllum* and turpentine oil against some bacteria and fungi. In a study, Sharma and Adhikari (1988) carried out an experiment to determine MIC values of *Allium sativum, Azadirachta indica, A. vulgaris* and *H. asiatica* against *E.coli.*

Risal (1994) studied antimicrobial activities of 32 indigenous plants against *S. aureus, B. pumilis, B. substilis, E. coli, S.* Typhi, *S. dysenteriae, C. albicans* and *S. cerevisiae.*

In the study, Tayler *et al*. (1996) found four out of 20 plants were active against *P. aeruginosa*. The extracts which showed broadest spectra of activity were *Eupatorium odoratum, Terminalia alata, M. philippensis* and *Rumex hastatus.*

The antimicrobial activities of nine medicinal plants of Nepal were evaluated against *P. aeruginosa, S. aureus, E. coli*, MRSA, *V. cholerae. S*. Typhi, *S. dysenteriae* and *S. fexneri* (Devkota *et al*., 1999). *Glycyrrhiza glabra* showed best antimicrobial activity among all tested plant extract.

Parajuli *et al*., (1998) examined the antibacterial activity of 29 medicinal plant used to treat skins ailments of Kaski district, Nepal. Out of them, 11 species were able to produce inhibitory activities.

Parajuli (2001) investigated antimicrobial properties of 29 medicinal plants against four test organisms, viz. *S. aureus, B. subtilis, E. coli* and *P. aeruginosa*. Out of 29, 12 species showed distinct ZOI with all types of test organisms.

Gautum (2002) selected 33 plants species used for curing respiratory disease in traditional society in Nawalparasi district of Nepal, among which 13 plants were able to produce inhibitory activity against all three test bacteria, *S. aureus, K. pneumonia*, and *P. aeruginosa*.

In the investigation of 20 different medicinal plants for their antimicrobial properties against ten microorganisms, seven (35%) were found active against 6 or more organisms and 4 plants (20%) were active against 4 test organisms. *Rhododendron anthopogon* and *Rhus javanica* were effective for all organisms, while, *Boerhavia diffusa* was ineffective to all the test organisms.

Some to the dissertation works done in Central Department of Microbiology are as follows:

Ranjit (1995) studied inhibitory activities of Neem extract on *S. aureus* and *E. coli*.

Thapa (1997) determined MIC values of *Moringa oleifera, Azadirachta indica, Momorrdica charantia* and *Teminalia chebula.*

Pokhrel (2000) screened and evaluated antimicrobial activity of 29 different medicinal plants of Nepal against 8 different microorganisms and reported that the extracts were more effective in Gram positive than Gram negative bacteria.

Sharma (2000) studied the antimicrobial activity of some of spices used in Nepal and found that essential oils from cinnamon, clove, and timur showed high degree of

inhibition whereas others like black pepper and coriander oil were found comparatively less inhibitory.

Baidya (2001) screened and evaluated antimicrobial activity of 20 different medicinal plants against 8 pathogenic organisms and found that extract from *Rubus ellipticus* was most effective.

Prasai (2002) evaluated the antibacterial activity of 8 different medicinal plants against 7 different Gram negative bacteria. *Alnus nepalensis, Ficus religisos, Myrica esculenta* and *Rhododendron arboretum* were effective against *E. coli, Klebsiella* spp, *P. vulgaris, S.* Typhi, *Shigella* spp. and *V. cholerae*.

Radha (2004) investigated antimicrobial activity of 6 species of lichens and 3 higher plants against 10 bacteria and one fungus. Gram positive organisms were most sensitive towards the ethanolic and methanolic suspensions of plant extracts whereas *C. albicans* and *E. coli* were found resistant.

In the study of antibacterial activity of 16 different medicinal plants against 14 bacteria, 12 plants (75%) showed activity against at least six or more test bacteria and 4 plants (25%) were active against 3 or less than 3 bacteria. Clove was the most active plant and *Acorus calamus* was the least effective against the test organisms (Thapa, 2006).

Bajaracharya (2007) screened and evaluated 8 different medicinal plants for their antimicrobial activity against the enteric bacteria isolated from water on the basis of their common use among the different ethenic groups for common disorder. He found that ethanolic extract of *Punica granatum, Woodfordia fruticosa, Psidium guajava, Syzygium cumini* were effective.

3.7.3. Agar diffusion test

For the preliminary test of the antimicrobial property of an essential oil and other fraction, agar diffusion test can be applied. This method was originally determined by

Dingle *et al.* (1953) for evaluation of enzymatic activity for degradation of pectin and other polysaccharides, which was modified for evaluating the antimicrobial activity of antimicrobials. In this test, the inoculated agar medium was made a well with a sterile cork borer. The antimicrobials diffuse in the media inhibiting the bacteria around the well depending on the concentration of the antimicrobials. Large clear zone of inhibition indicate most effective drugs where as no zone of inhibition indicates resistance to the drug.

3.7.4. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC refers to the minimum concentration of an antimicrobial agent that inhibits growth of the microorganism under test. MBC refers to the lowest concentration required to kill the microorganism. MIC and MBC are recorded in mg/L or µg/ml (Denyer *et al.*, 2004).

MIC and MBC can be determined my two fold broth dilution method or serial dilution method ad described by Baron *et al.* (1994). MIC gives the quantitative measure of the antimicrobial efficiency.

The concentration of the antimicrobial agent is lowered by two fold by using medium that supports the growth of the microorganism. A series of dilution are made usually ten dilution are prepared. After sufficient incubation, the tubes are observed for the turbidity. MIC is determined by comparing the turbidity of the tubes with that of the positive and negative control. The lowest concentration of the agent that inhibits growth of the organism, as detected by lack of the visual turbidity, is designated as MIC. The MIC value depends on different variables: innoculum size, rate of growth of the bacteria, the incubation period, the nature of the medium used, the stability of the antimicrobial agent and the solvent that is used to dissolve the agent.

MBC can be determined by subculturing the content of tubes on the agar surface. The concentration at which there is no growth is designed as MBC.

3.7.5. Antibiotic susceptibility pattern of the test bacteria

The bacteria used in the study are subjected to antibiotic susceptibility test to determine their sensitivity pattern by disc diffusion method of Modified Kirby-Bauer method.

3.7.6. Short description of bacteria involved in this study with their pathogenicity

3.7.6.1. *Staphylococcus aureus*

S. aureus are Gram positive cocci, nonsporing, nonmotile, usually non capsulated, aerobic and normally facultatively anaerobic cocci, characteristically arranged in clusters. They are catalase positive, oxidase negative, deoxyribonuclease positive, coagulase positive urease positive. They can grow readily on ordinary culture media under aerobic or anaerobic condition at temperature range of 10-42ºC, optimal temperature being 37ºC. In nutrient agar (NA), the colonies are large (2-4mm in diameter), round, smooth, raised shiny, opaque and are often pigmented (deep golden yellow, creamy, orange or yellow). In blood agar (BA), they produce beta haemolysis. Mannitol salt agar (MSA) is selective media. They can tolerate 5-10% of sodium chloride, Lithium chloride, tellurite and polymyxin. In Maconkey agar (MA), they form pink colonies (Chakraborty, 2000).

Pathogenicity

S. aureus contain different virulence facters such as exotoxin, leukocidin, exfoliatin, haemolycin, protein A, enzymes like coagulase, phosphatase, and deoxyribonuclease. They cause infection as;

Primary infection:-

Superficial infection like folliculitis, furuncles (boils), carboncles and impetigo

Deep infection like abscess, wound infection, pneumonia, osteomyelitis and septicaemia

Secondary infection:

Eczema and decubitus ulcers

Toxin mediated disease like food poisoning, toxic shock syndrome and scalded skin syndrome (Noble, 1998)

3.7.6.2. *Escherichia coli*

E. coli is a Gram negative, nonsporing, aerobic or facultative anaerobes, noncapsulated rod. They are generally motile. Some strains contain fimbriae and polysaccharide capsule. They are catalase positive, oxydase negative, indole positive, methyl red (MR) positive, Voges-Proskauer (VP) negative, citrate negative and urease negative. They produce gas from sugar fermentation.

Pathogenicity

E. coli have different virulence factor such as adhesive factor like AFA-I, AFA-II and surface O-antigen, enterotoxin (heat labile and heat stable), enterohaemorrhagic (verotoxin producing) *E.coli* and antiphagocytic capsule.

They cause infection as;

Urinary tract infection

Gastrointestinal disease caused by enterotoxic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC) and enteroinvassive *E. coli* (ETIC).

Septicaemic and pyogenic infection (Chakraborty, 2000; Cheesbrough, 1993)

3.7.6.3. *Salmonella* Typhi and *S.* Paratyphi A

S. Typhi and *S*. Paratyphi A are Gram negative rods, noncapsulated, nonsporing, usually motile by peritrichous flagella, primarily intestinal parasite. They are facultative anaerobes, oxidase negative, catalase positive, indole and VP negative; MR and citrate positive. *S.* Typhi ferment glucose, mannitol, maltose, and dextrin with the production of acid and no gas but *S.* Paratyphi A do so with the production of gas. They grow on ordinary culture media and in MA and deoxycholate citrate agar (DCA) and produce small, circular, translucent, colorless, nonlactose fermenting colonies. In Wilson and Blair bismuth sulphite medium, the colonies of *S.* Typhi are jet black with metallic sheen due to formation of hydrogen sulphide. *S.* Paratyphi A does not produce hydrogen sulphide.

Pathogenicity

S. Typhi and *S.* Paratyphi A posses different virulence factor such as endotoxin, invasions, Vi antigen and factor involved in resistance to phagocytosis like catalase, superoxide dismutase.

They cause infection:

Enteric fever (Typhoid fever and paratyphoid fever)

Septicaemia

Nephrotyphoid in those with urinary schistosomiasis

Osteomyelitis

Abscesses of the spleen and elsewhere

Meningitis and rarely pneumonia and endocarditis (Chakraborty, 2000; Cheesbrough, 1993)

3.7.6.4. *Pseudomonas aeruginosa*

P. aeruginosa are strict aerobes, nonsporing, noncapsulated, Gram negative bacillus. It is motile due to presence of one polar flagellum. Most strains are fimbriate. It is noncapsulated, but some strains produce an alginate capsule. They are catalase and oxidase positive, indole, MR, VP negative. All strains can utilize citrate and donot produce hydrogen sulphide. They are primarily aerobic but can grow anaerobically. *P. aeruginosa* can grow on NA, MA, BA and chocolate agar. It produces pigment such as pyocyanin, fluorescein and pyoverdin.

Pathogenicity

P. aeruginosa possess virurence factor such as alginate capsule, pilin and nonpilus adhesions, haemolysin, elastase, pyocyanin, endotoxin, and exotoxin.

They cause infection as

Urinary tract infection

Skin infection especially at burn sites, wounds, pressure sores, and ulcers, often as a secondary invader.

Respiratory infections, especially in patients with cystic fibrosis or conditions that cause immunosuppession

External ear infection (otitis externa)

Eye infections (usually hospital-acquired)

Septicaemia especially in persons already in poor health (Chakraborty, 2000; Cheesbrough, 1993)

3.7.6.5. *Klebsialla pneumoniae* and *K. oxytoca*

Klebsiella spp. are Gram negative, non-motile and usually capsulated (polysaccharide). They ferment sugars like glucose, lactose, sucrose, mannitol) with production of acid and gas and split by means of usease. *K. pneumoniae* is indole and MR negative; VP and citrate positive where as *K. Oxytoca* is indole and citrate positive; MR and VP variable. Capsular material is produced in greater amount in media containing a relative excess of carbohydrate. When much capsular material is produced, the growth on agar is luxuriant, grayish-white, mucoid and almost diffluent.

Pathogenicity

They posses several virulence factor, including endotoxin, capsule, adhesion proteins, and resistance to multiple antimicrobial agents. *K. pnuemoniae* is more pathogenic.

They cause infection as

Pneumonia

Urinary tract infection

Septicemia, wound infection and

Endemic diarrhea (Forbes *et al*., 2002; Chakraborty, 2000; Cheesbrough, 1993)

3.7.6.6. *Proteus mirabilis* and *P. vulgaris*

They are Gram negative actively motile, noncapsulated aerobic bacilli. They produce urease. *P. mirabilis* is indole negative where as *P. vulgaris* is indole positive. They are MR, citrate positive; VP negative; oxidase negative. Both produce hydrogen sulphide. They grow in ordinary media and produce characteristic putrefactive (fishy of seminal) odour. The colonies spread or swarm over the surface of NA and BA. They form pale or colorless colonies in MA and DCA medium and do not swarm on the surface of the medium.

Pathogenicity

P. mirabilis is medically important and *P. vulgaris* cause infection occasionally.

They cause infection such as

Urinary tract infection

Abdominal and wound infection

Septicaemia, and occasionally meningitis and chest infection

Acute otitis media (Chakraborty, 2000; Cheesbrough, 1993)

3.7.6.7. *Shigella dysenteriae*

S. dysenteriae are Gram negative, aerobic, nonmotile, nonflagellate and noncaplated bacilli. they are catalase positive axcept *S. dysenteriae* type 1; produce indole; and can utilize citrate. They ferment glucose with the production of acid only. They grow in ordinary medium like NA, in which the colonies are about 2mm in diameter, circular, convex, smooth and translucent. MA and Deoxycholate Citrate Agar (DCA) media are used to isolate from faeces, in which the colonies are colourless. In Shigella-Salmonella agar (SS agar), the colonies are colorless.

Pathogenicity

S. dysenteriae posses virulence factor such as invasion plasmid antigens, intracellular spread protein and shega toxin.

They cause bacillary dysentery (shigellosis) (Cheesbrough, 1993, Chakraborty, 2000)

CHAPTER IV

4. MATERIALS AND METHODS

4.1. Materials

All the materials used to accomplish this study are given in the appendix I

4.2. Method

This study was conducted from October, 2007 to July, 2008, partly in Department of Plant Resource (DPR), Thapathali and partly in Central Department of Microbiology, TU. The extraction part of the study was carried out in DPR and antibacterial activity of the spices was carried out in laboratory of Central Department of Microbiology.

During the period, six different types of spice-sample were processed for the extraction of essential oils and other extracts. These extracts were tested against Gram positive and Gram negative bacteria and MBC were determined for those showing zone of inhibition.

4.2.1. Sample collection

The spices used for the study were clove, black pepper, coriander, cinnamon, ajowan and turmeric. Spices of best quality were brought in market in packaged form. These spices were identified and confirmed in Department of Plants Resources (DPR), Thapathali.

4.2.2. Sample processing

The samples were grinded in a grinder. The powdered spices were used for extraction of essential oils and other extracts successively.

4.3. Extraction of spices material

4.3.1. Extraction of essential oils

Essential oils from spice were extracted by hydro-distillation method using Clevenger apparatus. It is of two types depending on the densities of oils:

(i) For oils with densities near or less than that of water, and

(ii) For oils with densities greater than that of water

The apparatus was designed by Sage and Fleck (1934).

