Occurrence of emerging food-borne pathogens in common fast foods and inactivation of pathogens with essential oil

A dissertation submitted for the partial fulfillment of the requirements for the degree of Masters of Philosophy in Microbiology

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DECLARATION

I do hereby declare that this dissertation submitted to the University of Dhaka for the Degree of Master of Philosophy is based on my own investigation, carried out under the supervision of Dr. Md. Mahfuzul Hoque, Professor, Department of Microbiology, University of Dhaka and that this or any part of this work has not been submitted for any other degree anywhere.

Afroz Jahan Taniya

CERTIFICATE

This is to certify that the thesis entitled **"Occurrence of emerging food-borne pathogens in common fast foods and inactivation of pathogens with essential oil"** submitted to the Department of Microbiology, Faculty of Biological sciences, University of Dhaka in fulfillment of the requirements for the degree of Master of Philosophy is a record of bonafied research work carried out by Afroz Jahan Taniya under my supervision. This is an original work and I believe that no part of the work has been submitted before for any other degree or diploma.

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TABLE OF CONTENTS

Content **Page No.**

2.2. Methods

LIST OF TABLES

LIST OF FIGURE

LIST OF ABBEREVATIONS

CFU	Colony forming unit
°C	Degree Celsius
Dist.	Distilled
etc.	Electra
hr	Hour
Fig.	Figure
e.g.	For example
g	Gram
kg	Kilogram
µ	Micron
µl	Microliter
µg	Microgram
mg	Milligram
ml	Milliliter
M	Molar
p^H	Negative logarithm of hydrogen ion concentration
p.	Page number
pp.	Page numbers
%	Percent
sp.	Species (singular)
spp.	Species (plural)
SD	Standard deviation

et al.	And others people
w/w	Weight per weight
w/v	Weight per volume
NA	Nutrient agar
MHA	Muller Hinton agar
SMA	Sorbitol MacConkey agar
MSA	Mannitol salt agar
MHB	Muller Hinton broth
TSA	Tryptone Soya agar
TSB	Tryptone Soya broth

Occurrence of emerging food-borne pathogens in common fast foods and inactivation of pathogens with essential oil

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Abstract

The present investigation can be classified into four major parts. The first part is the isolation and presumptive identification of *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus* from fast food samples collected from various parts of Dhaka city of Bangladesh. This was done following the criteria put forth in Bergey's manual of Determinative Bacteriology, 9 th edition. The second part is the detection of antibacterial activity of cinnamaldehyde against three food borne pathogens individually and combined by the disc diffusion method. The third part is to determine the effect of pH and temperature on antibacterial activity of cinnamaldehyde against three food borne pathogens. The fourth and the final part is inactivation of isolated organism applied in ground chicken meat with cinnamaldehyde.

For isolation of *Escherichia coli*, *Bacillus cereus* and *Staphylococcus aureus* from food samples three types of selective media was used such as Sorbitol MacConkey agar (SMA), *Bacillus cereus* selective agar base medium supplemented with egg yolk emulsion, Polymyxin B sulphate, and Mannitol salt agar (MSA), From the selective medium, the selected colonies were isolated and then sub cultured to maintain pure culture. From the collected food samples, 14 *Escherichia coli*, 09 *Bacillus cereus* and 17 *Staphylococcus aureus* were subjected to cultural, morphological, and microscopic and biochemical study for their presumptive identification. Antibacterial activity of

