ANTIBACTERIAL ACTIVITY OF CERTAIN SPICES ON SOME FOODBORNE INFECTION CAUSING BACTERIA

BY

KHORSHED D. BULSARA

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DECLARATION

I, Khorshed D. Bulsara, hereby declare that the work presented in this thesis is original. It was carried out by me in the laboratory of Department of Microbiology, Ahmadu Bello University, Zaria, Nigeria, under the supervision of Dr. J. B. Ameh and Dr. M. Galadima. No part of this work has been submitted for any degree or qualification before. The work of other workers were referred to during this work and were duly acknowledged.

CERITIFICATION

The thesis entitled " Antibacterial Activity of Certain Spices On Some Foodborne Infection Causing Bacteria" by Khorshed D. Bulsara, meets the regulation governing the award of the degree of Master of Science of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

Dr. J. B. Ameh
Chairman, Supervisory Committee

14/4/99
Date

Dr.. M. Galadima
Member, Supervisory Committee

14/4/99
Date

Dr. J. B Ameh
Head, Department of Microbiology
Faculty of Science

14/4/99
Date

Dean, Postgraduate School

20/4/99
Date

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ABSTRACT

Petroleum ether, methanol and aqueous extracts of clove garlic ginger, onion and fenugreek were tested on *Escherichia coli, Vibrio cholerae, Staphylococcus aureus, Shigella flexineri, Salmonella typhi, Pseudomonas aeruginosa* and *Bacillus cereus* for their antimicrobial properties. It was found that, in general, petroleum ether extracts were more active than methanol and aqueous extracts. Extracts of clove and garlic in particular were more active than the extracts of ginger, onion and fenugreek. Petroleum ether and methanol extracts of clove were more active against all the tested bacteria. The petroleum ether extracts of ginger were active against all the bacteria but the methanolic extract showed little activity against *B. cereus.* The aqueous extract of ginger had no activity at all against the bacteria tested. The greatest antimicrobial properties of onion were detected against *B. cereus* and S. *aureus(* Gram positive bacteria) and an unappreciable activity against Gram negative bacteria. Only the petroleum ether extract showed appreciable activity against the negative bacteria (except *E. coli).* The most sensitive bacteria were *B. cereus* and S. *aureus* while the most resistant were *E. coli* and *P.aeruginosa.*

The range of MIC and MBC values obtained for each of the spice extracts are: petroleum ether clove extract- MIC (0.251 mg - 2.3mg), MBC (0.356mg - 9mg); petroleum ether garlic extract- MIC (0.019mg - 2.5mg) and MBC (0.068mg - 2.5mg); petroleum ether onion extract- MIC (0.289mg - 7.2mg) and MBC (1.03mg - 9.5mg); petroleum ether ginger extract - MIC (0.639mg - 11.7mg) and MBC (0.358mg - 23mg); and petroleum ether fenugreek extract - MIC (0.523mg - 3.3mg) and MBC (1 .Omg - 13.5mg).

Petroleum ether methanol extracts also showed synergism with antibiotic such as ampicillin, nitrofurantoin and cloxacillin. The effectivity of spices was in order of: clove > garlic > ginger > onion> fenugreek. The effectivity of the solvents was in the order of: petroleum ether > methanol > water.

TABLE OF CONTENTS

LIST OF TABLES

ABBREVAITIONS

et al.	=	with other authors
g	=	grams
hr(s)	=	hour(s)
mg	=	milligrams
ml	=	milliliters
ppm	=	parts per million
rpm	=	revolution per minute
μg	=	micrograms

Chapter 1

INTRODUCTION

Spices obtained from the aromatic parts of plant, or their products such as extracts, essential oils, oleoresins and other seasoning forms are used to flavour foods and beverages. They include parts of the plant such as leaves, flowers, flower buds, bulbs, rhizomes and various other parts. They maybe used directly or in various forms to produce the desired results (Shelef,1983).

Garlic and onion (both bulbs) and such as (rhizome) are monocotyledonous herbs mostly cultivated in tropical areas. Fenugreek, a dicotyledonous herb and clove, a dicotyledonous tree, are widely grown in European and Asian countries (Purseglove, 1988).

The demand for onions is worldwide, and it is consumed by people of all nationalities it is widely used in powdered form in the food industry (Purseglove. 1988). Worldwide production of onion is more than 10 million tons per year and as they are harvested over a short season, they need to be supplied during the rest of the year. Spices and their products (apart from consumption as food) are in great demand by the food industries to be used as seasonings (Tainter, 1992).

Since ancient times, the spices have been well documented not only for their flavouring but also for their medicinal, preservation and antioxidant properties (Shelef, 1983). It is consistently evident from studies carried out on human being and animals, that strenuous physical exercise may induce a state wherein the antioxidants act together to evade such stress (Sen, 1995). A wide variety of spices are known to have antioxidant properties (Nakatani, 1994).

Apart from their antioxidant properties, spices are also considered to be of great value as antimicrobial agents. Certain active components in spices are known to inhibit bacteria, yeast and molds of medical importance (Giese, 1994). Of these, garlic and clove extracts have been recognised to be of great value as antimicrobial agents against some of multiple-drug resistant strains of bacteria (e.g *Shigella dysenteriae and Vibrio cholerae*) (Ahsan, *et al.*, 1995).

In Nigeria, spices are used in many forms. For instance, suya, a widely consumed meat product, is marinated with chillie powder, onion powder and other ingredients before roasting. The chillie powder and onion powder have an antimicrobial effect while the other ingredients serve to dehydrate the meat so that the bacteria cannot make use of the free water. Cloves, ginger, red and black pepper are used in kunun zaki, a beverage, as both flavouring and antimicrobial agent, although cloves is more effective than the other spices. Traditionally, powdered cloves are used to clean the mouth and teeth and to prevent infection. Onions are used as an antidote to scorpion bites while ginger is found effective after childbirth, by causing the muscles to take to their original shape (Ella, Personal Communication).

In some studies, essential oils of spices have been shown to produce synergistic effect with other salts and other preservatives. Studies are now encouraged to produce preservatives from natural food sources that have active antimicrobial components and to use less of the synthetic anti-microbial compounds (Gould, 1990).

Though studies have been carried out on the synergistic effect of spices and other preservative agents, no studies have been carried out on the effect of spices and antibiotics on pathogenic bacteria causing foodborne infection. The objective of this study, therefore, are :

(1) to study the antimicrobial effect of various spice extracts on some pathogenic food borne bacteria, and,

(2) to study the synergistic effect, if any, of spice extracts and antibiotics on those pathogenic bacteria.

Chapter 2:

REVIEW OF LITERATURE

2.1 History

Spices have been known to be as zealously sought after as gold and accepted as currency in the late 13th century. Nations competed globally in search of new trade route for it, and even went to war for that course. Spice trade was the impetus behind the discovery of new continents and the emerging of Eastern and Western civilization (Dziezak, 1989). The word "spices" was adopted from the Latin word "species" which mean "fruit of the earth" (Dziezak, 1989).

The first authentic record of spices and herbs may be dated from the pyramid age in Egypt, approximately 2600 to 2100BC (Rosengarten, 1973). The antiquity of the use of aromatic herbs and spices is well attested in ancient writings in the old Testament in early Greek and Roman documents, and in medieval manuscripts (Heath, 1973). The first records in Egypt stated that onions and garlic were fed to 100,000 labourers who toiled in the Codex Ebers, an Egyptian medical papyrus dating to about 1550 BC gives more that 800 therapeutic formulas, of which 22 mention garlic as an effective remedy for a variety of ailments including heart problems, headache, bites, worms and tumors (Block, 1985).

In the fifteenth century, the alternative to the lack of refrigeration was the utilization of spices to camouflage the bad flavours and odors of decomposing foods. Such was the interest in spices at that time that, in 1492, Columbus was not only looking for gold and a short cut to Indies, but also for spices (Rosengarten, 1973).

Many of the spices and herbs used today were known to the people of the ancient cultures throughout the world for preservative and medicinal purposes.

It is not know how the ancient people obtained this knowledge but recent scientific experiment have proved them to be true (Zaika .1988)

According to the International Organisation for Standardization (ISO), there are over 70 spices and herbs officially recognised, but more than 350 to 400 spices are used in different countries by different people with different religion and climate (Nakatani, 1994).

2.2 *Botany*

Onion, *Allium cepa,* belongs to the family liliaceae and genus *Allium* and is monocotyledonous. *A. cepa* is a biennial herb. All parts of the plant produce a strong onion odor when crushed. The seed produces the first primary root, after which all other roots are adventitious, arising from the short stem. A very short flattened stem is produced at the base of the plant, which increases in diameter as growth continues. The leaves are produced by the apical meristem.

The onion bulb which is the main edible part, is formed when the conditions are favourable (i.e. day length and temperature). Under these conditions, when the plant has reached a certain stage of growth, the thickening of leaf bases forms a bulb, a short distance above the stem. The outer leaf bases are thin, fibrous and dry, followed by leaves with thickened bases and the innermost leaves form thickened sheath bases aborted blades. Later, as the bulb matures, the leaf sheath weakens above the bulb and falls over (Brouk, 1975; Purseglove, 1988). Onion seeds deteriorate quickly in hot humid climate often losing their variability in less than a year.

Onion can be grown under a wide range of climatic condition, but they succeed best in mild climate without excessive rainfall or great extreme of heat and cold. Early growth is favoured by cool condition with an formation assists the process. For this reason, garlic may fail to produce bulbs in the tropics where it is usually grown at higher attitudes (Purseglove, 1988).

It has been suggested that the compound alliin may be synthesized in the leaves, because it was found that shoot forming callus tissues contained alliin although at a much lower concentration than in bulbs (Hayashi, *et al*, 1993).

It was found that garlic constituents, specially the antimicrobial agent, was maximum during post harvest after ripening, after which it decreased at a rate depending on cultivar and storage conditions. Activity was lost fairly rapidly in garlic paste and juice but was retained for more than a year in dried material (Hovadik, 1983).

Ginger, *Zingiber officinale,* is also a monocot belonging to the family Zingiberaceae. Ginger is a slender perennial bulb with robust branched rhizome borne horizontally near surface of soil, bearing leafy shoots close together. The rhizomes are thick and compressed, often palmately branched and pale yellow flesh, covered with small distichous scales with an encircling insertion and with fine fibrous roots in top layers of soil. Ginger is always propagated by portions of the rhizomes, usually about 2.5 – 5 cm long, which have at least one good bud. Ginger is mainly cultivated in the tropics from sea level to 1500m, but it can be grown successfully over more diverse conditions than most other spices. It requires an adequate rainfall of about 1500mm or more per annum, preferably with a short day season with light temperature for at least part of the year. It thrives best on medium loans with a good supply of humus, particularly after cutting down forest or bush (Purseglove, 1988).

Clove, *Syzguim aromaticum,* plant is a tropical evergreen tree native to the Moluccas. The tree is 9 – 12m tall. It bears its flowers in terminal cymes. The floral buds which are used as spices, are about 1.5m long and their bulk is formed by the fleshy calyx tube which terminates in four triangular tubes.