Procedure

100 gm of grinded spice was transferred to one liter round bottomed flask and distilled water was filled to half. Few glass beads and a drop of antifoam was added. The trap and condenser were set up with the help of the stands. The flask was heated with heating mantle and boiled moderately briskly for 6 hrs or more. The water was drained off and the oil was collected on a small well capped bottle. The oil was dried and made moisture free by adding anhydrous sodium sulphate. The dried oil was labeled and kept in dark and cool place at low temperature.

4.3.2. Soxhlet extraction with acetone and methanol

After extraction of essential oils, spices powered were dried at 45ºC and subjected to continuous extraction with solvent like acetone and methanol, by using Soxhlet extractor to obtain crude extracts, acetone extract and methanol extract respectively.

Procedure

Dried spices powder was loaded in a clean and dried thimble of soxhlet extractor. It was then fitted with clean and dried one liter round bottom flask, containing about 400 ml of solvent and glass beads. The condenser was fitted up on upper part with the help of stand. The flask was constantly heated with heating mantle. The solvent vapor, condensed by condenser, dropped on the powder of spices and was soaked it. The solvent soluble compounds were eluted in the conical flask through siphon tube (Tiwari *et al*., 1992). The process was allowed to run for 8-15 hrs or till the color solvent appeared in the siphon.

Removal of solvent

After complete extraction, the solvent was removed with the help of rotary vacuum evaporator. The extract was transferred to the clean and pre-weighed round bottom flask of evaporator. The flask was constantly heated in rotating condition by using water bath below 55ºC. The solvent was removed fast due to negative pressure. After complete removable of solvent, the flask containing extract was weighed and result was noted. To find out the exact weight of the extract yield, weight of the flask without extract was subtracted from the weight of flask with extract.

The crude extract of spices was transferred to a bottle. It was labeled and store in a refrigerator.

4.4. Preparation of stock/working solution

The stock solution of essential oil was made in 2% Tween80 in physiological saline (0.85gm of sodium chloride dissolved in 100 ml water, sterilized by autoclaving). 1gm of an essential oil was dissolved in 10ml of 2% Tween80.
The crude extract was dissolve in dimethyl sulphoxide (DMSO) (10-40%) by dissolving 1gm extract in 10ml of it. The concentration of the stock solution becomes 100mg/ml. The solutions were stored in refrigerator.

4.5. Collection of bacterial cultures

Ten different human pathogenic bacteria were selected for the study. The cultures were collected from Department of Microbiology, TUTH, and Central Public Health Laboratory, Teku. After obtaining the culture they were streaked in nutrient agar plates and incubated at 37ºC for 24 hours. Gram staining of the isolated colonies was performed. The organisms were grown on their selective media and biochemical tests were performed. Thus the organism were tested for their purity and confirmed by their morphological, cultural, and biochemical characteristics. The organisms included in this study were:

i. *Escherchia coli* ATCC 25922

ii. *Staphylococcus aureus* ATCC 25923

iii. *Klebsiella pneumoniae* ATCC 200603

iv. *K. oxytoca*

v. *Salmonella* Typhi

vi. *S.* Paratyphi A

vii. *Pseudomonas aeruginosa* ATCC 27853

viii. *Proteus mirabilus* ATCC 49132

ix. *P. vulgaris*

x. *Shigella dysenteriae*

The bacterial cultures were maintained on nutrient agar slant. The organisms were sub- cultured every two weeks.

4.6. Preparation of standard culture inoculums

Three or four colonies of bacteria were transferred to the test tube containing 5ml of sterile nutrient broth. It was incubated at 37ºC for 3 or 4 hrs. The tubes were compared with McFarland Nephelometer Standard 0.5 (turbidity standard) recommended by WHO (1991). A blank nutrient broth was used as a control.

4.7. Preparation of media

The media used in the study were prepared according to the manufacturer's recommendation. The detailed procedure for preparation of media is given in the appendix II

4.8. Screening and evaluation of antimicrobial activity

In this study, screening and evaluation of antimicrobial activity was performed agar well diffusion method and two fold dilution method. Agar well diffusion method, given by Dingle *et al.* (1953) was performed to screen the antimicrobial activity (determination of the zone of inhibition) while two fold dilution method was performed for the determination of the minimal inhibition concentration (MIC) and minimal bactericidal concentration (MBC)

4.8.1. Determination of zone of inhibition (ZOI)

Zone of inhibition was determined by agar well diffusion method as given by Dingle *et al.* (1953) which was performed as:

- Sterile Muller-Hinton Agar (MHA) plates of approximately 4mm thickness were prepared.
- Plates were dried under hot air oven to remove excess of moisture from surface of the media.
- The fresh inoculum, comparable with McFarland standard 0.5, was prepared as in section 4.6
- Sterile cotton swab was dipped into the prepared inoculum and rotated and pressed against the upper inside wall of the tube above the liquid level and swabbed carefully all over the surface of the plate three times rotating the plate through an angle 60º after each application. Finally, the swab was passed round the edge of the agar surface. The inoculated plate was left to dry for a few minutes at room temperature with the lid closed (WHO, 1991).
- Then with the help of sterile cork borer (no. 9 and no. 6), wells were made in the inoculated plate and labeled properly. 100µl and 50µl of the working suspensions of the spices extract were dispensed in the respective wells with the helps of the micropipette. The solvent itself was tested for its activity as a control as the same time.
- The plates were left for sometime with the lid closed for proper diffusion.
- Plates were incubated ad 37ºC for 18-24 hrs.
- After incubation (18-24 hrs) the plates were observed for the zone of inhibition (ZOI), which is suggested by clear area without growth of bacteria around the well. In case, if there were any clear zone then triplicate assays were performed. The ZOI were measured using scale and mean was recorded.

4.8.2. Determination of minimum bactericidal concentration (MBC)

The bacterial isolates, which were inhibited by spices extract, were subjected to broth dilution methods of Baron *et al*., (1994) to determine MBC. Media used was nutrient

broth (NB). Inoculum was prepared as given in section 4.6. It was performed as follows:

- A set of 12 screw capped test tube containing 1ml nutrient broth for each bacteria were taken. The test tubes were labeled as positive control, negative control and tube no. 1 to 10.
- In case of negative control, the nutrient broth was discarded. Then 1ml of crude extract (working suspension) of the particular spices was taken aseptically in negative control tube and tube no. 1.
- Tube no. 1 contains 1ml NB and 1ml extract. After complete homogenization, 1ml of it was transferred aseptically to tube no. 2.
- Similarly, after complete homogenization of the content in tube no. 2, 1ml of it was transferred to tube no. 3. Likewise, two fold dilutions were prepared up to the tube no. 10. From tube no. 10, 1 ml of the content was discarded. Hence all tubes contain equal volume i.e. 1ml with the concentration decreased by two fold. The table is given in appendix V
- With the help of micropipette, 50µl of inoculum (turbidity matched to McFarland standard 0.5) was added in each tube except negative control tube.
- All the tubes were incubated at 37ºC for 24 hrs.
- After incubation, all the tubes were subcultured in nutrient agar (NA) with proper labeling and incubated at 37ºC for 24 hrs, whereas for *Proteus* species, the tubes were subcultured in MacConkey agar.
- Then the plates were examined for the growth of bacteria and MIC and MBC were determined. The concentration at which there was no growth was taken as MBC and preceding concentration could be taken as MIC.

4.9. Antibiotic susceptibility test

The bacteria used in the study are subjected to antibiotic susceptibility test to determine their sensitivity pattern by disc diffusion method of Modified Kirby-Bauer method. The culture used were *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Salmonella* Typhi , *S.* Paratyphi A, *Pseudomonas aeruginosa*

(ATCC 27853), *Klebsialla pneumoniae* (ATCC 200603), *K. oxytoca, Proteus mirabilis* (ATCC 49132), *P. vulgaris, and Shigella dysenteriae.*

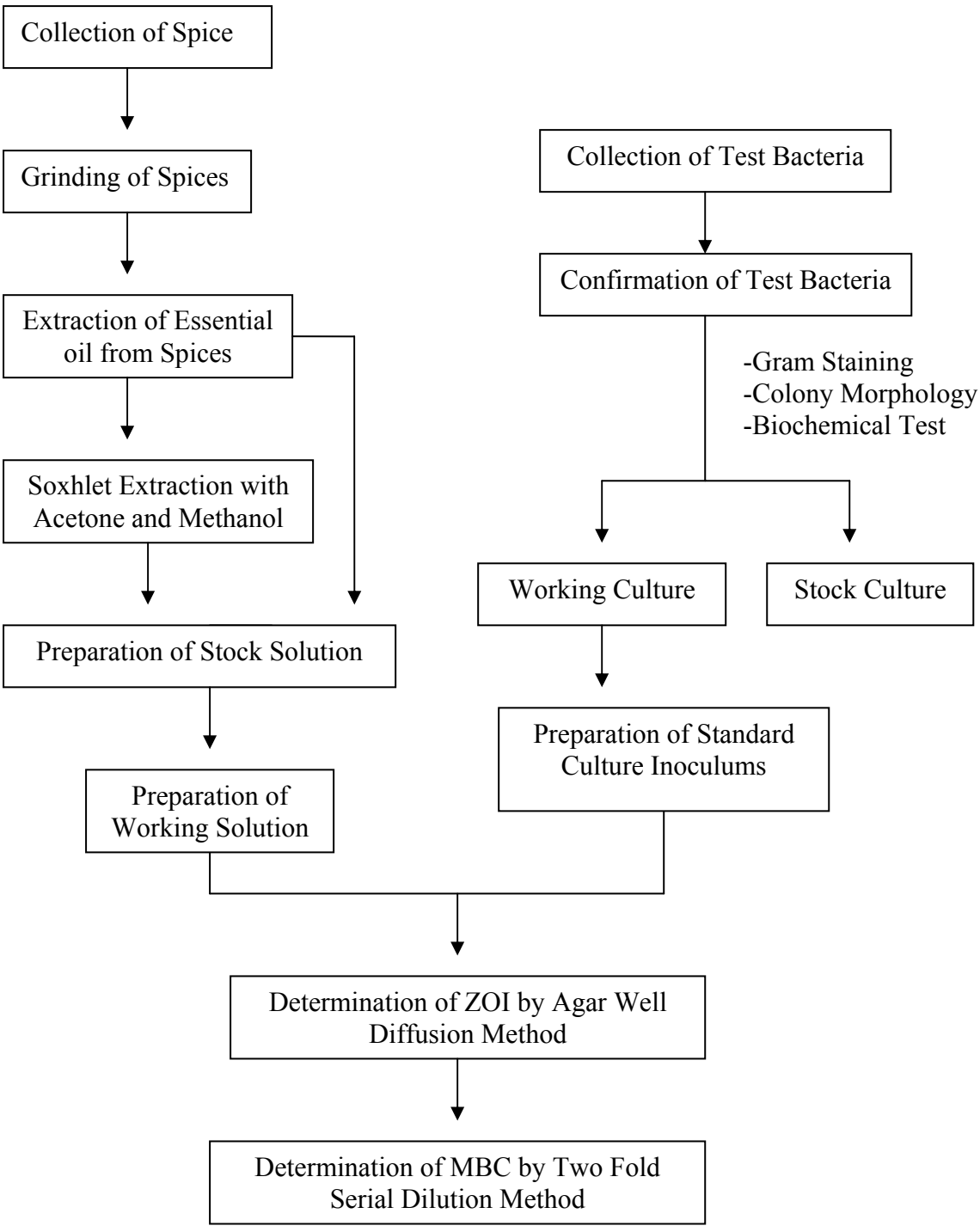
The antibiotic discs were selected according to the type of bacteria. The antibiotic discs used were penicillin G (10mcg), chloramphenicol (30mcg), amikacin (30mcg), ampicillin (10mcg), erythomycin (10mcg), cotrimoxazole (25mcg), gentamycin (10mcg), ofloxacin (5mcg), norfloxacin (10mcg), ciprofloxacin (5mcg), cephalexin (30mcg), nalidixic Acid (30mcg) and cloxacillin (5mcg).

For the antibiotic susceptibility test, sterile Mueller-Hinton Agar (MHA) plates were prepared as given in section 4.7. The inocolum of bacteria was given in section 4.6.

Sterile cotton swab was dipped into the prepared inoculum and rotated and pressed against the upper inside wall of the tube above the liquid level and swabbed carefully all over the surface of the plate three times rotating the pate through an angle 60º after each application. Finally the swab was passed round the edge of the agar surface. The inoculated plate was left to dry for a few minutes at room temperature with the lid closed (WHO, 1991).

Antibiotic discs were taken out from their respective vials with the help of a sterilized forceps and placed carefully on the plate, at least 15mm away from the edge of the zones of inhibition. The discs were pressed lightly with the forceps to make complete contact with the surface of the medium. All the plates were incubated at 37ºC for 18-24 hrs.

After proper incubation, the diameters of the zone of inhibition of each disc were measured. According to the size of the ZOI, the organism was interpreted as resistant, intermediate or sensitive to the antibiotic as referred to the standard chart of antibiotic susceptibility test given in the appendix IV



Flow Chart of Method of Extraction and Evaluation of Antibacterial Activity

CHAPTER V

5. RESULT

5. 1. Qualitative screening of antibacterial activity

Essential oils of spices were found to have better antibacterial activity against test bacteria followed by acetone fraction. 70% test bacteria were inhibited by clove oil, 90% bacteria were inhibited by ajowan oil and 100 % activity was shown by cinnamon oil. Essential oils of black pepper, turmeric and coriander showed weak inhibitory activity showing 10%, 20% and 20% inhibitory effects respectively. Acetone fraction of clove and cinnamon was found to have better antibacterial activity inhibiting 80% and 90% of bacteria respectively. Methanol fraction of clove was found to have better antibacterial activity inhibiting 60% of the bacteria (Table 1).