cinnamaldehyde, carvacrol and euginol was tested against *Escherichia coli*, *Bacillus cereus* and *Staphylococcus aureus* using disc diffusion method. Screening of cinnamaldehyde, carvacrol and euginol extracts showed antibacterial activity against the isolated organisms studied. The MIC values of cinnamaldehyde, carvacrol and euginol were determined by broth dilution method at 37 °C and p^H 7. MIC values of cinnamaldehyde, carvacrol and euginol against the test bacteria ranged from 2.5 to 10%. The effect of temperature and p^H on the antibacterial activity of cinnamaldehyde against *Escherichia coli*, *Bacillus cereus* and *Staphylococcus aureus* were determined. Cinnamaldehyde showed antibacterial activity after treatment at 100° C for 30 min treatment suggesting that a high temperature does not affect the activity but only reduced the activity insignificantly as showed by decreased zone diameter. The highest antibacterial activity was found at pH 7 for cinnamaldehyde against the test organisms. Cinnamaldehyde at each concentration of 2.5% (MIC level), 7.5% (three times of MIC) and 12.5% (five times of MIC) was applied separately in ground chicken meat inoculated with *Escherichia coli*, *Bacillus cereus* and *Staphylococcus aureus* and kept at -18° C for 10 days. The result showed that only a few log reduction of *Escherichia coli*, *Bacillus cereus* and *Staphylococcus aureus* was found in selective media used in case of MIC level of cinnamaldehyde (2.5%). 3 times (7.5%) and 5 times (12.5%) MIC level of cinnamaldehyde employed resulted 7 log reduction of *Escherichia coli*, *Bacillus cereus* and *Staphylococcus aureus*, respectively, growing on selective media after 24 hours of exposure. Therefore, cinnamldehyde could be useful to control *Escherichia coli*, *Bacillus cereus* and *Staphylococcus aureus* in ground chicken meat. From this experimental finding it was apparent that essential oils from herbs, sharps, trees and spices, especially cinnamldehyde from cinnamon could be fruitfully used to control food borne pathogens in various foods and food commodities.

Chapter One

Introduction

1. Introduction

1.1 Over the past 10-15 years, the main trends in food consumption worldwide have been increased as pre-prepared (convenience) food in the home and outside the home particularly in fast food restaurants (Gould, 1989).Food borne disease have been reported mainly from contaminated foods served in restaurants, of which outbreaks of food borne illness caused by *E. coli* are the result of under-cooking of hamburger meat. The largest and the most recent of outbreaks occurred in January 1993 in chain of fast food restaurants in USA (Tarr, 1993). Food borne pathogens are the leading cause of illness and death in less developed countries killing approximately 1.8 million people per annum. In developed countries, food borne pathogens are responsible for millions of cases of infectious gastrointestinal diseases every year, costing billions of dollars for medical care and lost productivity (Fratamico and Bayles, 2005).The presence of microorganisms in food is a natural and unavoidable occurrence. Cooking generally destroy most harmful bacteria but undercooked foods, processed ready to eat foods and minimally processed foods may contain harmful bacteria that are serious health threats. Meat, dairy and poultry products are important reservoirs for many of the food borne pathogens including *Salmonella, Camphylobacter, Listeria, Staphylococcus, Bacillus* and *Escherichia.* (Swaminathan and Feng, 1994). In spite of modern improvement in hygiene and food production techniques, food safety is an increasingly important public health issue (WHO, 2002a).). It has been estimated that as many as 30% of people in industrialized countries suffers from a food borne disease every year and in 2000 at least two million people died of diarrheal disease worldwide (WHO, 2002a). Microbiological food safety has given priority to every nation irrespective of developed or underdeveloped countries. From the earliest time, man has devised for preserving foods. In general, foods were preserved by use of heat, cold, drying, salting and fermentation. In recent years, chemicals such as food additives are extensively used and some advanced technology for long term storage is being developed. At the present time, the “Hazard Analysis Critical Control Point” (HACCP) approach to quality assurance is introduced in routine surveillance during food processing (Kokubo, 1994).