The calyx tube is termed as hypanthum and encloses the inferior ovary. The whole bud resembles a nail (Brouk, 1975). The green colour of the flower changes to dark brown on drying.

Fenugreek, *Trigonella foecum – graecum*, is a dicotyledonous annual herb belonging to the family Leguminosae. Its leaves and seeds are both used as food. The fruit is a sickle shaped, beaked legume, containing 10 – 20 hard seeds, 4 – 6mm long and 2 – 3mm wide. They are yellowish brown and a deep furrow divides them into two parts, the larger occupied by the cotyledons and the smaller by the radicle. Though fenugreek seeds are used as spice, they also have a very nutritive function as they are rich in proteins (Brouk, 1975).

It has been shown that herbs are best harvested on a clear day in the early morning, as the essential oils are greater at that time. The oils diminish as sunlight and temperature increases during the day. When exposed to open air, light and high temperatures, the essential oils decline, flower changes and the herb quality rapidly decreases (Splittstoesser, 1990)

2.3 *Antimicrobial and Medicinal Properties of some spices.*

A wide range of naturally occurring antimicrobials have been recognized and preferred for use in the food industry. The natural inhibitors are preferred more as component of systems that can together enhance the effectiveness of preservation with advantages in product quality (Gould, 1996).

2.3.1 Garlic – *Allium sativum* is known to be of great medicinal importance. Garlic oil and aqueous extracts of garlic are known to have a wide range of properties. They are antibacterial, antifungal and even have produced impressive results as possible anticancer agents.

Aqueous garlic extract, at 0.5, 0.7, 0.8 and 0.8% (w/v) was found to inhibit *S. typhimurium, B. cereus, B. stearothermophilus,* and *S. aureus* respectively. 8% of the extract was required for 100% lethality on *E.coli* and *S. aureus* while 10% of the extract required for 100% lethality on *B.stearothermophilus* and *S. typhimurium* and to kill 89.7% of *B.cereus* cells (El-Rahman, 1994). The inhibition of *B. cereus* was further confirmed by Gandhi and Ghodekar (1988) who found that garlic extract also inhibited *Micrococcus flavus, Enterococcus feacalis* and *Salmonella velteverdes.* Other bacteria that were inhibited by aqueous garlic extracts includes *Shigella dysenterieae, S.flexineri, V. cholerae* and *E.coli.* Sato, *et al.*, (1990) worked on a wide range of bacteria using aqueous garlic extract and found it to be inhibitory too. The test bacteria were *E.coli, P.pneumoniae, E.cloacae, P.morganii, P.vulgaris, S.enteritidis, S.epidermidis, S.agalactiae, B.cereus,* and *L. monocytogenes* which were inhibited by 1.25% (v/w) of extract solution. *S. typhimuium, Y.enterocolitica, V.mimicus, V. parahemolyticus* and *C.jejuni* were inhibited by 0.62% extract solution. *P.aeruginosa, P.fluorescens* and *P. cepacia* were inhibited by 5, 2.5 and 0.13% of the extract respectively.

Aqueous garlic extract was inhibitory to growth of *Xanthomonas campestris*, a common tomato pest. On soaking the tomatoes in culture suspension of *X. campestris* and then treated with garlic extract

(30g/100ml), and 85%, decrease in the disease was observed (Mangamma, *et al.,* 1991).

Aqueous garlic extract was also tested on soil nematode, *Meloidogyne incognita.* The aqueous extract was considered highly nematicidal for this and other spices as it killed the nematodes in less than 40mins. Infected plants were sprayed with the extract, nematodes killed and seeds sown in treated soil grew better then the control in untreated soil (Sukul, *et al.*, 1974).

Ajoene, another antimicrobial principle of garlic also has been found to inhibit bacteria and fungi. *B.cereus,* were inhibited at 5mg of ajoene/ml. *E.coli, K.pneumoniae* and *Xanthomonas maltophila* had MIC values of below 100 – 160 mg/ml. Ajoene also inhibited yeast growth at concentrations below 20mg/ml (Naganawa, *et al.*, 1996). It also inhibited Aspergillus *niger* at 20µg/ml (Yoshida, *et al.*, 1987).

Allicin was found to be nematicidal, and at 25 ppm for 5mins, as a root dip treatment for tomato seedlings is effective against *Meloidogyne incognita* (Gupta and Sharma, 1993).

Phytocinides from garlic and (to a lesser extent) onion, have been found to reduce the heat resistance of bacteria. As a result, bacteria considered almost resistant to heating were killed at a much lower temperature (Grzybowski and Lewicka, 1987).

Garlic also has antitumor properties. It is being used in studies against cancer with positive results (Unikrishnan and Kuttan 1980; Hayes, *et al.,* 1987; Sadek, *et al.,* 1994).

Although a large number of authors have established the bacteriostatic effect of garlic, it appears that the alcoholic garlic extract had no effect on

B. stearothermophilus and it spores, *B.coagulans, E.coli, S.aureus,* and *Salmonella* (Liu and Nakano, 1996). *S.aureus, S. subtilis, B. anthracis, K. peumoniae, P.vulgaris* and *P.morganii* were not inhibited by aqueous garlic extract at 1 – 10 mg/ml while *P.aeruginosa* and *E.coli* were inhibited at the same concentration (Lafont, *et al.*, 1984).

A mixture of garlic and other spices (garlic + oregano + pungent paprika) was found not to have improved the inhibitory effect of individual spices alone. The antimicrobial effect of garlic and oregano against some lactobacilli and pediococci involved in sausage fermentation was observed at concentrations higher than 2%;

the corresponding concentration for paprika and pepper was 5% (Gonzalez - Fandoz, *et al.*, 1996). 1% of garlic powder required 7 days to decrease Scott A strain of *L. monocytogenes* from 107 CFU/ml to <10 CFU/ml (Hefnawy, *et al.*, 1993).

Mycelial growth and toxin production by *Aspergillus parasiticus* were inhibited by garlic concentration of 0.3 – 0.4% in broth. However, when grown on rice, aflatoxin could still be detected at garlic concentrations of upto 1.25% while aflatoxin G, could be detected at concentrations of upto 1. 25% garlic. Furthermore, on bread containing no garlic, profuse fungal growth occurred, whereas on slices treated with garlic no growth was observed after 4 days of incubation at 30°C (Graham and Graham, 1987).

2.3.2 Cloves. Essential oil of clove flower buds was shown to have high antimicrobial activity. Essential oils containing phenols and phenolic compounds have most interesting inhibitory effects. Among all the spices having phenolic compounds, clove has the highest eugenol content, 91 – 95%. *S.aureus, B.subtilis, Mycobacterium smegmatis, E.coli, P.vulgaris* and *P. aeuiginosa*, were the test bacteria used to compare effectiveness for eugenol, clove oil and other phenolic compounds containing spices. Eugenol had the highest inhibiting activity (at 10 –100% µl/ml), followed by clove oil (Martinez- Nadal, *et al.*, 1973). The clove oil was also tested against *E.coli, P. aeruginosa* and *S.aureus* and they were all inhibited (Deans and Ritchie, 1987). Activity of clove oil against 25 strains of *L. monocytogenes* showed that levels of inhibition was found at 1– 10mg/ml of growth medium (Deans, *et al.*, 1995).

Farag, *et* al., (1989) showed that the Gram positive bacteria studied were inhibited by lower amounts of the antimicrobial compounds than Gram negative bacteria. Concentration of 0.25 –12mg/ml of clove oil was required to prevent microbial growth (both Gram negative and Gram positive bacteria). The Gram negative bacteria were *P.fluorescens, S.aureus. Micrococcuss spp., Sarcina spp* and *B.subtilis*.

Clove oil was found effective in killing many insects that are pest to cowpea beans. The best effect was achieved when the oil was in direct contact with the insect (Lale, 1991).

Though it has been established that clove oil inhibits many bacteria the inhibitory concentrations vary, as reported by various authors. This is probably due to the fact that different factors are involved when carrying out the antimicrobial studies. They include, method of study, test medium used, variety of spices, strain difference, etc (Zaika, 1988).

The activity of eugenol is not only antimicrobial, but eugenol and clove infusion showed sporostatic effect on the spores of *B.subtilis* spores when subcultured on nutrient agar, they did not grow into vegetative cells. However, when after storing them in clove infusion or with eugenol, the spores were observed to undergo germination (Al-Khayat and Blank, 1985). Eugenol was also found to be most effective, in comparison with other spice extract when tested against *S.typhimrium, S.aureus*, and *V. parahemolyticus* (Karapinar and Aktug, 1987).

Aqueous extract of cloves has good inhibiting properties too. Studies on kunun zaki, a Nigerian cereal drink showed that despite the presence of 4 spices (ginger, clove, black and red pepper), only cloves could inhibit the presence of *S. aureus* and *E.coli,* but the low pH of the drink was effective in keeping the population low (Onuorah, *et al.,* 1987).

Furthermore studies on aqueous extract of cloves showed that no inhibition was observed for *S. aureus, E.coli* and *K. pneumonia* while 40 – 70% inhibition was observed for *B.anthracis, V.cholerae* El Tor, *S.typhi, P.vulgaris* and *P.morganii* (Lafont, *et al.*, 1984).

Ethanol extracts also showed good inhibitory properties on *Y. enterocolitica.* It was also proved to be bacteriostatic in nature, the reduction of cell counts being in a concentration dependent manner (Bara and Vanetti, 1995).

It was also reported that both ethanol and aqueous extracts of clove were active against penicillin G resistant strain of *S.aureus* (Perez and Anesini, 1994).

Cloves and their extracts (i.e. eugenol and other eugenol compounds) showed antifungal activity too. Of the 21 isolates of *Fusarium oxysporum* from Japan and 9 other pathogenic fungi from Indonesia, the eugenol and its other compounds played a very significant role in suppression of growth of the tested pathogenic fungi. It was also found that eugenol inhibited radial growth and sporulation. The mycelial tips were swollen, branched and distorted due to eugenol treatment. These results suggest a favourable trial for field research to use these compounds as fungicides and organic treatment (Tombe, *et al.*, 1995).

Apergillus flavus and *A. vesicolor* were also inhibited by eugenol at 0.4mg/ml or less. However, no effect was observed to inhibit toxin production (Hitoko, *et al.*, 1980). *A. parasiticus* was completely inhibited (Farag, *et al.*, 1989). It was found that for clove oil, exposure to gamma – irradiation, caused the inhibitory activity of the clove oil to remain unaffected while that of cinnamon was affected, against *A.parasiticus* (Sharma, *et al.*, 1984).

The antiviral substance from cloves identified as euginiin was found to be active against herpes simplex virus at a concentration of 10µl/ml (Takechi and Tanaka, 1981).

2.3.3 Onions - Onion is one of the oldest home remedies. The active compound is a sulphurous compound. Though onion and garlic belong to the same genus, both vegetables have sulphurous compounds common to the genus, it is surprising to note that garlic has a very wide spectrum of activity compared to onion, which has a very slight activity or none at all. Onion although inhibitory to a few microorganism, can by no means be compared to the activity of garlic.