Table 1: Qualitative screening of the antibacterial activity of the spices

S. No.	Spices	Extracts	Organisms									
			Escherchia coli (ATCC 25922)	*S. aureus* (ATCC 25923)	*K. pneumoniae* (ATTC 200603)	*K. oxytoca*	*Salmonella* Typhi	*S.* Paratyphi A	*P. aerugi-nosa* (ATCC 27853)	*Proteus mirabilis* ATCC49132	*P. vulgaris*	*Shigella dysenteriae*
1.	Clove	E.O	+	+	+	+	+	-	+	-	-	-
		Ae.	+	+	+	-	-	+	+	+	+	+
		Me.	-	+	-	-	-	+	+	+	+	+
2.	Black Pepper	E.O	-	+	-	-	-	-	-	-	-	-
		Ae.	+	+	-	-	-	-	-	-	-	-
		Me	-	+	-	-	-	-	-	-	-	-
3.	Turmeric	E.O	+	+	-	-	-	-	-	-	-	-
		Ae.	-	+	-	-	-	-	-	-	-	-
		Me.	-	-	-	-	-	-	-	-	-	-
4.	Ajowan	E.O	+	+	+	+	+	+	-	+	+	+
		Ae.	-	-	-	-	-	-	-	-	-	-
		Me.	-	-	-	-	-	-	-	-	-	-
5.	Coriander	E.O	+	+	-	-	-	-	-	-	-	+
		Ae.	-	-	-	-	-	-	-	-	-	-
		Me.	-	-	-	-	-	-	-	-	-	-
6.	Cinnamon	E.O	+	+	+	+	+	+	+	+	+	+
		Ae.	+	+	+	+	+	+	-	+	+	+
		Me.	-	+	-	-	-	-	-	-	-	-

Note: E.O =Essential oils; Ae. =Acetone fraction; Me. =Methanol fraction
(+) indicates antibacterial activity and (-) indicates lack of antibacterial activity

5.2. Evaluation of antimicrobial activity

The diameter of the ZOI given by extracts against the test bacteria was measured for the estimation of the potency of the particular extract. Three reading of the diameter of ZOI were taken and their mean were tabulated in the result tables. No ZOI or very small zones, smaller than 12mm in case of 9mm diameter well and 9mm in case of 6mm diameter well were conferred as negative (-) in the tables.

5.2.1. Antibacterial activity of different fraction of Clove against tested bacteria

Table 2: Mean Diameter of Zone of Inhibition (ZOI) given by different fraction of Clove

S. No.	Organisms	Zone of Inhibition(mm)					
		Essential oils		Acetone extract		Methanol extract	
		50µl	100µl	50µl	100µl	50µl	100µl
1.	*E. coli* (ATCC 25922)	14	17	12	17	-	-
2.	*S. aureus* (ATCC 25923)	13	16	13	16	13	17
3.	*K. pneumoniae* (ATCC 200603)	13	17	13	17	-	-
4.	*K. oxytoca*	13	18	-	-	-	-
5.	*S.* Typhi	14	18	-	-	-	-
6.	*S.* Paratyphi A	-	-	12	17	12	18
7.	*P. aeruginosa* (ATCC 27853)	12	17	-	15	12	17
8.	*P. mirabilus* (ATCC 49132)	-	-	-	14	11	15
9.	*P. vulgaris*	-	-	-	-	11	16
10.	*S. dysenteriae*	-	-	-	13	15	22

Note :- (-) indicates no ZOI

Almost all fractions of clove were active against tested bacteria. Essential oil showed higher activity against *E. coli, K. oxytoca*, and *S.* Typhi and lowest activity against *S. aureus.* Acetone fraction was active against seven bacteria with higher activity against *E. coli, K. pneumoniae,* and *S.* Paratyphi A and lowest acivity against *S. dysenteriae.* Methanol fraction showed highest activity against *S. dysenteriae* and lowest activity against *P. mirabilus* (Table 2).

5.2.2. Antibacterial activity of different fraction of Black Pepper against tested bacteria

Table 3: Mean Diameter of Zone of Inhibition (ZOI) given by different fraction of Black pepper

S. No.	Organisms	Zone of Inhibition(mm)					
		Essential oils		Acetone extract		Methanol extract	
		50µl	100µl	50µl	100µl	50µl	100µl
1.	*E. coli* (ATCC 25922)	-	-	12	17	-	-
2.	*S. aureus* (ATCC 25923)	-	14	-	13	-	13
3.	*K. pneumoniae* (ATCC 200603)	-	-	-	-	-	-
4.	*K. oxytoca*	-	-	-	-	-	-
5.	*S.* Typhi	-	-	-	-	-	-
6.	*S.* Paratyphi A	-	-	-	-	-	-
8.	*P. aeruginosa* (ATCC 27853)	-	-	-	-	-	-
8.	*P. mirabilus* (ATCC 49132)	-	-	-	-	-	-
9.	*P. vulgaris*	-	-	-	-	-	-
10.	*S. dysenteriae*	-	-	-	-	-	-

Note :- (-) indicates no ZOI

Black pepper was found to have weak inhibitory activity at the tested concentration. Its essential oil was active against only one bacteria i.e. *S. aureus* with small ZOI. Acetone fraction was active against *E. coli* and *S. aureus* with higher antibacterial activity against *E. coli.* Methanol fraction showed inhibitory activity against *S. aureus* only (Table 3).

5.2.3. Antibacterial activity of different fraction of Turmeric against tested bacteria

Table 4: Mean Diameter of Zone of Inhibition (ZOI) given by different fraction of Turmeric

S. No.	Organisms	Zone of Inhibition(mm)					
		Essential oils		Acetone extract		Methanol extract	
		50µl	100µl	50µl	100µl	50µl	100µl
1.	*E. coli* (ATCC 25922)	10	15	-	-	-	-
2.	*S. aureus* (ATCC 25923)	-	14	-	13	-	-
3.	*K. pneumoniae* (ATCC 200603)	-	-	-	-	-	-
4.	*K. oxytoca*	-	-	-	-	-	-
5.	*S.* Typhi	-	-	-	-	-	-
6.	*S.* Paratyphi A	-	-	-	-	-	-
7.	*P. aeruginosa* (ATCC 27853)	-	-	-	-	-	-
8.	*P. mirabilus* (ATCC 49132)	-	-	-	-	-	-
9.	*P. vulgaris*	-	-	-	-	-	-
10.	*S. dysenteriae*	-	-	-	-	-	-

Note :- (-) indicates no ZOI

Turmeric was found to have weak inhibitory activity at tested concentration. Its essential oil showed activity against *E. coli*, and *S. aureus.* Acetone fraction was active against *S. aureus* only. Methanol fraction did show any inhibitory activity at tested concentration (100mg/ml) (Table 4).

5.2.4. Antibacterial activity of different fraction of Ajowan against tested bacteria

Table 5: Mean Diameter of Zone of Inhibition (ZOI) given by different fraction of Ajowan

S. No.	Organisms	Zone of Inhibition(mm)					
		Essential oils		Acetone extract		Methanol extract	
		50µl	100µl	50µl	100µl	50µl	100µl
1.	*E. coli* (ATCC 25922)	15	22	-	-	-	-
2.	*S. aureus* (ATCC 25923)	14	22	-	14	-	-
3.	*K. pneumoniae* (ATCC 200603)	-	15	-	-	-	-
4.	*K. oxytoca*	15	22	-	-	-	-
5.	*S.* Typhi	15	23	-	-	-	-
6.	*S.* Paratyphi A	-	15	-	-	-	-
7.	*P. aeruginosa* (ATCC 27853)	-	-	-	-	-	-
8.	*P. mirabilus* (ATCC 49132)	14	20	-	-	-	-
9.	*P. vulgaris*	15	21	-	-	-	-
10.	*S. dysenteriae*	11	19	-	-	-	-

Note :- (-) indicates no ZOI

Essential oils of Ajowan was found active against almost all bacteria except *P. aeruginosa,* where as other fraction were found to be inactive at the tested concentration. Essential oil showed higher antibacterial activity against *S.* Typhi and smaller antibacterial activity against *K. pneumoniae.*

5.2.5. Antibacterial activity of different fraction of Coriander against tested bacteria

Table 6: Mean Diameter of Zone of Inhibition (ZOI) given by different fraction of Coriander

S. No.	Organisms	Zone of Inhibition(mm)					
		Essential oils		Acetone extract		Methanol extract	
		50µl	100µl	50µl	100µl	50µl	100µl
1.	*E. coli* (ATCC 25922)	11	15	-	-	-	-
2.	*S. aureus* (ATCC 25923)	20	28	-	-	-	-
3.	*K. pneumoniae* (ATCC 200603)		-	-	-	-	-
4.	*K. oxytoca*	-	-	-	-	-	-
5.	*S.* Typhi	-	-	-	-	-	-
6.	*S.* Paratyphi A	-	-	-	-	-	-
7.	*P. aeruginosa* (ATCC 27853)	-	-	-	-	-	-
8.	*P. mirabilus* (ATCC 49132)	-	-	-	-	-	-
9.	*P. vulgaris*	-	-	-	-	-	-
10.	*S. dysenteriae*	-	15	-	-	-	-

Note :- (-) indicates no ZOI

Coriander was found to have weak inhibitory activity at the tested concentration. Essential oil showed antibacterial activity against three bacteria which include *E. coli*, *S. aureus*, and *S. dysenteriae.*

It showed higher antibacterial activity against *S. aureus* and similar activity was given against other bacteria. Acetone and methanol fractions showed no activity at tested concentration.

5.2.6. Antibacterial activity of different fraction of Cinnamon against tested bacteria

Table 7: Mean Diameter of Zone of Inhibition (ZOI) given by different fraction of Cinnamon

S. No.	Organisms	Zone of Inhibition(mm)					
		Essential oils		Acetone extract		Methanol extract	
		50µl	100µl	50µl	100µl	50µl	100µl
1.	*E. coli* (ATCC 25922)	20	26	10	15	-	-
2.	*S. aureus* (ATCC 25923)	29	35	13	19	10	15
3.	*K. pneumoniae* (ATCC 200603)	17	24	-	13	-	-
4.	*K. oxytoca*	21	27	-	14	-	-
5.	*S.* Typhi	22	32	12	17	-	-
6.	*S.* Paratyphi A	22	30	11	16	-	-
7.	*P. aeruginosa* (ATCC 27853)	18	25	-	-	-	-
8.	*P. mirabilus* (ATCC 49132)	20	28	10	16	-	-
9.	*P. vulgaris*	21	28	12	15	-	-
10.	*S. dysenteriae*	25	37	12	19	-	-

Note :- (-) indicates no ZOI

Cinnamon was found to show better antibacterial activity among the studied spices. Its essential oil was active against all bacteria at the tested concentration. It gave the higher antibacterial activity against *S. dysenteriae* which was 37mm in diameter and the smaller antibacterial activity was 24mm in diameter against *K. pneumoniae.* Acetone fraction did not show antibacterial activity against *P. aeruginosa.* However, higher activity against *S. aureus* and *S. dysenteriae* shown by acetone fraction of cinnamon. Methanol fraction showed antibacterial activity against only one bacteria i.e. *S. aureus.*

5.3. MBC of spice extract

In case of the essential oil and other fraction, that showed antibacterial activity against the tested bacteria, minimum concentration required to inhibit or kill were determined and tabulated in Table No. 8 and 9. The MBC value ranges from 0.39mg/ml to 25mg/ml. The Lowest concentration at which bacteria was completely killed was given by essential oil of cinnamon (0.39mg/ml).

Table 8: Minimum Bactericidal Concentration (MBC) of Different Fraction of Clove, Black pepper and Turmeric (mg/ml)

S. No	Name of Organism	Clove			Black Pepper			Turmeric		
		E.O	Ac	Me	E.O	Ac	Me	E.O	Ac	Me
1.	*E. coli* (ATCC25922)	6.25	6.25	-	-	6.25	-	25	-	-
2.	*S. aureus* (ATCC25923)	3.13	25	12.5	25	25	25	25	25	-
3.	*K. pneumoniae* (ATCC 200603)	3.13	25	-	-	-	-	-	-	-
4.	*K. oxytoca*	1.56	-	-	-	-	-	-	-	-
5.	*S.* Typhi	1.56	-	-	-	-	-	-	-	-
6.	*S* .Paratyphi A	-	25	6.25	-	-	-	-	-	-
7.	*P. aeruginosa* (ATCC 27853)	12.5	25	25	-	-	-	-	-	-
8.	*P. mirabilis* (ATCC49132)	-	25	25	-	-	-	-	-	-
9.	*P. vulgaris*	-	25	12.5	-	-	-	-	-	-
10.	*S. dysenteriae*	-	25	3.13	-	-	-	-	-	-

Note: - E.O= Essential oil; Ac= Acetone extract; Me= Methanol extract; (-) indicates not done

Table 9: Minimum Bactericidal Concentration (MBC) of Different Fraction of ajowan, coriander and cinnamon (mg/ml)

S. No	Name of Organism	Ajowan			Coriander			Cinnamon		
		E.O	Ac	Me	E.O	Ac	Me	E.O	Ac	Me
1.	*E. coli* (ATCC25922)	1.56	-	-	3.13	-	-	0.39	6.25	-
2.	*S. aureus* (ATCC25923)	1.56	25	-	3.13	-	-	0.39	12.5	25
3.	*K. pneumoniae* (ATCC 200603)	3.13	-	-	-	-	-	0.78	12.5	-
4.	*K. oxytoca*	1.56	-	-	-	-	-	0.78	12.5	-
5.	*S.* Typhi	1.56	-	-	-	-	-	0.39	3.13	-
6.	*S* .Paratyphi A	6.25	-	-	-	-	-	0.78	12.5	-
7.	*P. aeruginosa* (ATCC 27853)	-	-	-	-	-	-	0.78	-	-
8.	*P. mirabilis* (ATCC49132)	1.56	-	-	-	-	-	0.78	12.5	-
9.	*P. vulgaris*	1.56	-	-	-	-	-	0.78	12.5	-
10.	*S. dysenteriae*	1.56	-	-	3.13	-	-	0.78	12.5	-

Note:- E.O= Essential oil; Ac= Acetone extract; Me= Methanol extract; (-) indicates not done

5.4. Antibiotic susceptibility test of bacteria

Table 10: Antibiotic susceptibility test of the tested bacteria

S. No.	Antibiotics	Organisms									
		Escherchia coli (ATCC 25922)	*S. aureus* (ATCC 25923)	*K. pneumoniae* (ATTC 200603)	*K. oxytoca*	*Salmonella* Typhi	S. Paratyphi A	*P. aerugi-nosa* (ATCC 27853)	*Proteus mirabilis* ATCC49132	*P. vulgaris*	*Shigella dysenteriae*
1.	Amikacin	-	-	R	R	-	-	S	-	-	S
2.	Ampicillin	-	-	R	I	S	I	-	-	-	-
3.	Cephalexin	-	S	R	S	-	-	R	-	-	-
4.	Chloramphenicol	S	-	-	-	S	S	R	R	S	I
5.	Ciprofloxacin	S	-	R	R	S	R	S	S	S	R
6.	Cloxacillin	-	R	-	-	-	-	-	-	-	-
7.	Cotrimoxazole	S	S	I	S	-	-	-	I	S	I
8.	Erythromycin	-	S	-	-	-	-	-	-	-	I
9.	Gentamycin	-	-	R	S	-	-	S	-	-	S
10.	Nalidixic Acid	-	-	-	-	R	R	-	-	-	-
11.	Norfloxacin	S	-	-	-	S	S	S	S	S	-
12.	Ofloxacin	S	S	-	-	S	I	-	S	S	-
13.	Penicillin G	R	S	-	-	-	-	-	R	R	-

Note :- (-) indicates not included; S = Susceptible; R = Resistant; I = Intermediate