Among above mentioned techniques for controlling microorganisms in foods, some of chemical food additives have been questioned because of increased awareness of safety and the increased ability to evaluate safety (Branen, 1993). Thus, there is currently great interest in antibacterial compounds naturally present in foods and food ingredients (Beuchat and Golden, 1989). It has been known since ancient times that spices and their essential oils have varying degrees of antimicrobial activity (Shelef, 1983; Zaika, 1988; Beuchat and Golden, 1989; Ting and Deibel, 1992; Juven *et al*., 1994; Tassou, *et al*., 1995; Chang, 1995; Sivropoulou, *et al*., 1996; Wan, *et al*., 1998; Lachowicz, *et al*., 1998). The major antimicrobial components of spices and their essential oils are for example, eugenol in cloves, alicin in garlic, cinnamic aldehyde and eugenol in cinnamon, carvarcol and thymol in oregano and thyme and vanillin in vanillin beans. The antimicrobial activity of some essential oil components against food borne pathogens has also been tested (Bullerman, *et al*., 1977; Kim, *et al*., 1995; Thompson, 1996; Ultee, *et al*., 1999, 2001). More recently, plant extracts have been developed and proposed for use in foods as natural antimicrobial (Huhtanen, 1980; Ginesta-Peris, *et al.,* 1994; Holt and Gomez Almonte, 1995; Delaquis and Mazza, 1995; Park-Shin, 1999; Del Campo, *et al*., 2000; Hsieh, *et al*., 2001).

1.2. Food borne diseases

Food infection and intoxication are considered as the most common causes of food borne diseases worldwide. *Staphylococcus aureus, Salmonella* spp*., Escherichia coli* (Various serotypes), *Bacillus cereus* are common food borne pathogens that cause infection and intoxication (Lopez, *et al.,* 2003) among food spoilage organisms, which renders food unacceptable to consumers. Short shelf life of food products because of spoilage is one of the major problems of the food industry. Examples of food spoilage microorganisms include *Pseudomonas aeroginosa, Bacillus subtilis, Alcaligenes faecalis* etc (Jay, 2000 and Roy, 2001).

Food poisoning caused by *Listeria monocytogens, Staphylococcus aureus, Salmonella sp, Bacillus cereus, Aeromonus* spp*.* and *Escherichia coli* 0157:H7 is worldwide serious problem, especially in developing countries. Worldwide it has been reported that, there

were more than 1.3 billion cases of human salmonellosis annually, with three million deaths (Pang, *et al.,* 1995). Among the various diarrheagenic serotypes, enterohemorrhagic *E.coli* 0157:H7 is implicated in large number of food borne out breaks in many parts of the world (Mead, *et al.,* 1990*).* An estimated 7300 cases of infection and 61 deaths occurred in USA each year due to *Escherichia coli* 0157:H7 (USDA-FSIS). Most of this illness was associated with eating undercooked, contaminated ground beef (Gupta, *et al.,* 2005*). Listeria monocytogens* has been isolated from various environments and is reported to cause 25% of all the deaths resulting from food-borne outbreaks in the United States annually. *Aeromonas* spp. represents a group of ubiquitous microorganisms that have broad host range and have often been isolated from humans with diarrhea (Janda, *et al.,* 1998*)*. *Shigella dysenterae and Vibrio cholerea* are serious problem in Bangladesh. *Shigella dysenterae* type1 was isolated in 1897 from Japan. It is the most virulent *Shigella* with low infectious dose, high mortality. It is common in Bangladesh and to India, and common food and water borne pathogens. *Vibrio cholerea* 0139, spread rapidly in Bangladesh, producing disease in adults as well as children. It is mainly food and water borne pathogen but can survive for prolong time in water, possible in blue-green and replicating in fish and shellfish (Faruque, *et al*, 2003).

1.3. Historical background of plant antimicrobials

From the very beginning of human civilization, man has followed some processes and techniques with a trial and error basis without knowing the reason lying behind the processes. With the advancement of science and technology most of the processes adopted by the ancient people are regarded as very scientific and logical. New and modern technologies unveil the causes behind these processes. Interestingly, the newer technologists are to incorporate these primitive processes to include as eco-friendly approach. Herbs and spices are some exciting example of such approach. Spices are non-leafy parts used as a flavoring or seasoning, although many can also be used an herbal medicine. Herbs, a closely related term of spices to distinguish plant parts finding the same use but derived from leafy or soft flowering parts. The two terms

may be used for the same plants in which fresh leaves are used as herbs, while other dried parts are used as spices, e.g. coriander, cinnamon and cloves, etc.