Onion was found to have no effect against *E.coli, S.typhimurium, S. havana, S.paratyphi, S. flexineri* and *S. dysenteriae* while garlic inhibited all the above mentioned test bacteria (Mehrabian, *et al.*, 1992). However, onion did show activity in some studies. Certain fungi, *E.coli,* and *S.aureus* were slightly inhibited by onions (Boer-E-de, 1985; Hughes and Lawson, 1991). This slight inhibition is considered unimportant for preservative purposes. The seeds of onions are reported to have antimicrobial properties contained in the protein which is 10kDa and called *Ace-AMP1.* It inhibits growth but is active only in Gram positive bacteria but apparently non-toxic to Gram negative bacteria and cultured human cells (Cammue, *et al.*, 1995).

Although onion does not have an antimicrobial component, it does have many other medicinal properties, antimutagenic and antitumorigenic principals (Sadek, *et al.*, 1994). Onion has also been shown to be quite effective in the treatment of respiratory disease because it has the ability to liquefy phelgm and prevent its recurrence (Bakhru, 1996).

2.3.4 Ginger – *Zingiber officinale* does not have any specific active component. Moreover, the antimicrobrial properties of ginger are not well documented and the information available is quite scanty. Very few studies show ginger to give positive antimicrobial results. Ginger moderately inhibited *Aspergillus niger* at 37°C, but had either no effect or very insignificant effect against *B.cereus, L. acidophilus, Mycoderma* spp and *S. cerevisae* (Meena and Sethi, 1994). Ginger oil was reported to have good activity against *Aeromonas hydrophila, B. subtilis, Flavobacterium sauveolans, Leuconostoc cremoris, Serretia marcesans* and *S.aureus* in undiluted form (Deans and Ritchie, 1987). In diluted form, only *B. subtilis* was adequately inhibited. However, Lafont, *et al.,* (1984) showed that not inhibition was observed on *B. subtilis* by aqueous extract, although slight inhibition (20 – 40%) as observed for *S. aureus, P. aeruginosa*, and *V.cholerae* El Tor. Ginger also caused 70% inhibition of the sugarcane mosaic virus, but the effect reduced with dilution, with water, of the extract (Joshi and Prakash, 1978).

Gingerone A, isolated from ginger exhibited a moderate anticoccidium activity and a strong antifungal effect against *Pyricularia oryzae*, a plant pathogen (Nakatani, 1994). The pungent principle of ginger was shown to be responsible for the prevention of hatching of schistome eggs.

Tablets of ginger root powder and of ginger aqueous extract, as well as capsules for the ketonic fraction (pungent principle) have been given to school children suffering from schistosomiasis. The pupil's bloody urine was stopped by this treatment; the schistome egg counts in the urine also dropped, indicating some *in vivo* effectiveness of ginger on schistosomiasis (Sofowara, 1980).

Ginger has many medicinal properties and is even antioxidant nature. Ginger rhizomes are used in Brazil as a carminative, diaphoretic and antispastic against intestinal colic (Sertie, *et al.,* 1992). (6), (8), and (10) – gingerdiol were three compound identified to have cardiotonic activity. These compounds are also known as pungent principles of ginger (Shoji, *et al.,* 1982).

2.3.5. Fenugreek - Though the antimicrobial activity of fenugreek (*Trigonella foecum – graecum)* is not widely reported, there are a few studies carried out showing positive results. Fenugreek also has many medicinal and nutritive properties. Fenugreek showed good antibacterial activity against *B.cereus, B.subtilis, S.aureus, Sarcina lutea, E.coli,* and *P.vulgaris* at 50 – 100mg/ml of ethanol and aqueous extracts. Ethanol and aqueous extracts showed better activity than compared to hexane and chloroform extracts (Bhatti, *et al.,* 1996). Chloroform extract of fenugreek inhibited *V. cholerae* El Tor (Lafont, *et al.,* 1984).

Fenugreek has many medicinal properties too. Since fenugreek seed powder has a hypoglycemic effect, it is considered beneficial for diabetic mellitus, as a non-insulin substitute (Sharma, *et al.,* 1996). It is also used as an antihelmentic against common nematodes, as antipyretic, diuretic, supprative, for heart diseases, chronic coughs and enlargement of liver and spleen (Bhatti, *et al.,* 1996).

It has also been suggested that fenugreek tea, when taken during the early stage of respiratory tract infections such as bronchitis, influenza and catarrh is beneficial. The perspiring effect of fenugreek helps the body to dispel toxins and shorten the gestation period of fever (Bakhru, 1996).

2.3.6 Other Spices - Other spices, which show a high degree of inhibition are oregano, thyme, and mustard and to a lesser extent turmeric, curry leaves, mint and many other spices.

Oregano (*Origanum vulgare*) aqueous extract completely inhibited *S.aureus, P.aeruginosa* and *Proteus vulgaris* and those not inhibited completely were, *V.cholerae, K.pneumoniae* and *S.typhi* (Lafont, *et al.*, 1984). Its effectiveness against *P. vulgaris* and *S.aureus* was further confirmed by Akgul and Kivanc (1989), while *E.coli* and *Enterobacter aerogenes* were less sensitive than *S.typhi, P.aeruginosa* and *B.cereus*. However, it was also observed that though *S.aureus* and *P.vulgaris* were sensitive to oregano, *P.aeruginosa* was resistant to it (Kivanc and Akgul, 1986). Ethanol extract of oregano was found to be bactericidal to *Y.enterocolitica* at 4.1 – 4.4% w/v (Bara and Vanetti, 1995).

Thyme (*Thymus vulgaris*) aqueous extract completely inhibited *B. anthracis* and *P. vulgaris* but not *S.aureus, B.subtilis, V.cholerae, E.coli* and *K.pneumoniae* (Lafont, *et al.*, 1984). Hexane extract of thyme also showed that the volatiles were inhibitory against *P.aeruginosa* which was resistant to many other spices (Kivanc and Akgul, 1986). Thyme essential oils were required in as low as 0.25mg/ml for inhibition of *P.fluorescens, E.coli, Serretia marcesans, S.aureus,* and *B.subtilis* (Farag, *et al.*, 1989). Thymol, the active phenolic component of thyme showed high inhibitory and bactericidal activity against *E.coli, S.aureus* and *B.megaterium* (Lattaoui, *et al.*, 1994). Thymol and thyme oil were further demonstrated to have antagonistic effects against *S.typhimurium* and *S.aureus*. This effect was increased under anaerobic conditions and other environmental factors (Juven, *et al.*, 1994).

Kanemaru and Miyamoto (1990) showed that brown mustard (*Brassica juncea)* and its principal component, allyl isothiocynate (AIT) had high antibacterial activity. Concentration to mustard required to inhibit growth for 24hrs were 0.138, 0.104, 0.064, 0.043 and 0.089% - for *S.aureus, E.coli, P. seudomonas vulgaris, P.fragi* and *P.aeruginosa* respectively; corresponding values for AIT were 14.5,12.3, 6.5, 3.6 and 7.2 ppm respectively. It was also shown that at low concentrations it inhibited *Aspergillus oryzae, Saccharomyces cerevisae, B.subtilis* and *Daphnia pulex* (Kono, *et al.*, 1995). AIT shows promise as a food preservative.

Turmeric (*Curcuma longa*) contains the active principle, curcumin. Its rhizome is used as stimulant, tonic, carminative, applied to wounds and as antihelmentic and antiparasitic (Manzoor- I – Khuda, 1984). Alcoholic extracts of turmeric at concentration of 0.5 –5mg/ml as well as curcumin and essential oil, from turmeric, rhizome at 5 – 100µg/ml, inhibited species of *Sarcina, Corynebacterium, Clostridium, Staphylococcus, Streptococcus* and *Bacillus*. The alcoholic extract and essential oil showed bactericidal activity while curcumin acted as a bacteriostatic agent with respect to Staphylococci, (Lutomstki, *et al,* 1974). 0.5% of curcumin was known to cause a regression of pre–established cholesterol gallstones (CGS)., but it was found that feeding a controlled diet did not cause regression of CGS (Hussain and Chandrasekhara, 1994). The rhizome is also known to have anticancer activities. It inhibited the growth in Chinese Hamster Ovary at 0.4mg/ml and was cytotoxic to lymphocytes and Dalton's lymphoma cells at the same concentration (Kuttan, *et al.*, 1985).

2.4 *Active Principles of Spices and their Activity*

Spices may have one or more active principal constituents which may be active on different microorganisms.

Cloves have the active component called Eugenol. This may exist as eugenol, eugenol acetate or methyl eugenol. Other spices that have eugenol are bay, pimento leaves and berries.

Eugenol in cloves is one of the bound volatile compounds (Menon and Narayan, 1992). Eugenol, (as do a large group of natural products including many essential oil constituents) has the formula R-C-C-C, in which R is the phenol group or hydroxy phenyl radical (Ramstad, 1959). It has been observed that the normal chain is more active than the branched chain (Oster, 1971) It could possibly be due to this reason that eugenol has greater activity than chavicol, another phenolic compound (Martinez –Nadal, *et al.*,1973).

It has been hypothesized that physiochemical properties of the phenolic molecule may be due to the lipophilic and lipophobic nature of the molecule. The polar hydroxyl group have an affinity for water while the benzene ring and the alkyl group have affinity for lipid materials, therefore, by their orientation at the oil-water interface of the cell, results in a decreased activity. Phenolics are widely used as germicides and antiseptics and are effective provided they are used in proper concentrations (Oster, 1971). The extract of cloves reduced the histidine decarboxylase activity by 40%. However, the ethanol extracts had a higher inhibitory action upon histadine, lysine and ornithine decarboxylase (Wendakoom and Sakaguchi, 1995).

The active component of fenugreek are not known, but the seeds are known to contain sapogenins, flavonoids, betains, sugars and gums, acids, volatile oils and fixed oil about 7% (Parry,1969;Bhatti, *et al.,* 1996).

Onions have, in very few cases, exhibited their antimicrobial properties, but whatever their medicinal properties it is due to the sulphur compounds present, a characteristic of *Allium* group. Steam distillation of onion produces propionaldehyde and dipropydisulphide while solvent freon mixed with water at $0^{o}C$ yields the lacrimatory factor whose activity is that of irritation and repugnance to the animal (Block, 1985) The reason for the smudge disease and neck rot attacking white bulbs much more readily than coloured bulbs, is aimed at two phenol compounds

proto-catechus acid catechol which are present in the dry outer scales of coloured onions and are toxic to the fungi causing the diseases. These compounds appear to account for much of the resistance of coloured varieties (Jones and Mann, 1963).

Garlic contains two known compounds that are antimicrobial agents. They are allicin and ajoene. Of the two, allicin has been known to have more widespread effects while the effects of ajoene are known to be limited in inhibiting the microbial population. However, ajoene has higher antifungal activity than allicin. Much is not known about the mechanism of ajoene reaction, but the disulphide bond in ajoene appears to be necessary (Naganawa, *et al.,* .1996). Allicin is a very much studied compound. Steam distillation, the most harsh technique of extraction yields diallyl sulphide. A more gentle technique required pure ethyl alcohol at a subzero temperature to yield alliin, a precessor of allicin. Though allicin is responsible for the garlic odor, a whole garlic exhibits little or no odor until it is cut and the action of allinase converts alliin to allicin (Block, 1985).