The ZOI of antibiotics against each bacterium are in the appendix IV

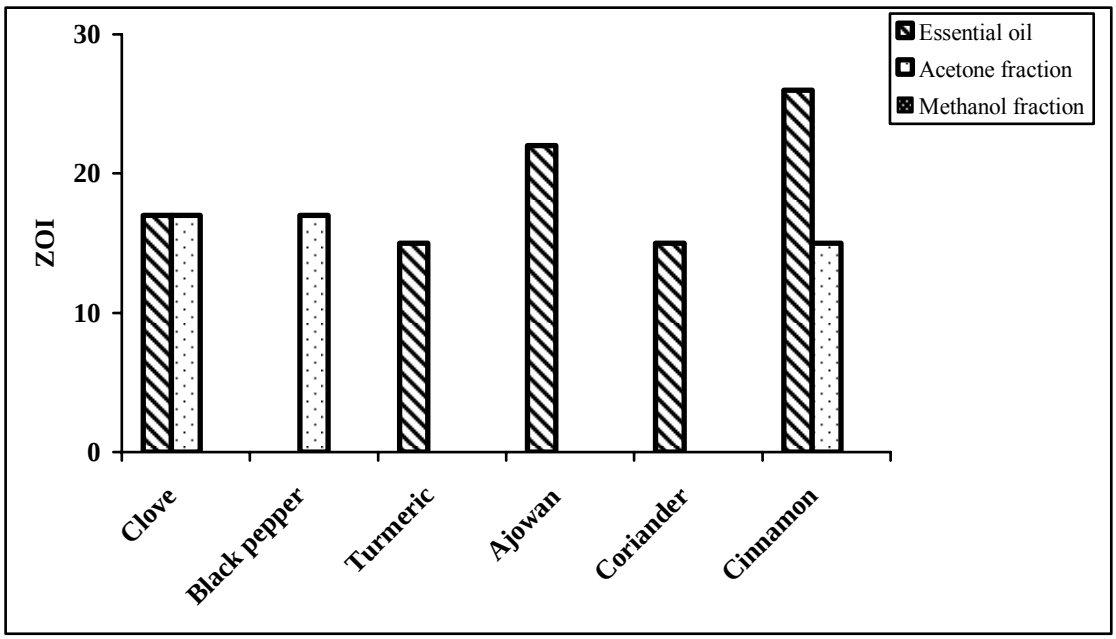


Figure: 1 Zone of Inhibition of *E. coli* (ATCC 25922) exhibited by different spices

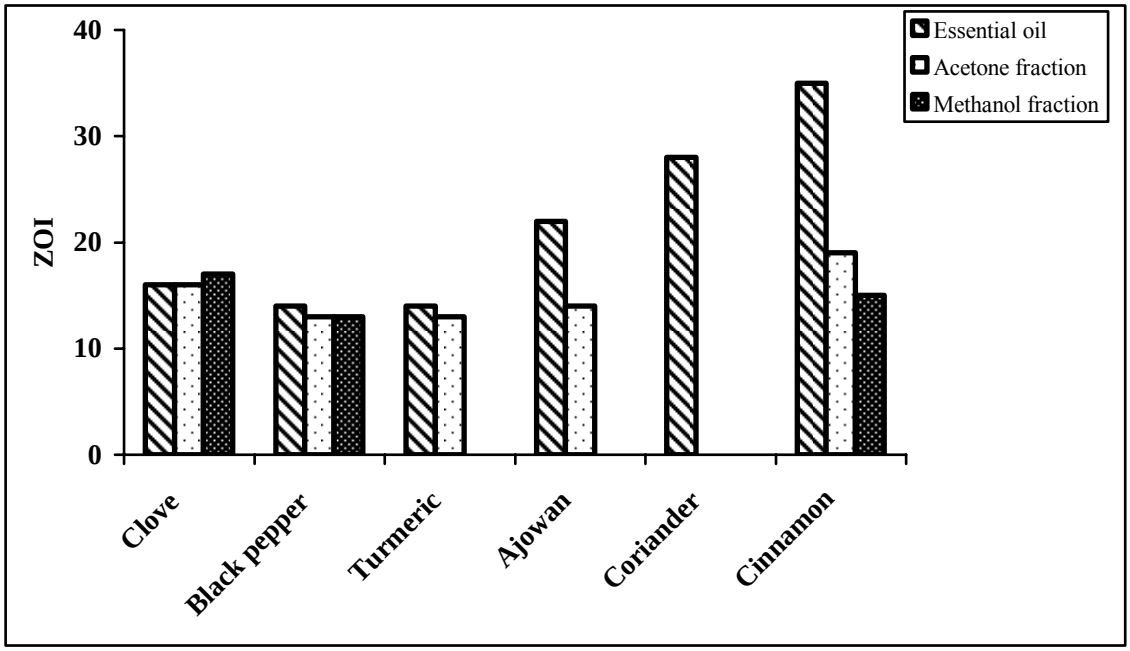


Figure: 2 Zone of Inhibition of *S. aureus* (ATCC 25923) exhibited by different spices

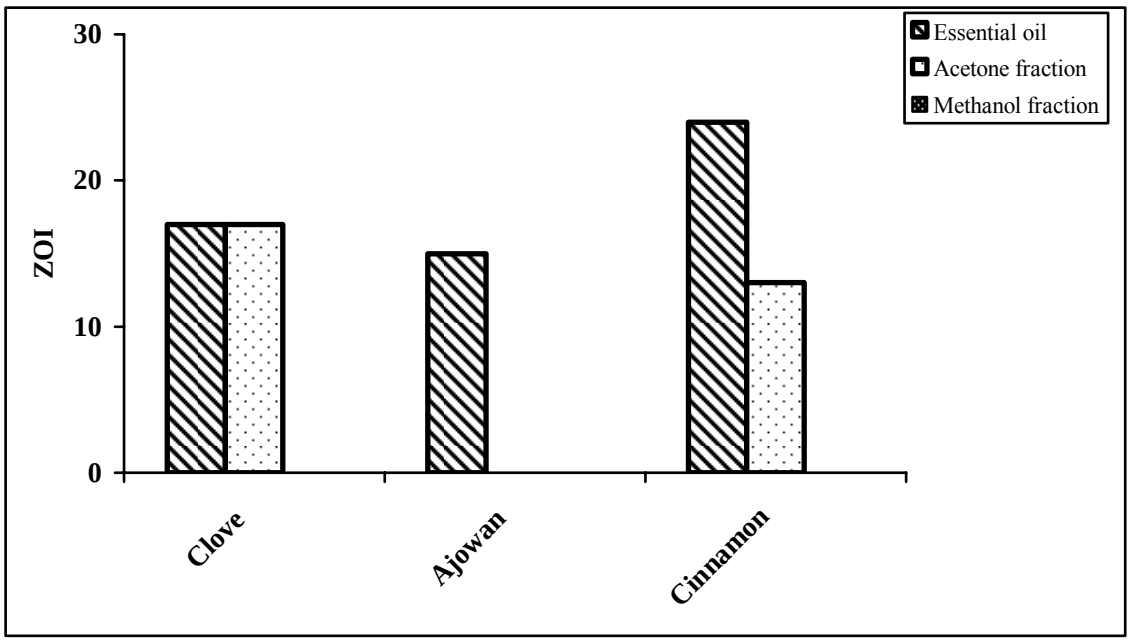


Figure: 3 Zone of Inhibition of *K. pneumoniae* (ATCC 200603) exhibited by different spices