Spices have a profound influence on the course of human civilization. They permeate our lives from birth to death. In everyday life, spices succor us, cure us, relax us and excite us. Ancient people such as Egyptian, the Arab and the Roman made extensive uses of spices, not only to add flavor to foods and beverages, but as medicine, disinfectants, incenses, stimulants and even as aphrodisiac agents. No wonder they were sought after in the same manner gold and precious metals (Chomchalow, 1996)

The use of herbs dates back over 5,000 years to the Sumerians. The ancient Romans and Greeks valued plants for various uses including food seasoning, dyes, cosmetics, and most notably as medicines. The use of herbs and spices in almost all the cultures is due to the flavor enhancement, preserving foods for their medicinal use.

Spices and herbs have been used traditionally for several centuries by many cultures in preserving foods and as food additives for aroma and flavor (Snyder, 1997). Scientific experiments since the late 19th century have documented the antimicrobial properties of some spices, herbs and their components (Snyder, 2005). The earliest description of antimicrobial effects of spice was made by Antony van Leeuwenhoek. In a letter dated 9 October 1676, van Leeuwenhoek described the decline in the number and activity of “animalcules” in a sample of well water following the addition of pepper (Dobell, 1960).By this time, it is well known that antimicrobial effects of herbs and spices lies basically in their active ingredient and these are the essential oil or extracts of the herbs and spices.

1.4. Antimicrobial activity of spices

Extract of plants, spices and herbs play an important role in promoting human health by their anticancer, anti-oxidative and anti-inflammatory properties. Flavanoids from tea beverages act as free radical-scavengers and anti-oxidants (Wiseman, *et al.,* 1997). Anthocyanins and flavonoids from teas and cherries possess anti-allergic, antiviral, anticancer, and anticarcinogenic properties and prevent cardiovascular diseases and aging (Balentine, *et al* 1997). Components in spices also possess colorant, bioactive

(i.e. antioxidant and antimicrobial), acidulant and sweetener effects (Chang *et al.,* 1977*;* Pszczola, 1999; Mansour and Khalil, 2000; Wang, *et al.,* 2000). Essential oils (volatile oils) are distilled parts of spices by mostly steam, and also by cold, dry and vacuum distillation methods (Giese, 1994). Oleoresins, solvent extracts of spices contain both volatile and nonvolatile fraction of spices (Farrell, 1985). Spices and herbs are used worldwide and their antimicrobial potential has been recognized since antiquity (Cervenka, *et al.,* 2006*).* The antimicrobial effectiveness of herbs and spices against various microorganisms was reviewed (Cowan, *et al.,* 1999).

Ueda, *et al.,* (1982) examined the ethanol extracts of spices and herbs for inhibiting of bacteria and fungi. Clove extract showed remarkable antibacterial activity against all organisms tested and oregano and cinnamon exhibited wide inhibitory spectrum. Gram-negative bacteria exhibited less sensitivity compared to Gram-positive bacteria. (Ouattara, *et al.,* 1997) examined the inhibitory properties of cinnamon, clove, cumin, garlic, oregano, black pepper, pimento and rosemary essential oils against meat spoilage bacteria, *Carnobacterium piscicola, L. curvatus, L. sake, Brochothix thermosphacta, Pseudomonas fluorescens and Serratia liquefaciens.* Clove, cinnamon, pimento and rosemary essential oils exhibited the most inhibitory activity. The inhibitory effects of cloves and pimento were attributed to the presence of eugenol and cinnamaldehyde in cinnamon oil. Although the essential oils of other spices with contained antimicrobial compounds such as eugenol, thymol, they were very small in quantity to exhibit antimicrobial activity. In general, Gram-positive bacteria are more sensitive than Gram-negative bacteria and *Pseudomonas* species are least sensitive to bioactive agents. The outer cell membrane of Gram-negative organisms has several lipid compounds that protect the cells from the antimicrobial agents (Shelef, *et al*., 1980, Branen, *et al.,* 1980, Farag, *et al.,* 1989b, Russell, 1991). However, *Brochothrix thermosphacta,* Gram-positive bacterium, was as resistant as Gram-negative bacteria to the spice oils (Ouattara, *et al.,* 1997). Gram-positive cocci (excluding sporulating Gram positive bacteria to many biocides (Russell, 1991). Proportional inhibitory activities of spices against bacteria are listed in table 1.1.