It was found that though oregano was not very effective in preventing staphylococcal growth it did affect synthesis of staphylococcal thermonuclease and enterotoxins. Oregano at 2% did not allow synthesis of enterotoxins B, C and D (SEB, SEC and SED) and partially inhibited that of enterotoxin A (SEA). Lower level of oregano (0.12% and 0.5%) actually simulated SEA . SEB and SED production (Gonzalez- Fandoz, *et al.*, 1996).

2.5 *Effect of Spices on Bacteria Resistant to Antimicrobial Agents.*

A great number of bacteria are emerging as multi-resistant bacteria in drug therapy. The occurrence of multi-resistant bacteria is an emerging problem in human medicine and the effects of resistance are being noted on an ever-increasing scale, whether it is the treatment of nosocomial bacteremia in New York City or community acquired dysentry in Central Africa.

Moreover, multi-resistant bacteria are diminishing the ability to control the spread of infectious diseases (Tenover and Hughes, 1996; Tenover and McGowan, 1996).

A few studies show that certain spices have synergistic effect with other spices or with spices and antimicrobiai drugs. This effect has also been observed with the drugs the microorganism is resistant to.

2.5.1 Mode of action of antimicrobial agents – Antimicrobial drugs are a class of compounds that owe their relative toxicity to fundamental biochemical differences between procaryotic and eucaryotic cells, their toxic effect being the consequence of their ability to inhibit one essential biochemical reaction specific either to the procaryotic or to the eucaryotic cells. The action may be by inhibiting synthesis of bacteria cell wall, inhibiting organelle function or by destroying the cytoplasmic membrane (Stainer, *et al.*, 1990).

An example of the penicillin group are cloxacillin (semi synthetic penicillinase resistant) and ampicillin (semisynthetic extended spectrum). The major antibacterial action of penicillin is from their ability to inhibit a number of bacterial enzymes namely, the penicillin binding proteins (PBPs), that are essential for peptidoglycan synthesis. Another mechanism of action is their ability to trigger membrane associated autolyic enzymes that destroy the cell wall. Other minor mechanism include inhibition of bacterial endopeptidase and glycosidase and enzymes involved in bacterial cell growth (Yao and Mollering, 1994).

Another drug, nitrofurantoin, belongs to a class of compounds consisting of a primary nitro group joined to a heterocycle ring. Its role in human therapeutics is limited to the treatment of the urinary tract infections. The precise mechanism of action is unknown, but the drug is believed to inhibit various bacterial enzymes and also damage the bacterial DNA (Yao and Mollering, 1994).

2.5.2 Resistance of bacteria to antimicrobial agents – For more than a few decades now, pathogens have been developing resistance to antibiotics, particularly to antibiotics of primary choice in treatment of diseases. This raises the risk of using alternate treatment with higher doses or more potent drugs that under normal circumstances may not be used or prescribed.

Pathogenic bacteria may be resistant to a single or more than one antibiotic in which case they are referred to as multiply resistant strains (Stainer, *et al.,* 1990). Though the search for new antibiotic continues, it is believed that most of really effective antibiotic have probably already been discovered (Stainer, *et al.,* 1990). Virtually all Staphylococci are now resistant to penicillin G. Many strains are also resistant to methicillin.

Several reasons have been attributed to the resistance of bacteria to antibiotics of choice. A common reason for emergence of multiply resistant strain is when patients are simultaneously treated with two or more drugs. In such cases, this may lead to treatment failure in the patient and spread of these resistant bacteria to the other host. One approach to this reasoning in that temporal fluctuation in the concentration of the antibiotics in course of normal treatment regime (Lipsitch and Levin, 1997).

The resistance problem is further complicated by the fact that the use of antibiotics is not limited to humans. It is also of much use in veterinary medicine, animal husbandry, agriculture and aquaculture. In the latter two cases, the risk of spread is even greater as it is directly, consumed by humans. Careful ministration of antibiotic therefore becomes imperative (Young, 1994; Tenover and Hughes, 1996). As antibiotics represent one of the most commonly prescribed medical therapies and also due to wide range of use, the administration of antibiotic is overly misused, Unnecessary administration of antibiotics also results in emergence of infection with antibiotic resistance of bacteria.

This again leads to treatment failure and thus patient mortality (Kollef, 1994).

The mechanisms used by bacteria to become resistant or multi-resistant are many. Resistance to certain antibiotics develop in bacteria through three basic processes: mutation, transduction and conjugation (Rickman, 1994). The mutation may be a chromosomal gene which modifies the structure of the cellular target. An example of this is the mutationally acquired streptomycin resistance (Stainer, *et al.*, 1990). Normally, this antibiotic deranges bacterial protein synthesis by attachment to one of the protein in the ribosome. Mutation of the gene that encodes this ribosomal protein destroys the ability of the protein to bind streptomycin, but they do not substantially affect ribosomal function (Stainer, *et al.*, 1990).

Resistance is also mediated by certain mobile elements (plasmids, phages, etc) Many plasmids carry genes that confer on the host cell resistance to antibiotics and other toxic agents. Plasmids of these types are called R- factors. They spread rapidly by conjugative transfer through a bacterial population and persist under the selective pressure of drug treatment. Some are self- transferable and other are mobilized by other cojugation plasmids (Stainer, *et al.*, 1990). An example of genetic transfer is the transfer from saprophytic, non pathogenic species to pathogenic *S. preumoniae* and *N. meningitidis* which introduced multiple changes in the penicillin target molecules, leading to rapidly growing penicillin resistance. Also in Enterobacteriaceae, a succession of minute mutations has generated new beta – lactamases with increasingly expended spectrum now covering practically all available beta –lactam antibiotics (Pechere, 1993; Livermore, 1996).

Another mechanism is the use of multidrug resistance pumps (MDR). They arise from three different gene families and are widespread in bacteria.

For example, in *E. coli* alone there are seven distinct MDRs, the commonest belongs to the major facilitator family of membrane translocases; this type of MDR is closely related to specific antibiotic extrusion pump such as the tetracycline/H+ antiporter. The similarity in design and the high incidence of the apparently independent evolution of MDRs suggested that the property of multidrug resistance might have resulted from a loss specificity in a specific hydrophobic drug efflux pump (Lewis, 1994).

The process of manufacturing antibiotics could be yet another way of acquiring resistance. It has been shown that a number of antibiotics preparations employed for human and animal use are contaminated with chromosomal DNA of antibiotic producing organism. The DNA contains identifiable antibiotic resistance gene sequences the uptake of which, by bacteria, leads to incorporation into bacteria relicons which leads to generations of antibiotic resistance (Webb and Davies,1993)

2.5.3. Synergism – Various types of microorganism are involved in food spoilage as various types of microorganism occur within a foodstuff at a time. If conditions are favourable for all, then bacteria usually grow faster than yeast, and yeast faster than molds. The most widely recognised types of association are the antibiotic (antagonistic) or symbiotic (mutually helpful) relationships. Two kinds of microorganisms, may be synergistic, i.e. when growing together, they bring about changes that neither could alone (Frazier and Westhoff, 1978).

The synergistic effect is not limited to the interaction between microorganisms alone. It may also be between two or more compounds to produce an effect on the microorganism. The synergistic effect has also been reported with salt and essential oils. This effect has been observed with 7 or 10% NaCl in combination with essential oils components such as cinnamaldehyde, perillaldehyde, critral citronellol and geraniol at less than 1mM,

whereas more than 10% and 1mM, respectively, or even higher were required to exhibit antimicrobial activity (Kurita and Koike,1982).

Test on mango pulp showned that in the presence of salt (16%) 0.3% cinnamon and 0.2% cloves were as effective in inhibiting mold spore germination as 3% cinnamon and 0.6% cloves in the absence of salt.

Cinnamon or cloves alone were also more effective as the salt concentration in the pulp increased (Shelef, 1983).

Anacardic acid isolated from the nut shell oil of cashew (*Anacardium occidentale)* is also found to have potent antibacterial activity against methicillin resistant *S. aureus*. Studies also show that combination of methicillin and anacardic acid showed synergistic effect. For example, methicilin and anacardic acid alone had MIC of 800μg/ml and 6.25μg/ml respectively, while in combination it was 1.56μg/ml and 3.136μg/ml respectively.

The mode of action of anacardic acid is hypothesized to be polar hypophylic carboxyl groups at one end of the molecule and hydrophobic chain at the other end. Furthermore, it was found that anacardic acid also markedly enhanced the activity of penicillin G against 2 penicillin resistant strain of *S. aureus* (Muroi and Kubo, 1996).

Synergistic effect has been observed between aqueous garlic extract and chloroquine resistant malarial parasites. Test on mice showed that a single dose of 50mg/ml of ajoene (a constituent of garlic) given on the day of infection, suppressed the development of parasitemia with no apparent toxic effects. However, combination of ajoene (50 mg/ml) and chloroquine (4.5mg/ml) given as a single dose on the day of infection, completely prevented the subsequent development of parasitemia in treated nice (Perez, *et al.*, 1994).

It has been suggested widely that the use of two or more compounds are superior to the use of a single compound, because apart from the synergistic effect, it is considered superior for the following reasons:

(1) to prevent or suppress the emergence of resistant strains,

(2) to decrease dose related toxicity as a result of reduced dosage, and

Chapter 3

MATERIAL AND METHODS.

The determination of antimicrobial activity of spices was carried out in two basic steps. The first step was the extraction of the spices used. This step includes extraction with petroleum ether which removes fats and oils whether volatile or fixed (Ribereau – Gayon, 1972). In this process, the extraction is more or less complete, while the extraction with methanol removes a wide group of compounds and lastly, the water extraction. The second step involves the antimicrobial testing of the extracts on bacteria.

3.1 *Collection of Spices*

The spices were obtained from Yankaba Local Market, Kano. The dried spices were bought and stored, while the fresh spices (onion, garlic, and ginger) were bought as required. The spices were brought in the period from February to May, 1998.

3.2 *Extraction from Spices*

Three solvents used for the extraction were petroleum ether (60 – 80°C), methanol and water. A general method of extraction was used. (Ribereau – Gayon, 1972).

3.2.1 Petroleum ether extraction – All the 5 spices were extracted in the same manner. Spices were washed (distilled water) and dried before use.

Cloves - Thirty grams of powdered cloves (*Syzgium aromaticum*) were shaken with 100ml of petroleum ether in 150ml conical flask for 18 – 20hrs and shaken in an environmental shaker at 140rpm. The mixture was then filtered using sterile Whatman filter paper. The filtrate was collected in a sterile container and the solvent was evaporated (desolventized) using the Rotavapour (Tainter, 1992). The remaining small amount of solvent (used to rinse the flask) was evaporated on the heating mantle. The extract was stored in sterile bottles at a temperature of 4°C, till required.

Garlic – The dried peel of garlic (*Allium sativum)* was removed before weighing. Then, 110.3g of garlic were cut into tiny cubes and shaken with 300ml petroleum ether in a sterile 500ml conical flask for 18 – 20hrs at 140rpm. The mixture was then filtered and the solvent evaporated, as carried out for cloves.