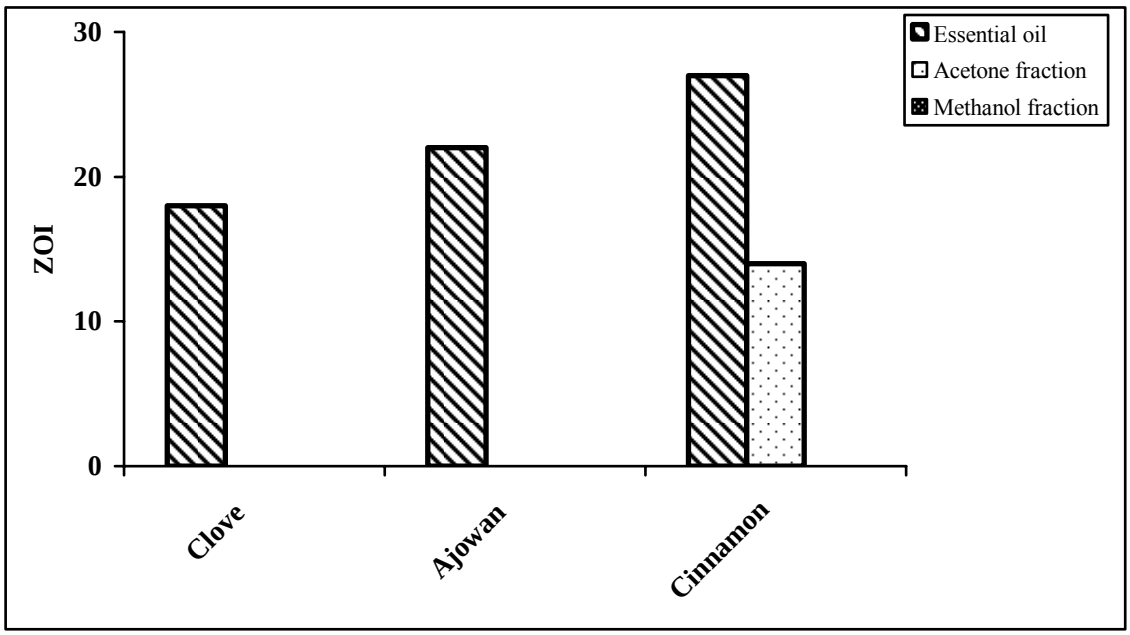


Figure: 4 Zone of Inhibition of *K. oxytoca* exhibited by different spices

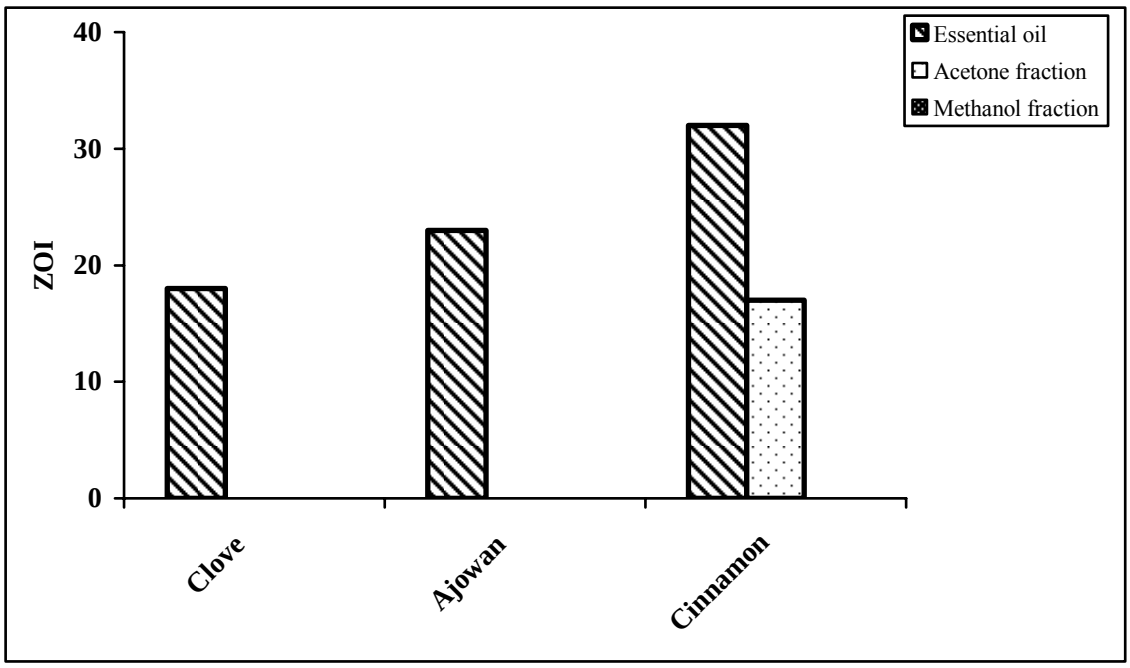


Figure: 5 Zone of Inhibition of *S.* Typhi exhibited by different spices

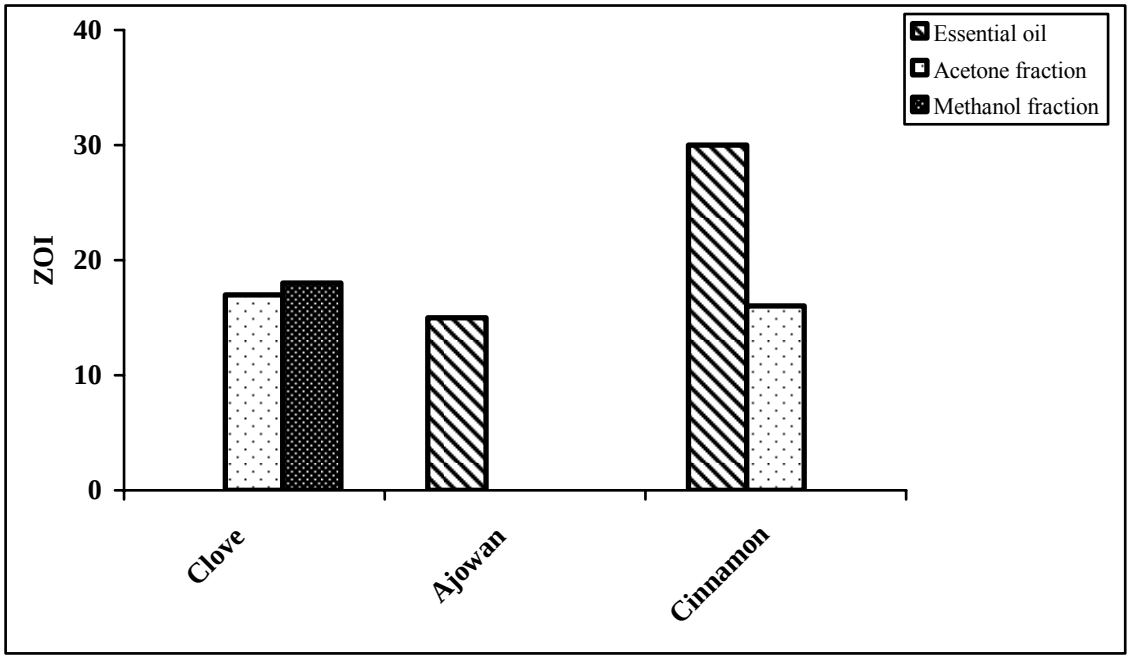


Figure: 6 Zone of Inhibition of *S.* Paratyphi A exhibited by different spices

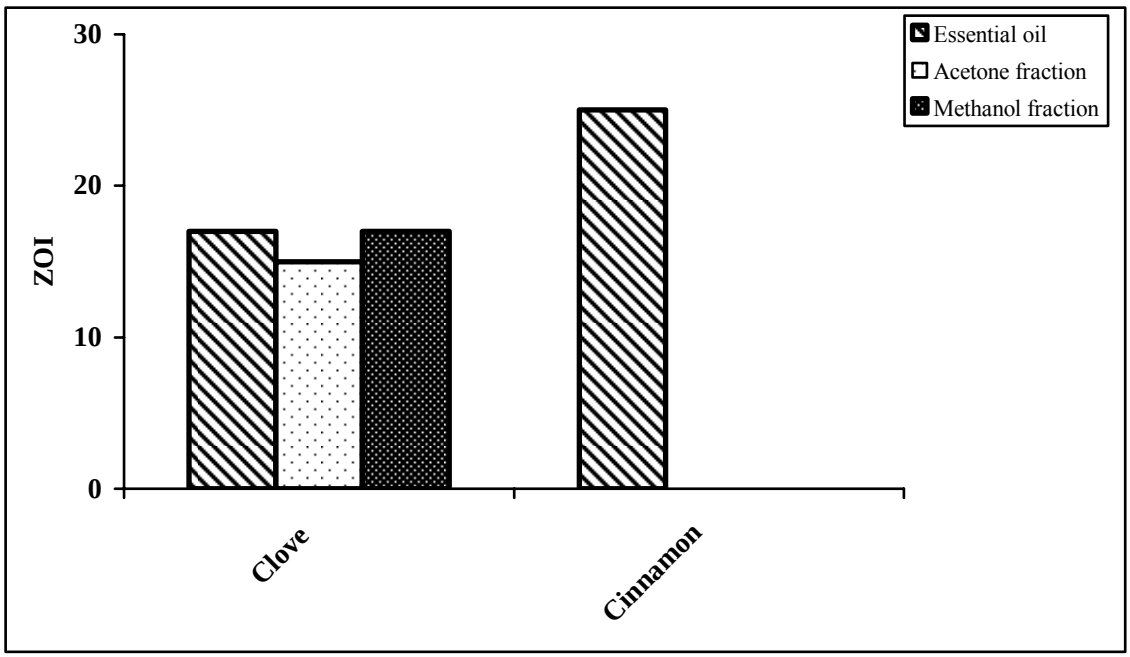


Figure: 7 Zone of Inhibition of *P. aeruginosa* (ATCC 27853) exhibited by different spices

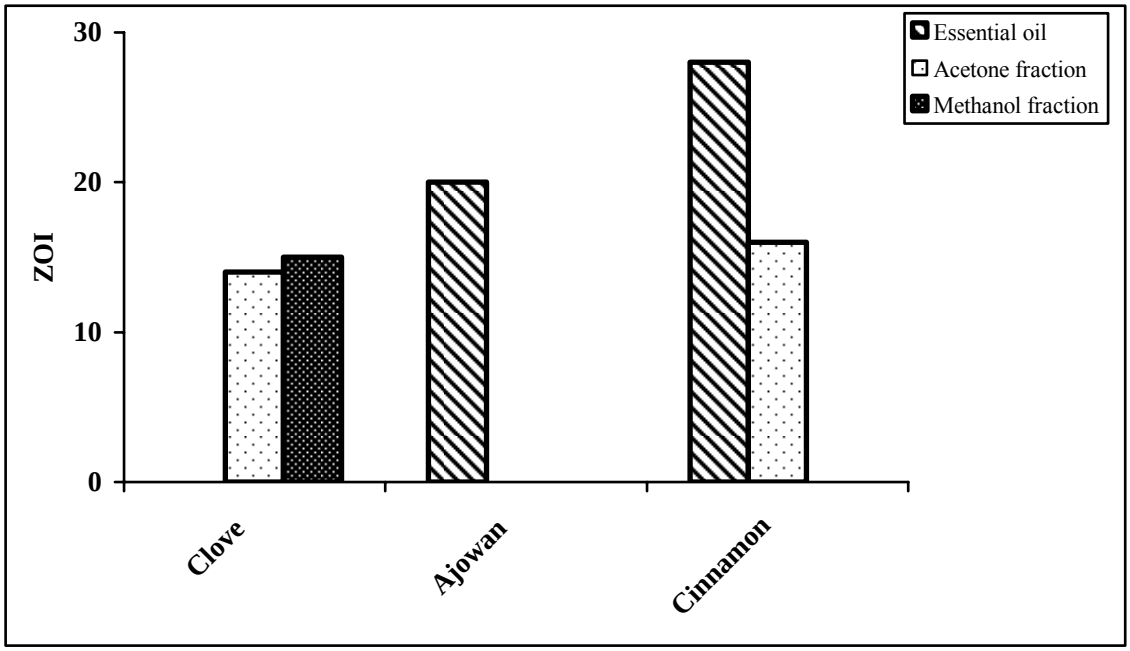


Figure: 8 Zone of Inhibition of *P. mirabilis* (ATCC 49132) exhibited by different spices

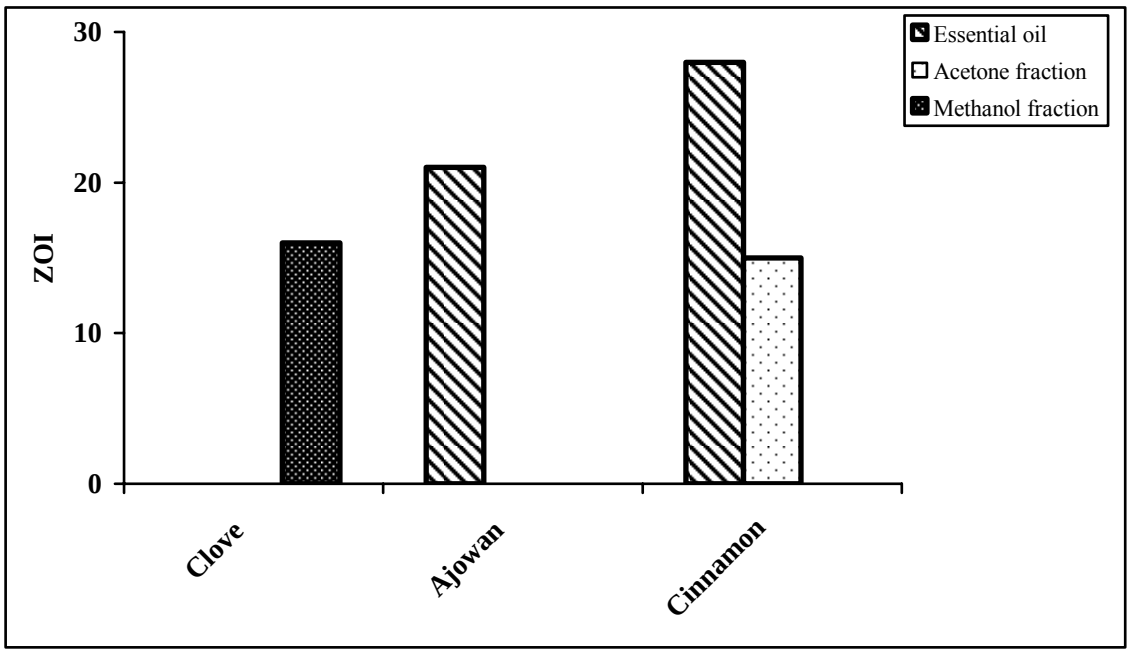


Figure: 9 Zone of Inhibition of *P. vulgaris* exhibited by different spices

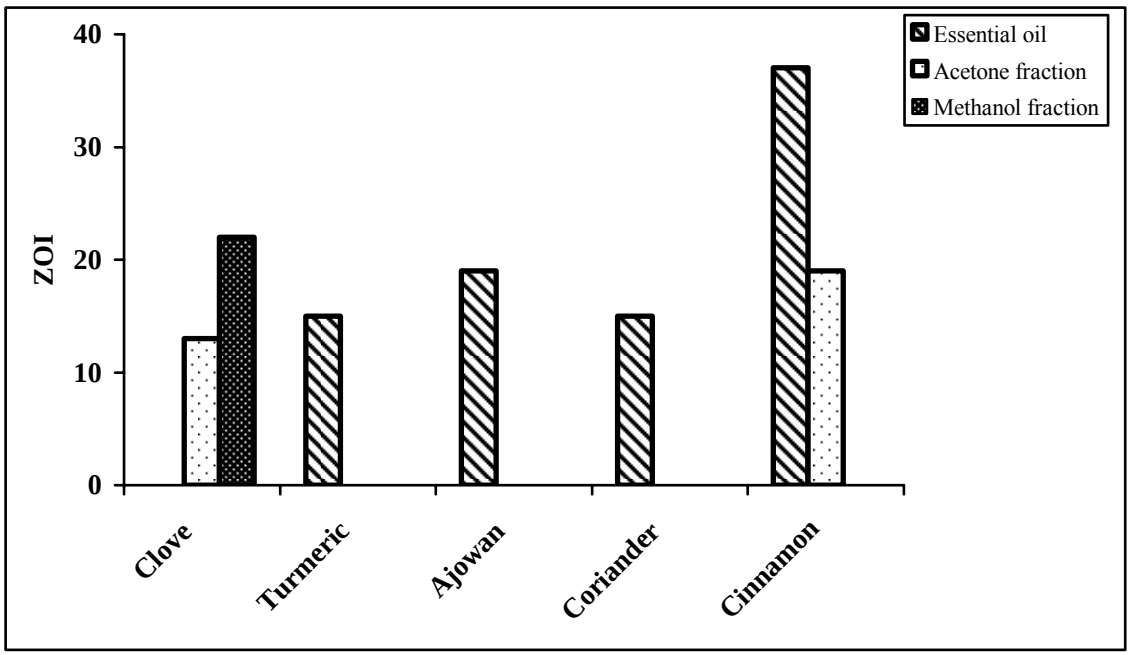
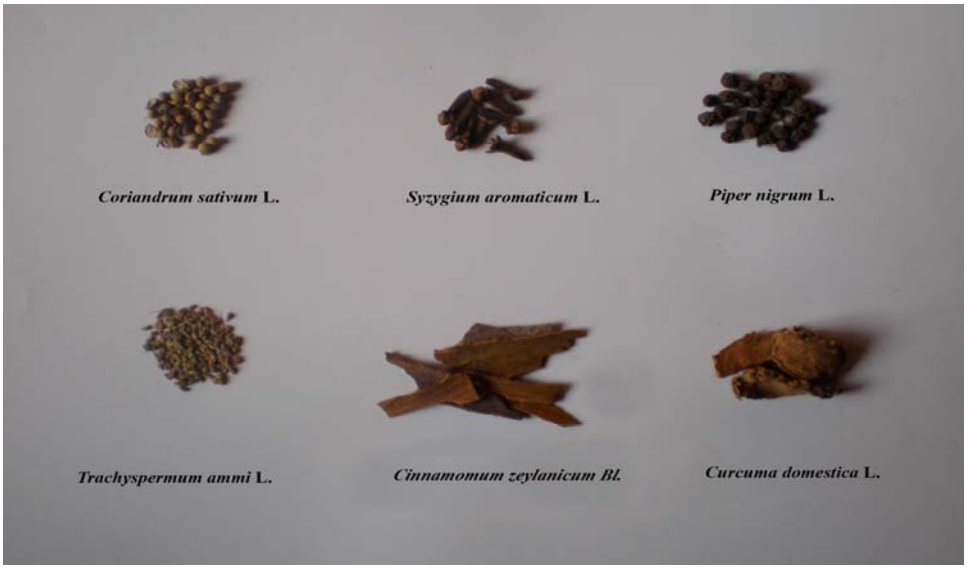


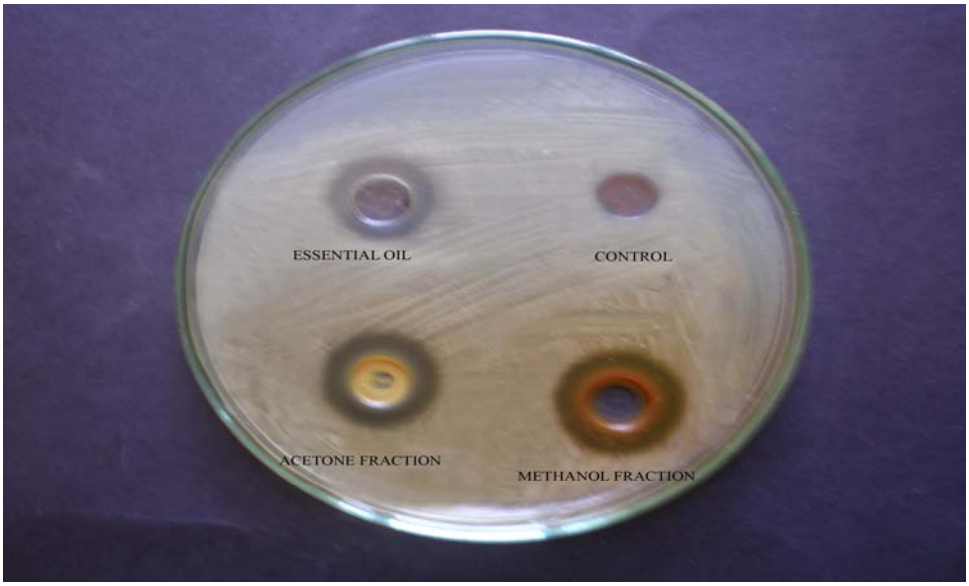
Figure: 10 Zone of Inhibition of *S. dysenteriae* exhibited by different spices