Table1.1: Proportional inhibitory properties of spices against bacterial strains (Billing, *et al.,* 1998)

Percent Bacterial Inhibition	Spices
75-100%	Garlic, onion, allspice,oregano, thyme, cinnamon,tarragon, cumin, cloves, lemon grass, bay leaf, capsicums and rosemary
50-75%	Marjoram. Mustard, caraway,mint, sage, fennel, coriander,dill and nutmeg
Less than 50%	Basil, parsley, cardamom, pepper (black and white), ginger, anise seed, celery seed, lemon and lime

1.5. Increasing awareness of people led to the discovery of antimicrobials from plants

Now-a-ways, consumers have growing concern about the food safety in relation to hormones, food additives, food preservatives etc (Brewer, *et al.,* 1994). The need for safe antimicrobial preservatives increased as alternatives of chemical preservatives in foods. For replacement of synthetic antimicrobial additives active components from herbs and spices are to be effective alternatives today (Brewer, *et al.,* 1994). For this reason, many studies on antimicrobial substance of spices as a natural food preservative against food borne pathogens have been reported (Smith, *et al.,* 1998). Moreover the onset of increased demand for minimally-processed, extended shelf-life of foods and report of chemical preservative as having potential toxicity demand food manufacturers to find alternative sources of antimicrobial compounds (Conner, 1993; Nychas, 1995). Therefore novel approach to the development of new antimicrobials

remains an important area of research. In recent years, multiple drug/chemical resistance pathogenic microorganisms have developed due to misuse of commercial antibiotics (Davis, 1994; Service, 1995). This situation has led scientists to search for new antimicrobials from various sources, including medicinal plants such as herbs and spices (Clark, 1996 and Cordell, 1998).

1.6. Use of the essential oil in food industry

As stated earlier, spices and herbs, aromatic vegetable materials, have long been used in foods not only for their flavoring, but also for their medicinal and preservation properties (Davidson, *el al.,* 1983). Spices also stimulate appetite by increasing salivation, carminative action and preserve the food by their antimicrobial and antioxidant properties (Lewis, 1984). More than 400 spices are used worldwide. Since ancient times, spices and herbs have been used for preventing food spoilage and deterioration and for extending shelf-life of food as well (Nakatani, 1994). Spices are used to enhance the flavor and palatability of food. Billing and Shermen (1998) evaluated several critical predictions in order to address the question of why people use spices.

Mild preservation techniques are becoming more important in modern food industries. As a consequence, spore-forming microorganisms are likely to proliferate and hence become a serious food safety risk. Mild processes are often combined to obtain safe products with improved organoleptic quality. A novel way to reduce the proliferation of microorganisms is the use of essential oils. The antifungal and antibacterial effects of these components on different microorganisms have been described in several studies (Conner, *et al*., 1993; Juven, *et al*., 1994; Hoque, *et al*., 2008). The current interest in the use of compounds derived from spices as antimicrobial agents was sparked in the 1980s by changes in consumer attitudes toward the use of preservative agents such as nitrates and NaCl in foods. However progress in the application of spices-derived compounds as antimicrobial agents in food products has been slowing. The major problems include accurate identification of active components and the apparent

requirement for concentrations that alter the sensory qualities of the food (Nychas, 2003; Roller, 2003).