Ginger – Two hundred and five grams of washed, unpeeled and grated ginger *(Zingiber officinale)* was shaken with 350ml of petroleum ether in 500ml sterile conical flash and shaken for 18 – 20hrs at 140rpm. The filtration of mixture and desolventization was carried out as for cloves.

Onions – Fresh onions *(Allium cepa)* (182.2g) was peeled, washed, dried, weighed, cut into tiny cubes and shaken with 250ml of petroleum ether, in 500ml conical flash for 18 – 20hrs at 140rpm. The mixture was filtered and desolventized as stated above.

Fenugreek – Sixty grams of fenugreek seeds *(Trigonella_foecum – graecum)* were finely ground in an electric grinder for about 15-20 seconds. The fine powder was mixed with 150ml of petroleum ether in a 250ml conical flask for 18 – 20 hrs and shaken at 140rpm. The mixture was then filtered and the filtrate desolventized as done for cloves.

3.2.2 Methanol extraction – The extraction procedure used as follows: Thirty grams of cloves in 150ml of methanol, 90.1g of peeled garlic cut into cubes were mixed with 200ml methanol, 147.6g of ginger in 400ml methanol, 170.5g of cubed onions in 300ml and 30g fenugreek seed powder in 150ml methanol. Each mixture was shaken in a flask for 18– 20hrs at 140rpm. The mixture were filtered, desolventized and dried to a constant weight using the heating mantle. The extracts were stored in sterile bottles and stored at 4°C, till required.

3.2.3 Aqueous extraction – The water extracts were used directly in the filtrate form. There was no evaporation of water involved.

Cloves – Twenty eight grams of powdered cloves were shaken with 150ml of sterile distilled water in a 250ml sterile conical flask for 18 – 20hrs at 140rpm. The mixture was then filtered over sterile Whatman filter paper, and the filtrate was collected in a sterile container. The filtrate was then sterilized by vaccum filtering through Seize filter paper. This sterilized filtrate was used directly for antimicrobial testing. The amount of extract per milliliter was obtained by evaporating separately, 5ml of the filtrate and then weighing the dried extract obtained. Freshly prepared extracts were used in all cases.

Garlic- Two hundred and ten gram of peeled garlic was ground in an electric grinder and mixed with 90ml of sterile distill water and was shaken for 5hrs at 140rpm. This was then filtered over sterile Whatman filter paper. Then the mixture was centrifuged as recommended by Ahsan, *et al.*, (1996) The supernatant was then vacuum filtered as done for cloves.

Ginger – Hundred and seventy grams of unpeeled ginger was mixed with 150ml of sterile distill water and shaken for 5hrs at 140rpm. The filtration and filter sterilization was carried out as for cloves.

Onion – Peeled, washed and dried onions (190.14g) mixed with 100ml of sterile distilled water and shaken for 5hrs in a modification of Lafont, *et al* (1994). The filtration of the mixture and the filter sterilization of the extract was done as for cloves.

Fenugreek – Coarsely ground fenugreek (7.6g) and 150ml of sterile distilled water were shaken in 250ml flask for 11/2-2hrs at 140rpm, in a modification of the method used by Lafont, *et al.*, (1984) and Bhatti, *et al.*, (1996). The filtration and filter sterilization was repeated as for cloves.

TABLE I Preparation Of Stock Solution Of Extracts And Antibiotics

a)	Petroleum ether clove extract. Extract – 1.63g Methanol (diluent) – 1ml Sterile distilled water – 21ml	Extract concentration = 74mg/ml
*b)	Petroleum clove extract Extract – 1g 0.2% agar suspension 21ml	Extract concentration – 47mg/ml
c)	Petroleum ether garlic extract Extract – 0.23g Methanol - 1ml Sterile distilled water - 22ml	Extract concentration – 0.01mg/ml (10mg/ml)
*d)	Methanol garlic extract Extract - 1g 0.2% agar suspension 21ml	Extract concentration – 48mg/ml
e)	Petroleum ether ginger extract Extract - 1.04g Methanol - 1ml Sterile distilled water - 21ml	Extract concentration - 47mg/ml
f)	Ethanol ginger extract. Extract - 1g Methanol - 1.5ml Sterile distilled water - 21ml	Extract concentration – 22.2mg/ml
g)	Petroleum ether onion extract Extract - 0.44g Methanol - 1ml Sterile distilled water - 22ml	Extract concentration – 19mg/ml
h)	Methanol onion extract Extract - 1g Methanol - 1ml Sterile distilled water -20ml	Extract concentration –23.8mg/ml
i)	Petroleum ether fenugreek extract Extract - 0.97g Methanol - 1ml Sterile distilled water - 21ml	Extract concentration –44mg/ml
j)	Methanol fenugreek Extract - 1g Methanol - 1.5ml Sterile distilled water - 20ml	Extract concentration –47mg/ml

k) Ampicillin solution
Ampicillin powder - 2mg
Sterile distilled water - 50ml
Concentration – 40µg/ml.

l) Nitrofurantoin solution
Nitrofurantoin powder - 2mg
Sterile distilled water - 50ml
Concentration - 40µg/ml

m) Cloxacillin solution
Cloxacillin powder - 0.1mg
Sterile distilled water - 50ml
Concentration - µg/ml

* -The 0.2% agar suspension was used whenever the extract did not dissolve with 1ml of methanol as diluent (Remmal, *et.al, 1993)*

3.3 *Test Bacteria*

Escherichia coli and *Staphyloccus aureus* cultures were obtianed from Mr. E. D. Jatau, *Shigella flexineri, Pseudomonas aeruginosa* and *Salmonella typhi* from Mr. A. A. Odowumi and *Bacillus cereus* from Mr. C. M. Whong all of the Department of Microbiology Ahmadu Bello University, Zaria. All the bacteria were checked for purity before use. The biochemical testing of the test bacteria was in accordance with that stated by Cowan and Steel (1965).

S. aureus was purified on mannitol salt agar *B. cereus* was purified on *B. cereus* selective agar, and the remaining organisms on McConkay agar (ICMSF,1975, Oxoid Manual,1982). The purified colonies were Gram strained before storing on slants for further studies.

3.4 *Preparation of Inoculum*

Bacterial suspension was prepared by taking a loopful of bacterial culture from the prepared slant and introducing it into 5ml of sterile nutrient both in a bijou bottle. This was incubated at $37^{0}C$ for 15 to 20hrs (specific value per bacteria is stated below)

Meanwhile, a No 1 Macfarland Standard was prepared in a same sized bijou bottles to be used in subsequent inoculum preparations. The preparation was left standing for about 4hrs or until no further precipitate was observed (Baron and Finegold, 1990).

The inoculated nutrient broth which was kept for incubation was then compared for its turbidity against that of the Macfarland Standard (Baron and Finegold,1990). This was done by taking a white paper with a dark line drawn across it. The two bijou bottles (i.e. the Macfarland Standard and the incubated bacterial suspension) were then observed together against lighted background. When the clarity of dark line is observed as the same through both the solutions, then that turbidity of the bacterial suspension was used for antimicrobial sensitivity testing. The time of incubation was noted and subsequent preparation (for the antimicrobial sensitivity testing) were incubated for the same time.

This turbidity obtained gives an approximate number of cells within the suspension (1×10^8 CFU/ml) whose extract number of cells varies with the organism. For this purpose, to find the extact concentration, serial dilution was carried out. 1ml of the bacterial suspension was serially diluted in 9ml of saline. Concentration of bacteria in each of the dilution was determined using the pour plate method. The bacterial suspension (0.1ml) was plated on nutrient agar and colonies counted and the number calculated using the formula given by Cheesborough (1985) (Appendix 1).

For subsequent use during antimicrobial sensitivity testing, 0.1ml of the culture from the previous cell suspension was incubated (at time mentioned below) in sterile nutrient broth to obtain the desired turbidity, and then serially diluted to 10^{-5} dilution to use as inoculum.

3.6 *Sensitivity to Antimicrobial Agents*

Prior to antimicrobial sensitivity testing, the bacteria were subjected to antimicrobial agent sensitivity using the multidisc method to determine which antimicrobial agents the bacteria are resistant to. The antimicrobial agents used and their concentration in the disc for Gram negative bacteria are: Ampicillin (25µg); Kanamycin (30µg); naladixic acid (30µg); nitrofurantoin (50µg); cotrimoxazole (25µg); tetracycline (25µg); sulphatriad (300µg); gentamicin (10µg). The disc for Gram positive bacteria contained: Ampicillin (2µg); Cephaloridine (5µg); enythromycin (5µg); cloxacillin (2µg); Clindamycin (2µg); Penicillin (1 i.u.); Tetracycline (25µg); and cotrimoxazole (25µg).

The resistance of bacteria to and antimicrobial agent was determined when no zone inhibition was observed and bacteria grew around the disc. It was observed that all the test bacteria were resistant to Ampicillin (except *S. flexineri*). Gram negative bacteria were resistant to nitrofurantoin (except *S.flexineri*) while Gram positive bacteria were resistant to cloxacillin. *S.flexineri* was inhibited by both the antibiotics and the MIC was 6.25mg for both.

3.7 *Combined Effect of Antimicrobial Agents and Extracts*

The method chosen for sensitivity testing was the broth dilution method. This method is preferred over the agar diffusion method as both MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) can be quantitated using the broth dilution method (Davidson and Parish, 1989; Sahm and Washington, 1990).

Three sets of Khan tubes were used, each set containing 8 khan tubes containing sterile Mueller Hinton broth. One batch of 3 sets was prepared for each of the seven test bacteria. One tube in each batch was used as control.

In the first set of tubes, only the extract (1ml) (Table 1) was added and then doubling dilution was carried out. After this 0.1ml of the inoculum was added to each tube. The tubes were then incubated at 37°C. Readings were taken after 18 – 24hrs of incubation.

In the second set, 1ml of the extract and the first antibiotic (Ampicillin – 0.63ml for Gram negative bacteria and 0.0 5ml Table 1) for Gram positive bacteria) was added to the first tube and then doubling dilution was carried out. Then, 0.1ml of the inoculum was added to each tube and the set was incubated at 37°C for 18 – 24hrs after which readings were taken.

In the last set, 1ml of the extract was added to the second antibiotic (i.e. nitrofurantoin - 1.25ml for Gram negative bacteria and cloxacillin - 0.3ml for Gram positive bacteria) and then doubling dilution carried out. Then, 0.1ml of the inoculum was added to each tube and incubated at 37°C for 18 – 24hrs after which readings were taken.

One tube containing the sterile broth was inoculated with 0.1ml inoculum. Extract or antibiotics were not added. This tube was incubated along with the rest of the batch(control).

The above procedure for the three sets (1 batch) was for one test bacteria. The same procedure was carried out for the seven test bacteria.

3.8 *MIC and MBC Determination*

MIC (minimum inhibitory concentration) is at the point when the lowest dilution shows no growth, but at this point the change (inhibition) is reversible. However, when the inhibition of growth is irreversible in the absence of the antimicrobial agent, then this is the bactericidal effect of the drug. The lowest point at which this occurs is the MBC (Davidson and Parish, 1989).