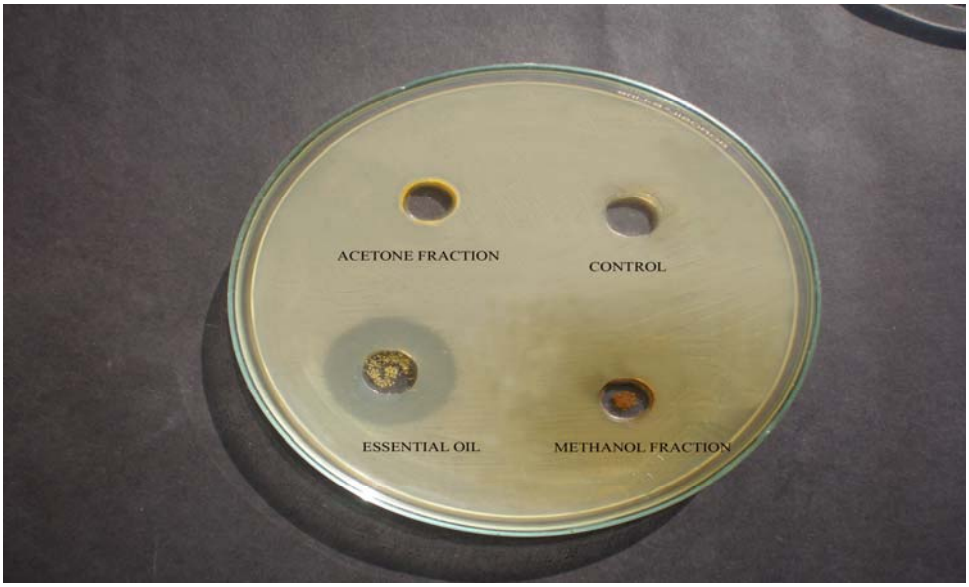
Photograph 1: Different spices sample

Photograph 2: Soxholet Extractor showing extraction of spice

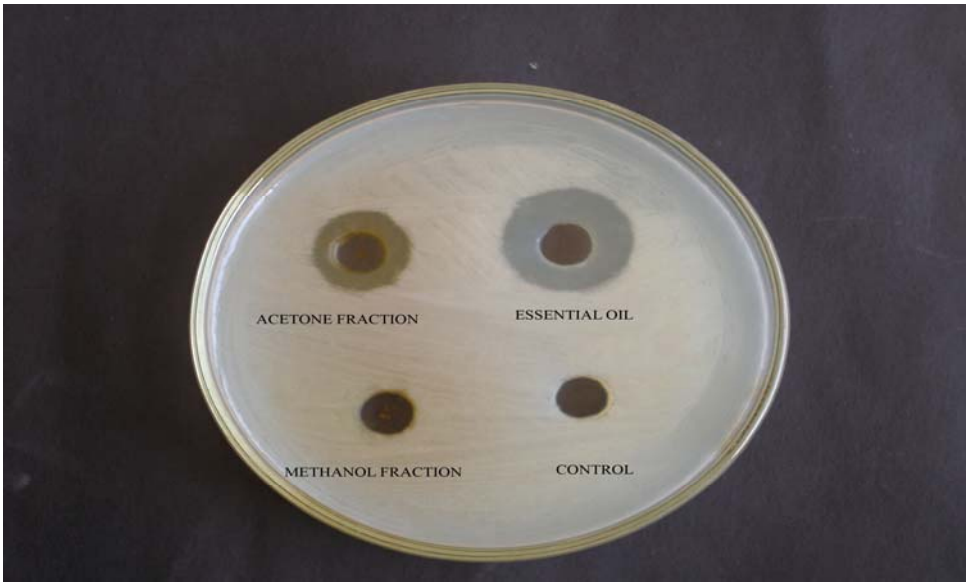
Photograph 3: Clevenger Apparatus and Rotary Evaporator



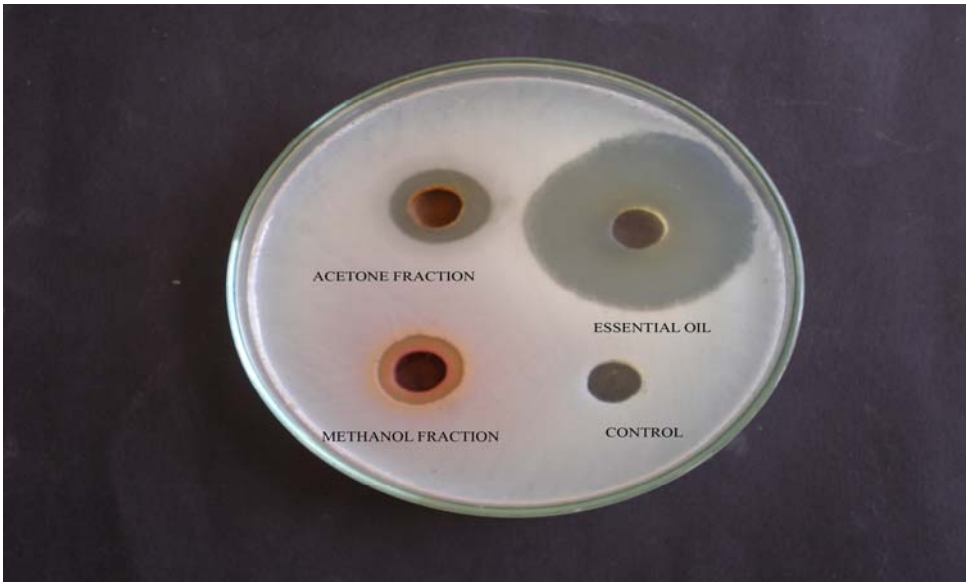
Photograph 4: Zone of Inhibition exhibited by clove against *Staphylococcus aureus*



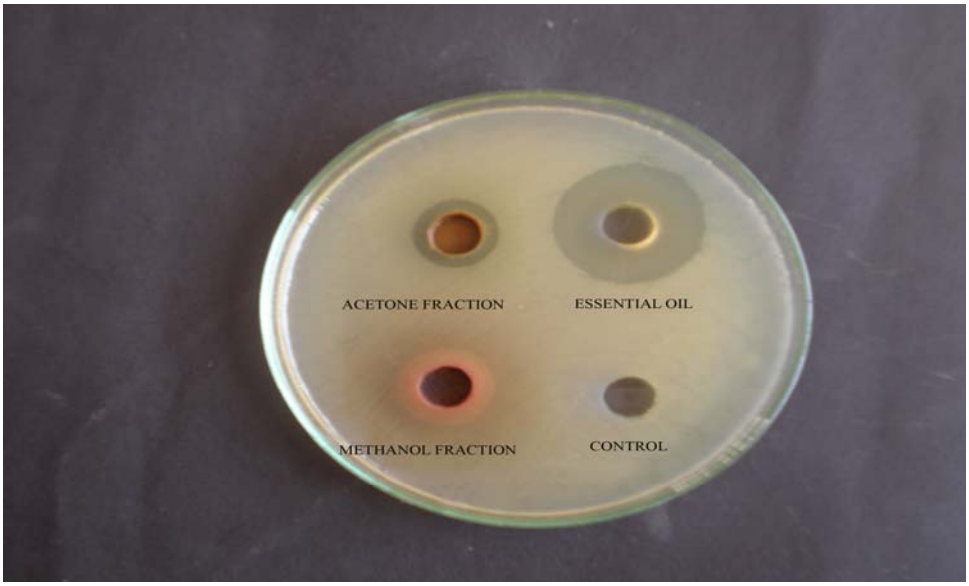
Photograph 5: Zone of Inhibition exhibited by black pepper against *Escherichia coli*



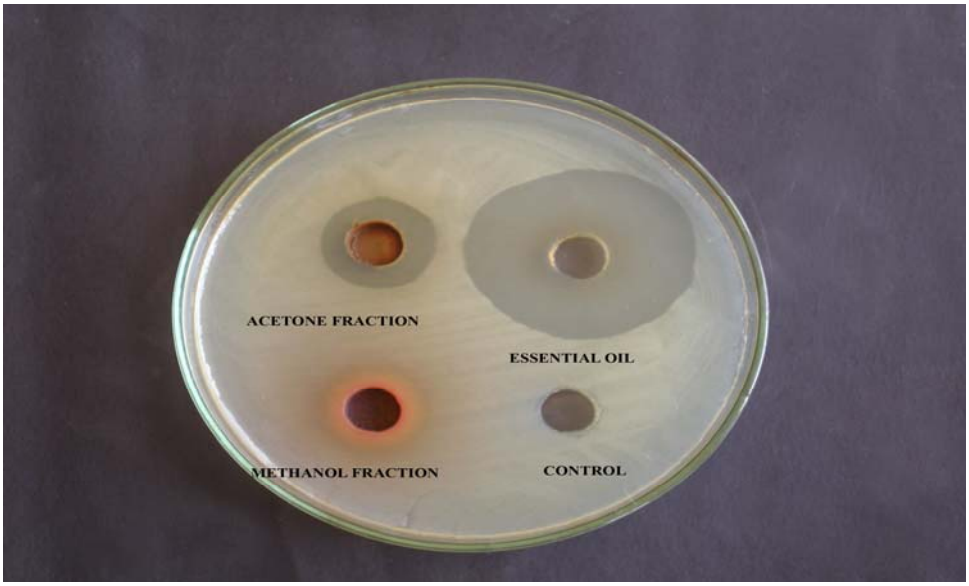
Photograph 6: Zone of Inhibition exhibited by ajowan against *Staphylococcus aureus*



Photograph 7: Zone of Inhibition exhibited by cinnamon against *Staphylococcus aureus*

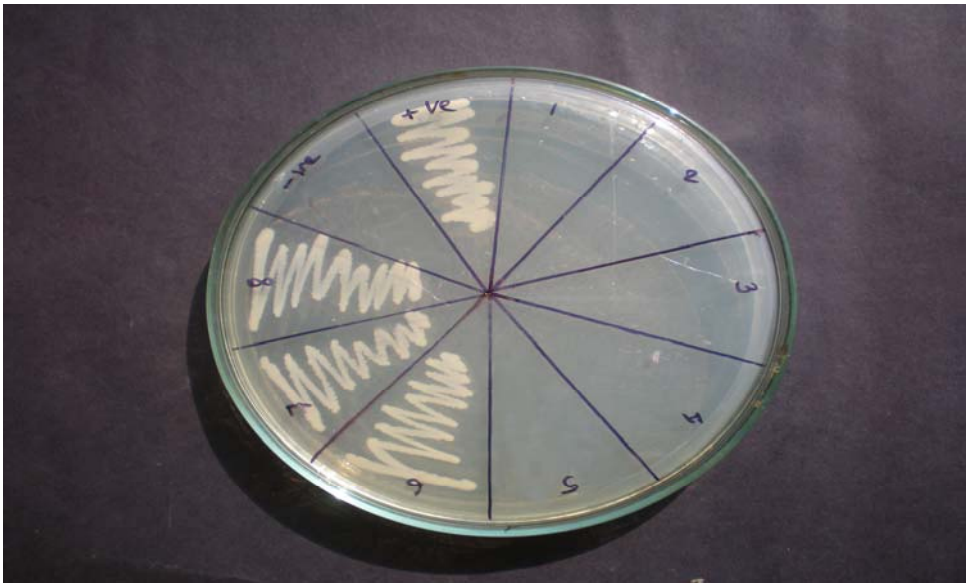


Photograph 8: Zone of Inhibition exhibited by cinnamon against *Escherichia coli*

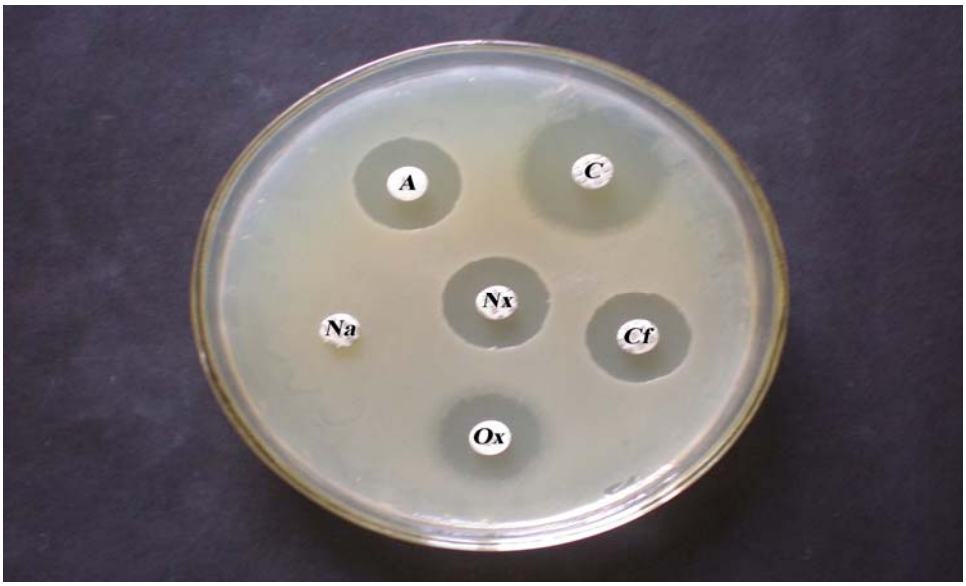


Photograph 9: Zone of Inhibition exhibited by cinnamon against *Shigella dysenteriae*

Photograph 10: Two fold serial broth dilution of essential oil in nutrient broth



Photograph 11: Minimum Bactericidal Concentration (MBC) test of essential oil of clove against *S. aureus* (No. 1,2,3… indicates the respective Tube No.)



Photograph 12: Zone of Inhibition exhibited by antibiotics against *Salmonella* Paratyphi A (the antibiotics include ampicillin, chloramphenicol, ciprofloxacin, nalidixic acid, norfloxacin and oflaxacin

CHAPTER VI

6. Discussion and conclusion

6.1. Discussion

This study was performed to analyze the antibacterial activities of the spices such as clove, black pepper, turmeric, ajowan, coriander and cinnamon which is widely used spices in every day's meal. Herbs and spices have been use for centuries to enhance the flavour, colour, aroma of the food. In addition, they are also known for their preservative and medicinal value (deSouza, 2005). Recently, modern science is concerned about the properties of spices especially, antimicrobial and antioxidative properties.

There is worldwide trend to go back to natural resources, mainly medicinal plants. The prescriptions of drugs of plant origin are gradually increasing. Plants continue to occupy an important place as raw materials for some important modern medicine as herbal medicine are generally safe and free of side effects (Abooleneil, 1982; Johri, 1994). So, large number of plants is used to treat disease. Thus continuous search for new drugs from plants should be carried out to face the challenges posed by resistant strains (Gibbons, 1992; Lavania and Lavania, 1956). The spices could be the alternative to modern drug and chemical additives.

In this study, spices are selected on the basis of their uses as household remedies in Nepal, in the treatment of certain ailments. They are also common spices used in food daily. These spices are claimed to have antimicrobial activities and used as preservatives. These spices are included in Medicinal plants of Nepal for Ayurvedic Drugs (1995) and in the Indian Materia Medica (1976). The spices of best quality were brought from the market "Ason Bazaar" Kathmandu. The spices were confirmed in Department of Plant Resources, Thapathali.

There is no any specific rule for the selection of solvent used for the extraction. The study was performed taking reference of the study done by Singh *et al.* (2007) in which antibacterial and antioxidative properties of essential oils and acetone extract of the spices were studied. In this study, further methanol is used to extract the more polar compound that were not extracted by acetone, so as to study if there is any antimicrobials left in the spice residue. The study is successful in showing antimicrobial activity of methanol extract of some spices like clove and cinnamon.

In this study, there were 18 fractions, obtained from the six spices that includes essential oil, acetone and methanol fraction. The antibacterial activity was carried out by dissolving essential oil in 2% tween 80 and acetone and methanol fraction in DMSO (10-40%) and testing against ten bacteria.