After incubation, the tubes were studied and the tubes showing no growth were selected. Then, 0.1ml of this test mixture was pipetted out and added to sterile Mueller Hinton broth and incubated for 24hrs. If growth was observed the effect was inhibitory, and if no growth was observed, then the effect was bactericidal. The lowest dilutions at which these effects (i.e. inhibitory or bactericidal) were observed were recorded as MIC and MBC points for the particular extract and bacteria (Davidson and Parish, 1989).

The antimicrobial activity varied with different spice extracts. In each table, the range between the lowest and highest MIC values varied, particularly in tables 3 and 4. In table 3, the lowest MIC is 0.167mg/ml while the highest is 2.3mg/ml.

It was generally observed that with most petroleum ether extracts, complete bactericidal effect was observed for most of the bacteria. This effect was more pronounced for petroleum ether clove extracts.
The synergistic effect was more pronounced for clove, garlic and ginger extracts but not so much for fenugreek and onion extract. This effect was sometimes observed for both antibiotics or sometimes only with one of the antibiotics. Also, it was sometimes with either the MIC value or the MBC value.

In table 3, petroleum ether extracts had a greater effect on Gram negative bacteria while methanol extract had greater activity on Gram positive bacteria. On the other hand, aqueous extracts showed greater effect on Gram positive bacteria than on Gram negative bacteria.

Table 4 showed that in general petroleum ether extracts had greater effect on bacteria. *B. cereus in* particular required lower amount of the extracts than the other bacteria and also showed synergism with both antibiotics. Methanol extract, on the other hand, required between 3.6mg/ml – 23mg/ml for MIC or MBC (except *P.aeruginosa*). Aqueous extracts were not effective on *E. coli* and *P.aeruginosa*.

Table 5 showed that only petroleum ether extracts of ginger had any effect. Methanol extracts had effect on *B. cereus* only. With extract alone, the MBC points were very high.

TABLE 2: YIELD OF EXTRACT OBTAINED AFTER EXTRACTION.

EXTRACT		WEIGHT OF SPICE(g)	VOLUME OF SOLVENT (ml)	WEIGHT OF EXTRACT (g)
Clove	Petroleum ether	30	100	1.63
Garlic	"	110.3	300	0.23
Ginger	"	205	350	1.04
Onion	"	182.2	250	0.44
Fenugreek	"	60	150	0.97
Clove	Methanol	30	150	7.57
Garlic	"	90.1	200	6.92
Ginger	"	147.6	400	2.86
Onion	"	170.5	300	13.18
Fenugreek	"	30	150	2.53
				weight of extract(mg/ml)
Clove	Aqueous	28	150	40
Garlic	"	210	90	100
Ginger	"	170	150	80
Onions	"	190.14	100	60
Fenugreek	"	7.6	150	30

TABLE 3. *ANTIBACTERIAL ACTIVITY (mg/ml) OF VARIOUS EXTRACTS OF CLOVE*

BACTERIA	PETROLEUM ETHER EXTRACT						METHANOL EXTRACT						AQUEOUS EXTRACT					
	E		E + Amp		E + A/C		E		E + Amp		E + A/C		E		E + Amp		E + A/C	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E.coli	-	0.578	-	3.5	-	2.8	0.744	2.9	0.566	2.2	1.8	3.6	-	-	-	-	-	-
S. typhi	-	1.15	-	0.439	-	1.4	0.372	2.9	0.283	1.1	0.458	7.3	20	-	1.9	10.6	3.07	12.31
P. aeruginosa	-	0.578	-	0.879	-	0.356	0.744	2.9	1.1	4.5	-	0.114	-	-	7.6	-	-	-
S.flexineri	-	1.15	-	0.439	-	0.712	0.372	0.744	0.283	0.566	0.29	0.458	10	-	7.6	-	1.53	-
V. cholerae	-	4.6	-	1.8	-	1.4	0.744	2.9	0.283	4.5	0.45	1.8	-	-	-	-	12.31	-
B. cereus	2.3	4.6	-	1.1	-	1.0	1.4	11	0.262	0.726	0.647	5.1	.3	2.5	2.5	-	0.5	7.39
S. aureus	0.578	2.3	2.2	9.0	0.251	2.0	0.186	0.372	0.181	0.726	0.162	0.647	5	20	20	19.5	8.7	-

KEY: E = Extract; Amp = ampicillin; A = Nitrofuration for Gram negative bacteria
and, C = cloxacillin for Gram positive bacteria
- = no effect of extract

TABLE 4. ANTIBACTERIAL ACTIVITY (mg/ml) OF VARIOUS EXTRACTS OF GARLIC

BACTERIA	PETROLEUM ETHER EXTRACT						METHANOL EXTRACT						AQUEOUS EXTRACT					
	E		E + Amp		E + A/C		E		E + Amp		E + A/C		E		E + Amp		E + A/C	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E.coli	2.5	5	1.9	-	1.5	-	-	-	-	-	23	-	-	-	-	-	-	-
S. typhi	1.25	-	0.951	-	0.769	-	23	-	4.5	-	0.916	14	50	-	15.4	-	15.38	-
P. aeruginosa	0.625	-	0.238	-	0.192	-	0.186	0.744	1.1	4.5	0.458	0.916	-	-	-	-	-	-
S.flexineri	-	0.156	-	0.059	-	0.096	11	-	9.05	18	3.6	7.3	3.12	50	7.69	15.4	15.38	30.76
V. cholerae	2.5	5	1.9	-	1.5	3	-	-	-	-	-	-	25	-	7.69	-	30.76	-
B. cereus	0.039	0.156	19	0.0152	0.034	0.068	2.9	23	1.4	-	2.9	11.6	12.5	50	12.2	6.1	21.7	43.47
S. aureus	0.156	2.5	0.152	0.609	0.136	2.1	1.4	23	1.4	11	2.5	10	25	-	48.78	-	43.47	-

KEY: E = Extract; Amp = ampicillin; A = Nitrofurantoin for Gram negative bacteria and, C = cloxacillin for Gram positive bacteria

-= no effect of extracts

TABLE 5. ANTIBACTERIAL ACTIVITY *(mg/ml)* OF VARIOUS EXTRACTS OF GINGER

	PETROLEUM ETHER EXTRACT						METHANOL EXTRACT					
BACTERIA	E		E + Amp		E + A/C		E		E + Amp		E + A/C	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E.coli	11.7	-	4.4	-	3.6	-	-	-	-	-	-	-
S. typhi	2..9	23	1.1	2.2	3.6	14	-	-	-	-	-	-
P. aeruginosa	1.4	-	4.4	-	3.6	-	-	-	-	-	-	-
S.flexineri	1.4	11.7	-	0.558	-	0.904	-	-	-	-	-	-
V. cholerae	11.7	-	4.4	-	3.6	-	-	-	-	-	-	-
B. cereus	-	0.734	-	0.358	0.639	20	1.3	-	0.678	-	0.604	-
S. aureus	2..9	11.7	1.4	2.8	2.5	10	-	-	-	-	-	-

KEY: E =Extract; Amp = ampicillin; A = nitrofurantoin for Gram negative bacteria

and, C = cloxacillin for Gram positive bacteria

\- = no effect of extracts

Aqueous extract did not show any inhibition.

Petroleum ether extracts of onion seemed to show reasonable activity only on *S. aureus* and *B. cereus*, though no particular effect was observed for Gram negative bacteria (Table 6). Methanol extracts had no mentionable effect while aqueous extract could only inhibit *S. aureus*

Petroleum ether extract of fenugreek could only inhibit the bacteria, except for *S.typhi* and *S. aureus*(Table 7). Methanol extracts could only inhibit *S. flexneri* and *S. aureus* .Aqueous extract showed no effect.

The effects of various spices and their extracts on each of the bacteria is discussed now. The greatest action on *E. coli* was observed by the clove extract (table 3). The petroleum ether extract had a complete bactericidal effect only in combination with antibiotics, but not with the extract alone.

Synergism was observed with methanol extract with ampicillin only. Within this extract, synergism with ampicillin was observed for both MIC and MBC. The MIC with extract alone was 0.744mg/ml and MBC was 2.9mg/ml. However with ampicillin in combination the MIC value was lower (0.566mg/ml) and also the MBC value (2.2mg/ml), but an increase in the MIC and MBC point with nitrofurantoin was observed, where the MIC value was 1.8mg/ml and MBC value was 3.6mg/ml (Table 3). Though complete bactericidal effect was observed for the petroleum ether extract, no synergism was observed. The MBC values, were 0.578mg/ml for extract alone, 3.5mg/ml for extract with ampicillin and 2.8mg/ml for extract with nitrofurantoin. The clove extract did not have any effect on *E. coli.*

The garlic extracts were not very active compared to the clove extracts. Methanol and garlic extract did not have any effect on *E. coli* (Table 4). With petroleum ether ginger extract, only inhibitory effect was observed at 11.7mg/ml. Methanol and aqueous extract of ginger had no effect. Onion and fenugreek extract had no inhibitory effect (Table 6 and 7).

Amongst all spices used, the lowest MIC value was observed for methanol clove extract in combination with ampicillin (0.566mg/ml) while lowest MBC value was observe for petroleum ether extract of clove (0.578mg/ml) which had a complete bactericidal effect.

TABLE 6. ANTIBACTERIAL ACTIVITY (mg/ml) OF VARIOUS EXTRACTS OF ONION

BACTERIA	PETROLEUM ETHER EXTRACT						METHANOL EXTRACT						AQUEOUS EXTRACT					
	E		E + Amp		E + A/C		E		E + Amp		E + A/C		E		E + Amp		E + A/C	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E.coli	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. typhi	-	-	7.2	-	-	2.9	-	-	-	18	-	14	-	-	22.8	-	-	-
P. aeruginosa	-	-	3.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S.flexneri	-	-	-	-	2.9	-	-	-	-	-	14	-	-	-	-	-	18.5	-
V. cholerae	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B. cereus	0.594	1.18	0.289	1.15	0.516	2.06	-	-	23	-	-	20	-	-	-	-	-	-
S. aureus	0.594	9.5	0.289	4.6	1.03	8.2	-	-	11	23	5.5	-	7.5	-	14.6	-	26.1	-

KEY: E = Extract; Amp = ampicillin; A = nitrofurantoin for Gram negative bacteria
and , C= cloxacillin for Gram positive bacteria
- = no effect of extracts

TABLE 7. ANTIBACTERIAL ACTIVITY (mg/ml) OF VARIOUS EXTRACTS OF FENUGREEK

BACTERIA	PETROLEUM ETHER EXTRACT						METHANOL EXTRACT					
	E		E + Amp		E + A/C		E		E + Amp		E + A/C	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E.coli	-	-	-	-	-	-	-	-	-	-	-	-
S. typhi	11	-	0.523	1.0	6.7	13.5	-	-	-	-	-	-
P. aeruginosa	22	-	16	-	1.3	-	-	-	-	-	-	-
S.flexineri	11	-	16	-	6.7		11	-	8.8	-	7.1	-
V. cholerae	11	-	16	-	3.3	-	-	-	-	-	-	-
B. cereus	22	-	21.4	-	19	-	-	-	-	-	-	-
S. aureus	22	-	1.3	1.0	4.7	-	5.8	-	5.6	-	10	-

.KEY: E = Extract; Amp= ampicillin; A=nitrofurantoin for Gram negative bacteria

and, C = cloxacillin for Gram positive bacteria

\- = no effect of extracts

Aqueous extract did not show any inhibition.

S. typhi was inhibited better, in general, in combinations with ampicillin. Synergism was observed with the following combination: ampicillin + petroleum ether clove extract (Table 1); petroleum ether ginger extract + ampicillin (Table 3); and petroleum ether fenugreek + ampicillin (Table 5). The MIC value of petroleum ether extract of clove was 11.5mg/ml and MBC was 2. 3mg/ml while in combination with ampicillin, MIC 0.439mg/ml and MBC was 0.879mg/ml. The MIC and MBC values of petroleum ether clove with nitrofurantion was greater than for the extract alone, synergism was observed in combination with methanol clove extract and ampicillin which had MIC and MBC values of 0.372mg/ml and 2.9mg/ml respectively. Tough synergism was observed with both petroleum ether extracts of both ginger and fenugreek, the MBC values were very high (as much as 2.3mg/ml of petroleum ether extract of ginger). Synergism with both antibiotics was observed for petroleum ether and methanol garlic extract and also for petroleum ether and methanol extracts of onion although onion had higher values than garlic. Nitrofurantoin , in combination with garlic extracts had greater synergistic effect on *S. typhi*. The petroleum ether extract alone had a MIC value of 1.2mg/ml while in combination with ampicillin and introfuratoin it was 0. 951mg/ml and 0.769mg/ml respectively . Similarly, methanol extract alone inhibited at 2.3mg/ml while with ampicillin and introfuratoin , MIC was 4.5mg/ml and 0.916mg/ml respectively. In both these extracts, MBC was not observed, but for methanol garlic extract, in combination with nitrofurantoin, it was 1.4mg/ml. Synergistic and also complete bactericidal effect was observed with petroleum ether onion extract and methanol extract with nitrofurantoin and methanol onion extract with ampicillin. In case of aqueous extract aqueous clove extract produced the best effect, followed by garlic aqueous extract. Once again ampicillin produced better effect with clove extracts, while nitrofuratoin produced better effect with garlic extract. The lowest MIC value obtained was observed with methanol clove extract combined with ampicillin (0.283mg/ml) while the lowest MBC value was with petroleum ether extract in combination with ampicillin(0.879mg/ml)

Aqueous extract of any of the spices did not inhibit *P. aeruginosa* except for aqueous clove extract in combination with ampicillin which only inhibited at 3.5mg/ml . Nitrofurantoin showed better synergistic effect in combination with most extracts. The MBC value for petroleum ether clove extract alone and with nitrofuratoin was 0.578mg/ml and 0.356mg/ml respectively, while for methanol clove extract it was 2.9mg/ml and 0.114mg/ml respectively. A similar observation was made with MIC values of petroleum ether garlic extract – nitrofurantoin (0.192mg/ml) had better synergistic effect than ampicillin (0.238mg/ml) compared to the extract alone (0.625mg/ml). Petroleum ether fenugreek extract also produced synergistic effect with both antibiotics, though the values required were higher than garlic or clove extracts. Other extacts either did not produce any effect or no synergistic effect was observed. The lowest MIC and MBC values recorded were for methanol clove extract combined with nitrofurantoin, 0.114mg/ml and 0.229mg/ml respectively.

Clove, garlic and ginger extracts were observed to have synergistic effect with both antibiotics on *S. flexineri* . It was observed that ampicillin produced greater synergistic effect with petroleum ether extracts while nitrofurantoin produced greater synergistic effect with methanol extracts. The MBC values of petroleum ether extracts of clove, garlic and ginger alone and in combination with ampicillin, were 1.15mg/ml, 0.156mg/ml and 1.4mg/ml and 0.439mg/ml, 0.059mg/ml and 0.558mg/ml respectively. Methanol extract of clove was more active than the garlic extract, the MIC value of clove methanol extract was 0.372mg/ml while for garlic methanol extract it was 11mg/ml. The petroleum ether extract of ginger alone was active, but no effect was observed with methanol extract. Aqueous clove and garlic extracts produced a synergistic effect, but no action was observed by ginger, onion and fenugreek aqueous extracts. The lowest MIC and MBC values amongst all the extracts were found to be for clove methanol extract in combination with nitrofurantoin (0.114mg/ml and 0.229mg/ml).

Onion extract had no effect, while only the fenugreek Petroleum ether extract showed inhibitory effect at 11.70mg/ml (extract alone) 16mg/ml (with ampicillin) and 3.3mg/ml (with nitrofurantoin). The lowest value MIC amongst all the spice extracts were observed for methanol clove extract in combination with ampicillin (0.283mg/ml) while, the lowest value for MBC was observed for clove methanol extract with nitrofurantoin (1.8mg/ml)

Complete bactericidal effect was observed only for clove and ginger petroleum ether extracts (with synergism) on *B. cereus*. For petroleum ether clove extract complete bactericidal effect was observed only in combination with ampicillin and cloxacillin. MIC value with extract alone was 23mg/ml while MBC values were 4.6mg/ml (extract alone), 11mg/ml (with ampicillin) and 1mg/ml (with cloxacillin). The MIC and MBC value of methanol extract were 1.4mg/ml and 1.1mg/ml (extract alone), 0.362mg/ml and 0.726mg/ml (with ampicillin) and 0.647mg/ml and 5.1mg/ml (with cloxacillin). Aqueous extracts too, showed good bacteriostatic and bactericidal activity. MIC and MBC value with extract alone, with ampicillin and with ampicillin and with cloxacillin were 1.5mg/ml and 12mg/ml, 2.8mg/ml (MIC), and 0.6mg/ml and 81mg/ml respectively. Here, though synergism was observed with cloxacillin for the MIC value, it was not so for the MBC value.

Garlic petroleum ether extract was required in very low amount. The MIC values with extract alone, with ampicillin and with cloxacillin were 0.039mg/ml, 0.019mg/ml and 0.034mg/ml while MBC were 0.156mg/ml, 0.152mg/ml and 0.068mg/ml. The methanol extracts required low concentrations while aqueous extracts required low concentrations: MIC and MBC with extract alone were 0.29g/ml and 1.17g/ml; with ampicillin (0.28g/ml and 0.57g/ml) and with cloxacillin it was (0.51g/ml and1.01g/ml).

The MBC value with ginger petroleum ether extract were 0.734mg/ml (extract alone), and 0.358mg/ml with ampicillin while for cloxacillin the MIC was 0.639mg/ml and 0.604mg/ml respectively, no MBC was observed. Aqueous extracts did not show any effect. Complete bactericidal effect was showed by methanol extract of onion with cloxacillin at 20mg/ml. The petroleum ether extract showed the highest activity among onion extracts. The MIC and MBC values with extract alone (0.594mg/ml and 1.18mg/ml),with ampicillin (0.289mg/ml and 1.15mg/ml) and with cloxacillin (0.516mg/ml and 2.06mg/ml). Onion aqueous extracts had not effect. No appreciable effect was observed with petroleum ether fenugreek extract while methanol and aqueous extracts had no effect at all. The lowest value of MIC obtained was for petroleum ether garlic extract with ampicillin (0.019mg/ml) while lowest value for MBC was for petroleum ether garlic extract with cloxacillin (0.068mg/ml).

Complete bactericidal effect was not observed for any of the spice extracts on *S. aureus* though synergism was observed. All the spices were found to be active on *S.aureus*. All the extracts of cloves, garlic and onion were active while only the petroleum ether extract of ginger was effective. Aqueous extract of fenugreek showed no effect. The lowest value of MIC obtained was for garlic Petroleum ether extract in combination with cloxacillin (0.136mg/ml) while the lowest MBC value was with clove methanol extract (0.372mg/ml).

Chapter 5

DISCUSSION

Spice extracts showed great effect when tested singlely and also when tested in combination with antibiotics. Extracts alone, sometimes showed greater activity then in combination with antibiotics. For instance, *S.aureus* was inhibited better and with a lower concentration then in combination with petroleum ether clove extract and ampicillin (Table 3). In general, clove extracts showed greater activity among all the spices, followed by garlic, ginger, onion and fenugreek. For example, for *E coli,*. 0.578mg/ml of petroleum ether clove extract was required for complete killing of the bacteria and 2.5mg/ml of petroleum ether garlic extract (Table 4) was required for inhibition alone while 11.7mg/ml of petroleum ether ginger extract was required for inhibition (Table 5). Onion and fenugreek petroleum ether extracts exhibited no effect on *E. coli* (Table 4 and 5). In this study the effectivity of spices was in the order of: clove > garlic > ginger > onion > fenugreek. The effectivity of solvents was the order of: petroleum ether > methanol > water.

However, no particular pattern was observed for the synergistic effect. Some bacteria were synergistic with ampicillin and some with nitrofurantoin/ cloxacillin while some were synergistic with both, for a particular extract. The same bacteria were not necessarily synergistic with the same antibiotic and another extract. For example, synergism was observed with *P. aeruginosa* for both ampicillin and nitrofurantoin for petroleum ether clove extract while this was not so for petroleum ether ginger extracts and the same antibiotic (Table 1 and 3).

The results obtained in this study show a variation in the findings compared to the information obtained from the literature. The results obtained in the study compare favourably as far as qualitative analysis is concerned. For example, extracts of clove, garlic, ginger, onion and fenugreek which have been reported to be bacteriostatic, have been shown in this work to be so for some Gram negative and Gram positive bacteria. However, there were some differences in the susceptibility of microorganisms to some spices as seen in the reports of other workers. For example, aqueous garlic extract has been reported to inhibit and kill a wide range of bacteria, but in this study, no inhibition was noted for *E. coli* and *P.aeruginosa* while 12.5mg of the extract was required for *B. cereus*. However, El- Rahman (1994) reported that 0.7% of extract was required to inhibit *B. cereus* while Sato, *et al.*, (1990) reported that 1.25% extract was required to inhibit the bacteria. Similarly, in this study, such non-correlation was found for other spices and bacteria.

The reasons for variation of results using the same spices are many. The main reason may be due to the concentration of active compounds within the spice which differs as a result of climatic variations in different geographic areas, time of harvest, season of harvest, etc. (Zaika, 1988). Strain difference also plays an important role in the variation of results. For instance, different concentration of aqueous garlic extract were required to inhibit different strains of *V. parahemolyticus* and *E. coli* (Zaika, 1988; Sato, *et al.*, 1990).

The extracts obtained in this study were by the use of cold infusion. Very little heat was used (for evaporation). This method, therefore, varies from the one used for obtaining the essential oils. Lafont, *et al.*, (1984) used the reflux method of extraction which also require heat and extracted the volatile component only, whereas it is possible that the cold infusion may extract other active components. Heat has been known to inactivity many active component of spices (Davidson and Parish, 1989; Sato, *et al.*, 1990).