In this study, it was found that clove was effective against all test bacteria. The essential oil showed antibacterial activity against six bacteria including *E. coli, S. aureus, K. pneumoniae, K. oxytoca. S.* Typhi and *P. aeruginosa*. Acetone extract of clove was active against 7 bacteria including *E. coli, S. aureus, K. pneumoniae, S.* paratyphi A, *P. aeruginosa, P. mirabilis* and *S. dysenteriae*. Similarly, methanol extract of clove was effective against 6 bacteria including *S. sureus, P. aeruginosa, S.* paratyphi A, *P. mirabilis, P. vulgaris* and *S. dysenteriae*. Sharma (2000) reported that essential oil of clove showed antibacterial activity against *S. dysenteiae*. However, Thapa (2006), reported that ethanolic extract of clove was active against *E. coli, S, aureus, K. pneumoniae, P. mirabilis, P. vulgaris,* and *S. dysenteriae*. So S. dysenteriae and *P.vulgaris* are inhibited by clove. Our result also supports the results obtained by Agaoglu (2006) and Agnihotri and Vaidya (1995). The antimicrobial activity of clove was reported to be due to the action of eugenol (The Wealth of India, 1976).

Black pepper was found to have weak inhibitory activity at the tested concentration. It inhibited two bacteria. Essential oil was active against *S. aureus* only while Acetone extract was also found active against *E. coli* and *S. aureus*. According to Singh *et al.* (2005) and Pradhan *et al.* (1999), black pepper was effective against to *S. aureus* which supports with the result of study. The antimicrobial activity of black pepper was reported to be due to the action of piperine (The Wealth of Indian, 1969).

The fraction of turmeric was also found to have weak inhibitory activity at tested concentration. It inhibited only two bacteria. Essential oil was active against *E. coli* and *S. aureus* while acetone extract was effective to only *S. aureus*. Singh *et al.* (2007) reported that essential oil of turmeric was effective against *E. coli, S. aureus*, and *Bacillus* species but acetone fraction didn't show any antibacterial activity. The contradiction of the result may be due to concentration variation. The antimicrobial activity of turmeric was reported to be due to the action of curcumin (The Wealth of India 1950).

Ajowan was found to show antibacterial activity against 9 bacteria. Essential oils of ajowan were found to have high inhibitory activity. It was active against all bacteria except *P. aeruginosa*. Acetone fraction was active against *S. aureus* only. Singh *at al.* (2007) reported that essential oil was also active against *P. aeruginosa* that contradicted with our result and acetone fraction was didn't showed any antibacterial activity that supports our result. The antimicrobial activity of ajowan was reported to be due to the action of thymol (The Wealth of India, 1976).

The fractions of coriander showed weak inhibitory activity. It was active against 3 bacteria. Essential oils of coriander were active to *E. coli, S. aureus* and *S. dysenteriae*. Both acetone and methanol fractions did not show any activity against tested bacteria at tested concentration. *S. aureus* was most susceptible bacteria. The result supports the finding of the study done by Singh *et al.* (2007) but contradict with that of Sharma (2000) who found that coriander oils showed antibacterial activity against *S. aureus* only. The

antimicrobial activity of coriander was reported to be due to the action of linalool (The Wealth of India, 1950).

Cinnamon was the most effective spices among studied spices. Essential oils of cinnamon were active against all tested bacteria with higher antibacterial activity against *S. dysenteriae* and lower activity against *K. pneumoniae*. Acetone fraction of the cinnamon was also active against all test bacteria except *P. aeruginosa*. The result supports the results of the study done by Sharma (2000), Rajendhran *et al.* (1998), Sofia *et al*. (2007), Fabio *et al*. (2007) and Agaoglu *et al*. (2007). The antimicrobial activity of cinnamon was reported to be due to the action of trans-cinnamaldehyde and eugenol (The Wealth of India, 1950).

The MBC was determined to know the exact efficacy of the antimicrobials by two fold serial dilution method where the concentration was decreased by two times. The ZOI may be maximum with low MBC value which suggests that the fraction was highly diffusible but has low bactericidal activity. Sometimes the substance has high MBC value with smaller ZOI suggesting the substance is highly bactericidal with minimum diffusible property. The MBC ranged from 0.39mg/ml to 25mg/ml in case of essential oils, 6.25mg/ml to 25mg/ml in case of acetone and methanol fractions. The essential oils showed better antibacterial activity against test bacteria at tested concentration (100mg/ml).

In case of essential oil, the minimum MBC value was given by cinnamon essential oil against *E. coli, S. aureus* and *S*. Typhi. The MBC value of essential oils of cinnamon ranged from 0.39 to 0.78mg/ml. Thus cinnamon oil was found bactericidal at very low concentration. So, it is considered as the best antibacterial among the spices tested. The MBC value of clove oil ranged from 1.56 to 12.50mg/ml. MBC value of ajowan oil ranged from 1.56 to 6.25mg/ml. MBC value of coriander oil against three bacteria was 3.13mg/ml and that of turmeric and black pepper oils were 25mg/ml.

In case of acetone fraction, MBC value of all spice ranged from 3.13 to 25 mg/ml. The minimum MBC value was given by acetone fraction of cinnamon against *S.* Typhi followed by acetone fraction of clove, cinnamon and black pepper against *E. coli*.

In case of methanol fraction, the MBC value ranged from 3.13mg/ml. The minimum MBC value was given by *S. dysenteriae* (3.13mg/ml).

Thus in the study, we found that the highest and broadest antibacterial activity is that of cinnamon among the spices tested followed by clove and ajowan.

The tested bacterial strains were also subjected to antibiotic susceptibility tests which revealed that there is appearance of resistance among the test bacteria. The development of resistance is a natural phenomenon which is accelerated by the extensive use of the antibiotics. It helps in comparing the susceptibility of bacteria to antibiotic and spices.

E. coli (ATCC 25922) was sensitive to all the antibiotics including chloramphenicol, ciprofloxacin, cotrimoxazole, norfloxacin and ofloxacin, except penicillin G to which it was resistant. It was inhibited by all spices and 8 out of 18 test suspensions. Essential oil of clove, turmeric, ajowan, coriander and cinnamon and acetone extract of clove, black pepper and cinnamon showed antibacterial activity against it. The MBC value ranged from 0.39mg/ml that of cinnamon oil to 25mg/ml that of turmeric oil. The MBC value of essential oil of ajowan was 1.56mg/ml that of clove was 6.25mg/ml, that of coriander oil was 3.13mg/ml and that of acetone fraction of effective spices was 6.25mg/ml.

S. aureus (ATCC 25923) was sencitive to cephalexin, cotrimoxazole, erythromycin, ofloxacin and penicillin G and resistant to cloxacillin. However, it was inhibited by all spices and 13 out of 18 test suspensions. Essential oils of all spices except turmeric, acetone fraction of clove, black pepper, turmeric and cinnamon and methanol fraction of clove and cinnamon were found to show antibacterial activity against it. The lowest MBC value was 0.39mg/ml that of cinnamon oil in case of essential oil and 6.25mg/ml that of

acetone fraction of cinnamon in case of acetone fraction. The MBC value of essential oil of ajowan was 1.56mg/ml and that of clove was 3.13mg/ml.

K. pneumoniae (ATCC 200603) was resistant to all antibiotic including cephalexin, ciprofloxacin, ampicillin, gentamycin and amikacin, and moderately sensitive to cotrimoxazole. It is inhibited by 5 out of 18 test suspensions. Essential oil of clove, ajowan and cinnamon, acetone fraction of clove and cinnamon were found to antibacterial activity against it. The lowest MBC value was 0.78mg/ml that of cinnamon oil. The MBC value of essential oil of clove and ajowan was 3.13mg/ml.

K. oxytoca was sensitive to cephalexin and cotrimoxazole, moderately sensitive to ampicillin and resistant to ciprofloxacin and amikacin. It was inhibited by 4 out of 18 test suspensions i.e. essential oil of clove, ajowan and cinnamon and acetone fraction of cinnamon. The lowest MBC value was 0.78mg/ml that of cinnamon oil. The MBC value of essential oil of clove and ajowan was 1.56mg/ml.

S. Typhi was sensitive to chloramphenicol, ciprofloxacin, norfloxacin, ofloxacin and amikacin and resistant to nalidixic acid. However, it was inhibited by 3 spices and 4 out of 18 test suspensions i.e. essential oil of clove, ajowan and cinnamon and acetone fraction of cinnamon. The MBC value ranged from 0.39mg/ml that of cinnamon oil to 3.13mg/ml that of acetone fraction of cinnamon. The MBC value of essential oil of clove and ajowan was 1.56mg/ml. Acetone fraction of cinnamon gave lowest MBC value (3.13mg/ml) against it.

S. Paratyphi A was sensitive to chloramphenicol and norfloxacin, moderately sensitive ofloxacin and amikacin and resistant to nalidixic acid and ciprofloxacin. It was inhibited by three spices and 5 out of 18 test suspensions that include essential oil of ajowan and cinnamon, acetone fraction of clove and cinnamon and methanol fraction of clove. The

MBC value ranged 0.78mg/ml that of cinnamom oil to 25mg/ml that of acetone fraction of clove.

P. aeruginosa (ATCC 27853) was sensitice to ciprofloxacin, norfloxacin, gentamycin and amikacin and resistant to chloramphenicol and cephalexin. However, it was inhibited by two spices and 5 out of 18 test suspensions that include essential oil of clove and cinnamon and acetone fraction of clove. The MBC value ranged from 0.78mg/ml that of essential oil of cinnamon to 25mg/ml that of acetone and methanol fraction of clove.

P. mirabilis (ATCC 49132) was sensitive to norfloxacin, ofloxacin and ciprofloxacin,

moderately sensitive to cotrimoxazole and resistant to chloramphenicol and penicillin G. However, three spices were found to be effective against it and it was inhibited by 4 out of 18 test suspension that include essential oil of ajowan and cinnamon, acetone fraction of clove and cinnamon and methanol fraction of clove. The MBC ranged from 0.78mg/ml that of cinnamon oil to 25mg/ml that of acetone and methanol fractions of clove. The MBC value of ajowan oil was 1.56mg/ml.

P. vulgaris was sensitice to norfloxacin, ofloxacin ciprofloxacin, cotrimoxazole and chloramphenicol and resistant to penicillin G. It was inhibited three spices and 5 out of 18 test suspensions that include essential oil of ajowan and cinnamon, acetone fraction of clove and cinnamon and methanol fraction of clove. The MBC value ranged from 0.78mg/ml that of cinnamon oil to 25mg/ml that of acetone fraction of clove.

S. dysenteriae was sensitive to gentamycin and amikacin, moderately sensitice to chloramphenicol, cotrimoxazole ans erythromycin and resistant to ciprofloxacin. It was inhibited by essential oil of ajowan, coriander and cinnamon, acetone fraction of clove and cinnamon and methanol fraction of clove. Cinnamon essential oil gave highest ZOI measuring 37mm but the MBC value was 0.78mg/ml. The MBC value ranged from 0.78 to 25mg/ml. The MBC value of ajowan oil was 1.56mg/ml.

From the study, we found that gram positive bacteria were more susceptible to spices than gram negative bacteria. *S. aureus* was inhibited by all spices and 13 out of 18 suspensions and relatively low test suspension inhibited gram negative bacteria. E. coli was inhibited by all spices but by 8 out of 18 suspensions. Other gram negative bacteria were inhibited by two or three spices. This supports the results obtained by Chandarana et al. (2005). The variation in inhibition may be due to difference in the composition and structure of the cell wall. In addition, gram negative contains extra outer membrane composed of phospholipids that may prevent the entry of antimicrobial.

However, the result differed from those of former researcher. The differences may be due to various test environments and methods, the quality of spices and their age etc. The antimicrobial activity is also affected by farming, harvesting, storage condition and extraction procedure. The volatilility and poor solubility of most essential oils are problematic especially with diffusion and dilution of the test substance in a microbiological medium. Antimicrobial activity of spices depend on several factors, including kinds of spices, composition and concentration of spices, microbial species and its occurrence level, substrate composition and processing conditions and storage (Shelef, 1983).

6.2. Conclusion

From present study we can conclude that the essential oils of spices especially that of cinnamon can be a good antimicrobials followed by essential oil of clove, ajowan and acetone fraction especially that of clove and cinnamon. They were found to possess better antibacterial activity. So essential oils which were found highly inhibitory to certain bacteria could be tried as effective remedies against disease caused by them. Spices were also effective against spoilage bacteria. So they could be the preservatives and may be used alternative to chemical additives that alter the quality of food and also affect the health of consumers.

CHAPTER VII

7. SUMMERY AND RECOMMENDATIONS

7.1. Summary

1. Six different types of spices which are used daily in food to alter the taste of the food and also use in household medical purpose. The spices under study include clove, black pepper, turmeric, ajowan, coriander and cinnamon. The parts used are buds of clove, fruits of black pepper, rhizome of turmeric, seeds of ajowan and coriander and barks of cinnamon.

2. Essential oils were extracted by hydrodistillation, acetone and methanol fraction were extracted using Soxhlet extractor.

3. The method used to evaluate the antibacterial activity was agar well diffusion given by Dingle *et al.* (1953) and two fold serial dilution method.

4. The antibacterial activity increased with the increase in volume of the suspension i.e. increase in the content of antimicrobial agents.

5. Essential oil of cinnamon, clove and ajowan were found to show higher antibacterial activity inhibiting 100%, 70% and 80% test bacteria respectively whereas essential oil of black pepper, turmeric and coriander were found to show weak antibacterial activity inhibiting 10%, 20% and 20% bacteria respectively. Most active essential oil was that of cinnamon with the MBC value ranging from 0.390 to 0.781mg/ml.

6. Acetone fraction of cinnamon and clove showed higher antibacterial activity inhibiting 9 and 7 test bacteria respectively whereas that of black pepper, turmeric and coriander were found to have very weak antibacterial activity inhibiting 1 or 2 bacteria.

7. Methanol fraction of clove showed higher antibacterial activity inhibiting 6 test bacteria whereas that of cinnamon and black pepper inhibited only one bacterium. *S. aureus* was the most susceptible bacteria inhibited by all spices and 13 out of 18 test suspensions followed by *E. coli* inhibited by all spices and 8 out of 18 test suspensions. *P. aeruginosa* was most resistant bacteria inhibited by 2 spices.

8. Gram positive bacteria are more susceptible to the spices than Gram negative bacteria.

7.2. Recommendation

Based on the study following recommendation were made:

1. Essential oil of cinnamon, clove and ajowan were found active against the pathogenic and spoilage bacteria. So, it can be suggested to use them for therapeutic and as preservatives.
2. Antibacterial activity of only six spices has been studied. So, the study should be carried with other spices.
3. In this study, only antibacterial activity of spices has been studied. So, antiviral, antifungal and antiparasitic activity should be studied.
4. Spices, which showed higher antibacterial activity during evaluation, should be analyzed in detail to identify the active antimicrobials and also their mode of action.
5. *In vivo* test should be performed before they can be prescribed for therapeutic uses.
6. Hydrodistillation yields less amount of oil so steam distillation may be recommended.