Yet another factor could be that, different form of extractions remove different, but limited, component and there are reported to be thousands of phytochemicals within every fruit and vegetable, many of which have different antimicrobial properties (Begley, 1994). It could also possibly be due to this reason, combined with fact that no heat was used for extraction procedure, that petroleum ether extracts had better effect than essential oils and also the methanol extract compared to ethanol extracts.

It could be assumed from the hypothesis stated by Oster (1971), that phenolic compounds play a role in the destroying the cell membrane of the bacteria. The synergistic property could possibly be due to this effect – then, once the cell wall activity is reduced, then it becomes possibly for the antibiotic to exert its effect on the organelles, or other activities, to destroy the bacteria cell. The mechanism of action of allicin is not known, but it has been suggested that the disulphide bond in allicin, when decomposes, produces a highly reactive compound which groups on the surface of the target, thereby deactivating the cell wall (Block, 1985). Thus, when another antibiotic substance is added, it hastens the break down of the cell in the combined process. Synergism, achieved by combination of two or more compounds is widely recommended as it decreases dose – related toxicity as a result of reduced dosage and to attain broad spectrum of activity (Eliopoulos, 1989).

However, as observed in this study, synergism may occur either with both antibiotics or only with one or not at all. This variation may depend on the different chemical compound combinations of extract, antibiotic and the interaction with different bacteria.

The complete bactericidal activity observed, without the inhibition point occurring can be due to the high potency of the spice extract (along with the lack of heat treatment). It is highly possible that the time required for inhibition was lower than 18-24 hours as used in this study. This similar observation was made by Muroi and Kubo (1996) who found that the highly potent component of cashew nut shell oil could inhibit bacteria in only 6 hours. However, this effect has never been reported for spices which normally takes upto 18 –24 hours for inhibition and at a high concentration.

When used in combination with antibiotics, it was found that very low concentrations could still bring about the inhibition of the test bacteria. These concentrations were lower than when the extract, were used alone. For example, the antibiotic concentrations required to be bacteriostatic was found to be required in as low 0.013µg/ml of ampicillin (for Gram positive bacteria and 0.15µg/ml to inhibit Gram negative bacteria) while 0.12µg/ml of nitrofurantoin and 0.003µg/ml of cloxacillin were required in combination of extracts to bring about inhibition whereas the bacteria were resistant to the antibiotics alone.

Medically, spices are of great importance both singly and in combination with antibiotics. For instance, Sofowara (1980) showed the effectivity of ginger by treating school children with ginger pills for schistosomiasis. Garlic oil potential has been recognised for many decades and now garlic oil capsules are commercially available as health boosters/tonic. Furthermore, onion and fenugreek have been used in curing fever (Bakhru, 1996). The effectivity of this spices may be obtained as spice alone or in synergism with antibiotics as found by Perez, *et al.,* (1994) on malarial parasites. The use of spices (whether alone or with antibiotics) need not be limited to multi-resistant bacteria but can also be used on strains that are not resistant, as combined therapy is found more effective.

From the industrial viewpoint, possible applications are still many. Though antibiotics are banned from use in the food industry, the possible implications of its potential with naturally produced antimicrobial agents are many. For instance, many bacterial strains liberate plasmid encoded proteinaceous toxins, bacteriosins which are active only against closely related strains of bacteria (*Stainer, et al.,* 1990). For instance, various strains of *B. cereus* and *B. subtilis* were found to inhibit the growth of some fungi (Pleban, *et al.,* 1993). Strains of *S.aureus* producing antibacterial agent has also been reported (Crupper and Iandolo, 1996). Lactic acid bacteria have been known for a long time to produce antimicrobial substance. Five psychrotrophic strains of lactic acid bacteria were found to inhibit mesophilic bacteria and coliforms (Vescovo, *et al.,* 1996). Apart from using natural bacteriosins and extracts for food preservation further studies can be carried out on preservation with antibiotics and extracts as used in very, very small quantities in combined formed, and also, combined therapy is considered for more safer than a single antibiotic alone (Eliopoulos, 1989). These natural antimicrobial agents combined with spice extract show a great possibility in improving the preservation technology. Of such natural combinations, Gould (1996) stated that: "....while naturalness alone is not necessary a sufficient object for these developments, the use of natural inhibitors as component of system that can together enhance the effectiveness of preservation, with advantages in product quality and safety justifies pursuit". For preservation purposes, it would be more effective to use spices in combination with natural antimicrobial agents than alone, because spices (or their extracts) are found to be ineffective in the concentration used for cooking, and large concentration of spice alone may effect the food flavour.

The possibility of using spice extract as antiseptics or disinfectants is plausible. As garlic extracts are very odoriferous, they may be unacceptable as disinfectants, but extracts of cloves may be useful, provided cost effective means of extraction are derived (desolventization procedure can be very expensive). Extract may also be tested for synergism with other chemical disinfectants or antiseptics. It can be concluded that antimicrobial activity of natural products including spices, either as spices alone or synergistically with other antimicrobial agents presents a great challenge in both the medical and industrial fields. Furthermore, as the effect of some spices alone have already been in use for centuries, it is safe to recommend them for the cure of certain ailments while the use of spices with other antimicrobial agent can be recommended for further research to produce an all natural cure.

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B. Methanol extract of ginger.

a)	Extract alone	22.2mg/ml
b)	Extract +Ampicillin (Gram negative bacteria)	16.88mg/ml
	" " (" positive ")	21.6mg/ml
c)	Extract +Nitrofurantoin	13.66mg/ml
d)	Extract + Cloxacillin	19.3mg/ml

C. Aqueous extract of ginger.

a)	Extract alone	40mg/ml
b)	Extract +Ampicillin (Gram negative bacteria)	30.4mg/ml
	" " (" positive ")	39.02mg/ml
c)	Extract +Nitrofurantoin	24.6mg/ml
d)	Extract + Cloxacillin	34.79mg/ml

Concentration of onion extract

A. Petroleum ether extract of onion.

a)	Extract alone	9.5mg/ml
b)	Extract +Ampicillin (Gram negative bacteria)	7.2mg/ml
	" " (" positive ")	9.2mg/ml
c)	Extract +Nitrofurantoin	5.8mg/ml
d)	Extract + Cloxacillin	8.2mg/ml

B. Methanol extract of onion.

a)	Extract alone	23.8mg/ml
b)	Extract +Ampicillin (Gram negative bacteria)	18.1mg/ml
	" " (" positive ")	23.3mg/ml
c)	Extract +Nitrofurantoin	14.6mg/ml
d)	Extract + Cloxacillin	20.7mg/ml

C. Aqueous extract of onion.

a)	Extract alone	30mg/ml
b)	Extract +Ampicillin (Gram negative bacteria)	22.8mg/ml

	" " (" positive ")		29.27mg/ml
c)	Extract +Nitrofurantoin		18.46mg/ml
d)	Extract + Cloxacillin		26.09mg/ml

Concentration of fenugreek

A. Petroleum ether extract of fenugreek.

a)	Extract alone	22.mg/ml
b)	Extract +Ampicillin (Gram negative bacteria)	16.7mg/ml
	" " (" positive ")	21.4mg.ml
c)	Extract +Nitrofurantoin	13.5mg/ml
d)	Extract + Cloxacillin	19.1mg/ml

B. Methanol extract of fenugreek

a)	Extract alone	23.5mg/ml
b)	Extract +Ampicillin (Gram negative bacteria)	17.81mg/ml
	" " (" positive ")	22.93mg/ml
c)	Extract +Nitrofurantoin	14.3mg/ml
d)	Extract + Cloxacillin	20.2mg/ml

C. Aqueous extract of fenugreek.

a)	Extract alone	15mg/ml
b)	Extract +Ampicillin (Gram negative bacteria)	11.41mg/ml
	" " (" positive ")	14.63mg/ml
c)	Extract +Nitrofurantoin	9.23mg/ml
d)	Extract + Cloxacillin	13.04mg/ml

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APPENDICES

APPENDIX I

The following standard formula was used to enumerate the test bacteria:

$$C = \frac{(\text{count} \times 10^{n})}{0.1}$$

Where, C= number of cells in inoculum/ml,

Count = the number of colonies counted,

n= the number of dilution steps from the standard inoculum.

APPENDIX 2

CONCENTRATION OF EXTRACTS

The extracts were tested in three sets. In the first set, the extract alone was tested. In the second set, the extract was tested along with the first antibiotic(ampicillin) was tested. In the third set, the extract along with the second antibiotic(nitrofurantoin/cloxacillin) was tested. Since the volume of the antibiotic solutions varied, the concentrations of the extracts also varied. The different concentrations in each of the extracts are given below.

Concentration of clove extract

A. Petroleum ether of extract of clove.

a)	Extract alone	37mg/ml
b)	Extract + Ampicillin (Gram negative bacteria)	28.1mg/ml
	" " (" positive bacteria)	36mg/ml
c)	Extracct + Nitrofurantoin	22.8mg/ml
d)	Extract +Cloxacillin	32.1mg/ml

B. Methanol extract of clove.

a)	Extract alone	23.8mg/ml
b)	Extract + Ampicillin (Gram negative bacteria)	18.1mg/ml
	" " (" positive ")	23.2mg/ml
c)	Extract +Nitrofurantoin	14.6mg/ml
d)	Extract + Cloxacillin	20.7mg/ml

C. Aqueous extract of clove.

a)	Extract alone	20mg/ml
b)	Extract + Ampicillin (Gram negative bacteria)	15.21mg/ml
	" " (" positive bacteria)	19.5mg/ml
c)	Extract + Nitrofurantoin	12.31mg/ml
d)	Extract + Cloxacillin	17.39mg/ml

Concentration of garlic extracts.

A. Petroleum ether extract of garlic.

a)	Extract alone	50mg/ml
b)	Extract +Ampicillin (Gram negative bacteria)	38.0mg/ml
	" " (" positive ")	48.0mg/ml
c)	Extract +Nitrofurantoin	30mg/ml
d)	Extract + Cloxacillin	43.0mg/ml

B. Methanol extract of garlic.

a)	Extract alone	23.8mg/ml
b)	Extract +Ampicillin (Gram negative bacteria)	18.1mg/ml
	" " (" positive ")	23.2mg/ml
c)	Extract +Nitrofurantoin	14.65mg/ml
d)	Extract + Cloxacillin	20.69mg/ml

C. Aqueous extract of garlic.

a)	Extract alone	0.05g/ml(50mg/ml)
b)	Extract +Ampicillin (Gram negative bacteria)	38mg/ml
	" " (" positive ")	48mg/ml
c)	Extract +Nitrofurantoin	30.76mg/ml
d)	Extract + Cloxacillin	43.47mg/ml

Concentration of ginger extracts

A. Petroleum ether extract of ginger.

a)	Extract alone	23.5mg/ml
b)	Extract +Ampicillin (Gram negative bacteria)	17.8mg/ml
	" " (" positive ")	22.9mg/ml
c)	Extract +Nitrofurantoin	14.4mg/ml
d)	Extract + Cloxacillin	20.4mg/ml