CHAPTER VIII

8. REFERENCE

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Appendix I

List of the materials used for the study

Spice sample

S. No.	Spices	Scientific name	Family	Parts used
1.	Clove	*Syzygiun aromaticum* Linn.	Myrtaceae	Fruit 'Mother of cloves
2.	Black pepper	*Piper nigrum* Linn.	Piperaceae	Fruits
3.	Turmeric	*Curcuma domestica* Linn.	Zingiberaceae	Rhizome
4.	Ajowan	*Trachyspermum ammi* Linn.	Umbelliferae	Leave, Roots, Seeds, Fruits
5.	Coriander	*Coriandrum sativum* Linn.	Umbelliferae	Whole plant
6.	Cinnamon	*Cinnamomum zeylenicum* Blume.	Lauraceae	Barks (inner parts of stems)

Hydro distillation

- Round bottom flask
- Clevenger type oil trap
- Condenser
- Heating mantle
- Stands

Soxhlet extraction

- Condenser
- Grinder
- Heating mantle
- Rotatory Vacuum Evaporator
- Round bottom flask
- Sieve
- Soxhlet extractor
- Stands

Media

- Eosin Methylene Blue Agar
- Mannitol Salt Agar
- MacConkey Agar
- Mueller-Hinton Agar
- Nutrient Agar
- Nutrient Broth
- Triple Sugar Iron Agar
- Sulphide Indole Motality
- MRVP Broth (Glucose, Phosphate, Peptone Broth
- Simmon's Citrate Medium
- Urease

Chemical Reagent

- Absolute Ethanol
- Tween 80
- DMSO
- Crystal Violet
- Gram's Iodine
- Kovac's Reagent
- Methyl Red
- α napthol solution
- Barium Chloride
- Conc. H_2SO_4
- Potassium hydroxide
- Physiological salice
- Blood plasma
- Saffranin

Apparatus and equipments

- Aluminium foil
- autoclave
- Compound microscope
- Cork borer
- Cotton roles
- Electric balance
- Filter paper
- Forceps
- incubator

- Inoculating loop
- Laminar flow hood
- Micropipette
- Measuring scale
- Refrigerator
- Sticker
- Water distillation plant
- Vortex shaker

Glass ware

- Beaker
- Conical flask
- Funnels
- Glass rod
- Measuring cylinder
- Glass slides
- Petri dishes
- Pipette
- Screw capped test tubes
- Test tubes

Antibiotics discs

Antibiotic discs from companies Hi-media Laboratory

Appendix II

Composition of media

1. **Nutrient Agar (NA)**

Ingredients	gm/litre
Peptone	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Agar	15.0
Final pH (at 25oC)	7.4±0.2

Procedure: 28gm of media was dissolved in 1000ml of distilled water and heated to dissolve the media. The media was autoclaved at 15lbs pressure at 121oC for 15 minutes.

2. **Nutrient Broth (NB)**

Ingredients	gm/litre
Peptone	5.0
Sodium Chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Final pH (at 25oC)	7.4±0.2

Procedure: 13gm of media was dissolved in 1000ml of distilled water and heated to dissolve the media. The media was autoclaved at 15lbs pressure at 121oC for 15 minutes

3. **Mueller-Hinton Agar (MHA)**

Ingredients	gm/litre
Beef infusion broth	300.0
Casein acid hydrolysate	17.0
Starch	1.0
Agar	17.0
Final pH (at 25oC)	7.0±.0.2

Procedure: 38gm of media was suspended in 1000ml distilled water, boiled to dissolve and sterilized by autoclaving at 121oC for 15 minutes. It was poured while at 45-55oC in sterile 9cm diameter plates in 25ml quantities. To ensure the uniformity in depth of medium, the plates were placed over level surface and the medium was poured into it.

4. **Deoxycholate Citrate Agar (DCA)**

Ingredient	gm/ml
Lab-Lemco powder	5.0
Peptone	5.0

Lactose	10.0
Sodium citrate	8.5
Sodium thiosulphate	5.4
Ferric citrate	1.0
Sodium deoxycholate	5.0
Neutral red	0.02
Agar	12.0

Procedure: the medium was used at a concentration of 5.2gm in every 100ml of distilled warer. The media should not boil but heat to dissolve the media.

5. **Xylose Lysine Deoxycholate (XLD) Agar**

Ingredient	gm/ml
Yeat extract powder	3.0
L-lysine HCl	5.0
Xylose	3.75
Lactose	7.5
Sodium deoxycholate	1.0
Sodium chloride	5.0
Sodium thiosulphate	6.8
Ferric ammonium citrate	0.8
Phenol red	0.08
Agar No. 1	12.5

Procedure: 5.3gm was dissolved in 100ml if distilled water and heated to dissolve.

6. **MacConkey Agar (MA)**

Ingredient	gm/ml
Peptone	20.0
Lactose	10.0
Bile salts	5.0
Sodium chloride	5.0
Neutral red	0.075
Agar	12.0

Procedure: 5.2gm was dissolved in 100ml of distilled water.

Appendix III

Preparation of McFarland Nephelometer Standards

Principle- A chemically induced precipitation reaction used to approximate the turbidity of a bacterial suspension

Method

1. Set up 10 test tubes or ampoules of equal size and of good quality. Use new tubes that have been thoroughly cleaned and rinsed.
2. Prepare 1% chemically pure sulphuric acid.
3. Prepare a 1.175% aqueous solution of barium chloride ($BaCl_2.H_2O$)
4. Slowly, and with constant agitation, add the designated amounts of two solutions to the tubes as shown in table to make a total of 10ml per tube.
5. Seal the tubes or ampoules. The suspended barium sulphate precipitate corresponds approximately to homogenous E. coli cell densities per ml through out the range of standards, as shown in the following table.
6. Store the McFarland standard tubes in the dark at room temperature. They should be stable for 6 months

	McFarland Nephelometer Standards										
	0.5	1	2	3	4	5	6	7	8	9	10
Barium Chloride (ml)	0.05	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1
Sulphuric acid (ml)	9.95	9.9	9.8	9.7	9.6	9.5	9.4	9.3	9.2	9.1	9
Approximate cell density (x 10^8/ml	1.5	3	6	9	12	15	18	21	24	27	36

The table shows the approximate no. of bacteria/ml equivalent to the opacity standard, but the numbers vary with the size of the bacteria. With yeast they would be only about 1/30 of those shown.

Use

- In a tube of the same internal diameter as standard, prepare a uniform suspension.
- Shake the standard prepared.
- Compare the bacterial suspension with the standard. View with oblique illumination against a dark background or over a printed page.

Appendix IV

Zone Size Interpretation Chart for Antibiotic Sensitivity Test

S. No.	Antimicrobial Agents	Symbol	Strength mcg	Diameter of Zone of Inhibition (mm)		
				Resistant	Intermediate	Sensitive
1.	Amikacin	Ak	30	14	15-16	17
2.	Ampicillin	A	10	13	14-16	17
3.	Cephalexin	Cp	30	14	15-17	18
4.	Chloramphenicol	C	30	12	13-17	18
5.	Ciprofloxacin	Cf	5	15	16-20	21
6.	Cloxacillin	Cx	5	11	12-13	14
7.	Cotrimoxazole	Co	25	10	11-15	16
8.	Erythromycin	E	15	13	14-22	23
9.	Gentamycin	G	10	12	13-14	15
10.	Nalidixic Acid	Na	30	13	14-18	19
11.	Norfloxacin	Nx	10	12	13-16	17
12.	Ofloxacin	Ox	5	12	13-15	16
13.	Penicillin G	P	10	14	-	15

Source: HiMedia Laboratories Pvt. Limited

The diameter of zone of inhibition of given by antibiotics against test bacteria

No.	Antibiotics	Zone of Inhibition (mm)									
		Escherchia coli (ATCC 25922)	*S. aureus* (ATCC 25923)	*K. pneumoniae* (ATTC 200603)	*K. oxytoca*	*Salmonella* Typhi	S. Paratyphi A	*P. aerugi-nosa* (ATCC 27853)	*Proteus mirabilis* ATCC49132	*P. vulgaris*	*Shigella dysenteriae*
1.	Amikacin	-	-	14	14	-	-	21	-	-	20
2.	Ampicillin	-	-	0	15	25	16	-	-	-	-
3.	Cephalexin	-	21	0	20	-	-	0	-	-	-
4.	Chloramphenicol	25	-	-	-	28	22	12	0	19	14
5.	Ciprofloxacin	30	-	14	14	21	13	25	22	22	11
6.	Cloxacillin	-	10	-	-	-	-	-	-	-	-
7.	Cotrimoxazole	24	25	13	21	-	-	-	14	18	12
8.	Erythromycin	-	31	-	-	-	-	-	-	-	18
9.	Gentamycin	-	-	12	15	-	-	15	-	-	19
10.	Nalidixic Acid	-	-	-	-	0	0	-	-	-	-
11.	Norfloxacin	28	-	-	-	22	17	25	31	24	-
12.	Ofloxacin	29	29	-	-	22	13	-	26	23	-
13.	Penicillin G	0	29	-	-	-	-	-	0	0	-

Appendix V

Chart of Two fold serial dilution

Code name of test tube	-ve tube	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	+ve tube
Volume (ml)	1	1	1	1	1	1	1	1	1	1	1	1
Concentration mg/ml	100	50	25	12.5	6.25	3.13	1.56	0.78	0.39	0.2	0.1	0.05

Appendix VI

Chemical composition of spices

1. Cove

Analysis of dried cloves contains moisture 25..2%; protein 5.2%; fat 8.9%; fibre 9.5%; other include carbohydrate 46% and mineral matter 5.2%; calcium 740; phosphorus 100; iron 4.9mg and iodine 50.7µg/100g. The vitamins reported to be present at (per 100g): carotene 253µg; thiamine 0.08mg; riboflavin 0.13mg; nicotinic acid 1.51mg. The clove also contains 13% tannin and oleanolic acid.

Clove bud oil: - Steam-distillation of clove buds yield (14-23%) a colorless or pale-yellow oil, with the characteristic odour and taste of cloves. It contains free eugenol (70-90%), eugenol acetate (2-17%) and caryophyllene as its main constituents. Methyl-n-amyl ketone is present in trace amount (The Wealth of India, 1976).

2. Black pepper

Analysis of the black pepper gave the following value: moisture 8.7-14.1%; total nitrogen 1.55-2.60%; volatile ether extract 0.3-4.2%; alcohol extract 4.4-12.0%; starch 28.0-49.0%; crude fibre 8.7-18.0%; crude piperine 2.8-9.0%; piperine (spectrophotometrically) 1.7-7.4%; total ash 3.6-5.7% and acid insoluble ash 0.03-0.55%. The oil of pepper is almost colourless to slightly greenish liquid with a characteristic odour of pepper. It consists chiefly of the terpenes, L-phellandrene, caryophyllene and perhaps dipentene (The wealth of India, 1969).

Stem contain alkaloids chavicine, piperrine, hentriacontane-16-one, hentriacontane and β-sitosterol, α-cis-bergamotene from essential oil, pepercide from fruits (Joshi, 2000).

3. Turmeric

Analysis of Indian turmeric gave the following values: moisture 31.1%; protein 6.3%; fat 5.1%; mineral matter 3.5%; fibre 2.6%; carbohydrate 69.4%; carotene calculated as Vitamin A 501 U/100g. The essential oils (5.8%) obtained by steam distillation of dry rhizomes, which contain d-α- phellandrene 1; d-sabinese 0.6; lineol 1; borneol 0.5; zingiberene 25; sesquiterpenes (turmerones) 58% (The wealth of India, 1950).

It contains campesterol, stigmasterol, β-sistosterol, cholesterol and fatty acids from rhizome, an essential oil, resin, an alkaloid, curcumin, turmeric oil. Aqueous extract suppressed carrageenin-induced oedema and showed very potent activity in granuloma pouch test (Joshi, 2000).

4. Ajowan

Analysis of the fruits gave the following values: moisture 7.4%; protein 17.1%; fat 21.8%; fibre 21.2%; carbohydrate 24.6%; and mineral matter 7.9%; calcium 1.525; total phosphorus 443; phytin phosphorus 296; iron 27.7; sodium 56; potassium 1.390; thiamine 0.21; riboflavin 0.28 and nicotinic acid 2.1mg/100g; carotene 71µg/100g. Other

constituent are sugar, tannins, glycosides, saponin, yellow-crystalline flavones and a steroidal substance.

Ajowan owes its characteristic odour and taste due to the presence of an essential oil (2-4%). Ajowan oil is used in medicine and is official in Indian Pharmacopoeia. The oil for a long time was the principal source of thymol. The principal constituents of the oil are phenols, mainly thymol (35-60%) and some carvacrol. The remainder of the oil is called 'thymene' ehich constitute 45% of the oil and has the following composition: p-cymene, 50-55; γ-terpinene, 30-35; α- and β-pinenes, 4-5; and dipentebe, 4-6%. Prensence of minute amounts of camphene and myrcene is also reported (The wealth of India, 1976).

5. Coriander

Analysis of the fruit gave the following values: moisture 11.2%; protein 14.1%; fat (ether extract) 16.1%; carbohydrate 21.6%; fibre 32.6%; mineral matter 4.41; calcium 0.63 and phosphorus 0.37 and iron 17.9mg/100g. The leaves constitute a rich source of the vitamin C (250mg/100g) and carotene (5.200µg/100g). Coriander oil is a colorless, pale yellow liquid, having the characteristic odour and taste of coriander. The chief constituent of the oil is coriandrol, terpene tertiary alcohol, now known to be identical with d-linalool. The other constituent of the oil are α & β- pinene, p-cymene, dipentene, γ-terpinene, phellandrene, terpinolene and traces of geraniol, borneol, n-decyclic aldehyde and ester of acetic and decyclic acid (The Wealth of India, 1950).

Aflatoxin B1 and B2 are found in sample of coriander. Leaves are a rich source of vitamin C and carotene. Oxalic acid and calcium content of leaves are 0.01% and 0.172%. Fruit contain β-sitosterol, chlorogenic and caffeic acids, rutin, umbelliferone and scopoletin. Seeds contain 19-21% fatty acid and essential oil which causes irritation when in contact with skin for a long time. Seed oil contain α-pipene, limonene, β-phellandrene, linalool, borneal, citronellel, thymol, geraniol, linalyl acetate, geranyl acetate, caryophellene oxide, elemol and methylheptenone (Joshi, 2000).

6. Cinnamon

The analysis of the cinnamon gave the following value: moisture 5.50-11.4%; volatile oil 0.3-2.8%; fixed oil 0.3-1.9%; fibre 25.6-30.5%; carbohydrate 16.6-22.6%; protein 3.0-4.5%; total ash 3.4-6.0%. The most important constituent is volatile oil (The Wealth of India, 1950).

Cinnamon bark contains 0.5-1.0% oil which contains a considerable amount of eugenol. It also contains cinnamaldehyde, benzaldehyde, methyl amyl ketone, linalool, cumic aldehyde, caryophyllene, phellendrene, pinene, cymere nonylaldehyde and ester of isobutyric acid (The wealth of India, 1950).