1.7. Essential oil

Essential oil is a highly concentrated cocktail of volatile compounds consisting of the natural chemicals found in plants. They are also known as volatile or ethereal oils, or simply as the "oil of" the plant material from which they were extracted. Essential oils are usually lipophilic (Literally: "oil-loving") compounds that usually are not miscible with water. Instead they can be diluted in solvents like ethanol and polyethylene glycol. Essential oils are produced using several techniques.

As stated earlier, essential oils have been shown to possess antibacterial, antifungal, antiviral insecticidal and antioxidant properties (Burt, 2004; Kordali, *et al*., 2005). Some oils have been used in cancer treatment (Sylevestre, *et al*., 2006). Some other oils have been used in food preservation (Faid, *et al*., 1995) aromatherapy (Buttner, *et al*., 1996) and fragrance industries (Braak*, et al*., 1999). Essential oils are rich source of biologically active compounds. There has been an increased interest in looking at antimicrobial properties of extracts from aromatic plants particularly essential oils (Valentin, *et al*., 1997). Therefore it is reasonable to expect a variety of plant compounds in these oils with specific as well as general antimicrobial activity and antibiotic potential (Darokar, *et al*., 1998)

1.8. Antimicrobial properties of essential oil components

Antimicrobial activity of the oils depends on the major constituents and their concentration. The inhibiyory effects of essential oils are mainly due to the major component (Farag, *et al.,* 1989a). The small amounts of minor components might also contribute the antimicrobial activity of the oils (Ouattara, *et al.,* 1997). The essential oils of spices are composed of a complex composition of compounds. Bioactive ingredients in spices such as sulfides, thiols, terpenes and their derivatives, phenols, glycosides, alcohols, aldehydes and ester have been investigated for their properties. Although

some phytochemicals have been identified there are still many questions to answer (Uhl, 2000). The majority of the antimicrobial components of spices are phenol compounds with a hydroxyl group (-OH) (Beuchat and Golden, 1989). The phenolic compounds such as carvacrol, eugenol and thymol are very active antimicrobial agents (Nadal, *et al*., 1973; Mahmoud, 1994; Kim, *et al*., 1995a; Sivropoulou, *et al.,* 1996; Suresh, *et al.,* 1992). The antimicrobial property of oregano and thyme has been attributed to the essential oils terpenes, carvarol and thymol (Beuchat, 1976). Kim, *et al*., (1995a) evaluated the antimicrobial activity of citral, carvacrol, geraniol, α-terpineol, perillaldehyde, eugenol, linalool, o-ionone, limonene, nerolidol and citronellal against five food borne pathogens, *E.coli, E.coli* 0157:H7. *Salmonella typhimurium, Listeria monocytogenes* and *Vibrio vulnificus*. Carvacrol was the most inhibitory and citral and geraniol had potent antibacterial effect against the five pathogens tested. Thymol was more effective against Gram-negetive bacteria than carvacrol (Sivropoulou, *et al.,* 1996). *Pseudomonas aeruginosa* has been found very resistant to many antibacterial agents but was found to be sensitive to puleogone, isopulegol and piperitone (Sivropoulou, *et al*., 1995).

Antimicrobial activity of the spices components was different for each bacterial culture and the response of these cultures varied toward each component. In general, bacteria are more resistant than fungi (Morozumi, 1978; Zaika, 1988) and Gram-negative bacteria are more resistant than Gram-positive bacteria (Farag*, et al.,* 1989b; Zaika, 1988).

1.9. Extraction of essential oil

Plant oils are obtained from their plant hosts through a variety of processes commonly called Extraction Methods. Essential oils are generally extracted by distillation. Other processes include expression and solvent extraction but the method of steam distillation is most commonly used for commercial production. Standard extraction processes used to obtain essential oil is given in Fig 1.

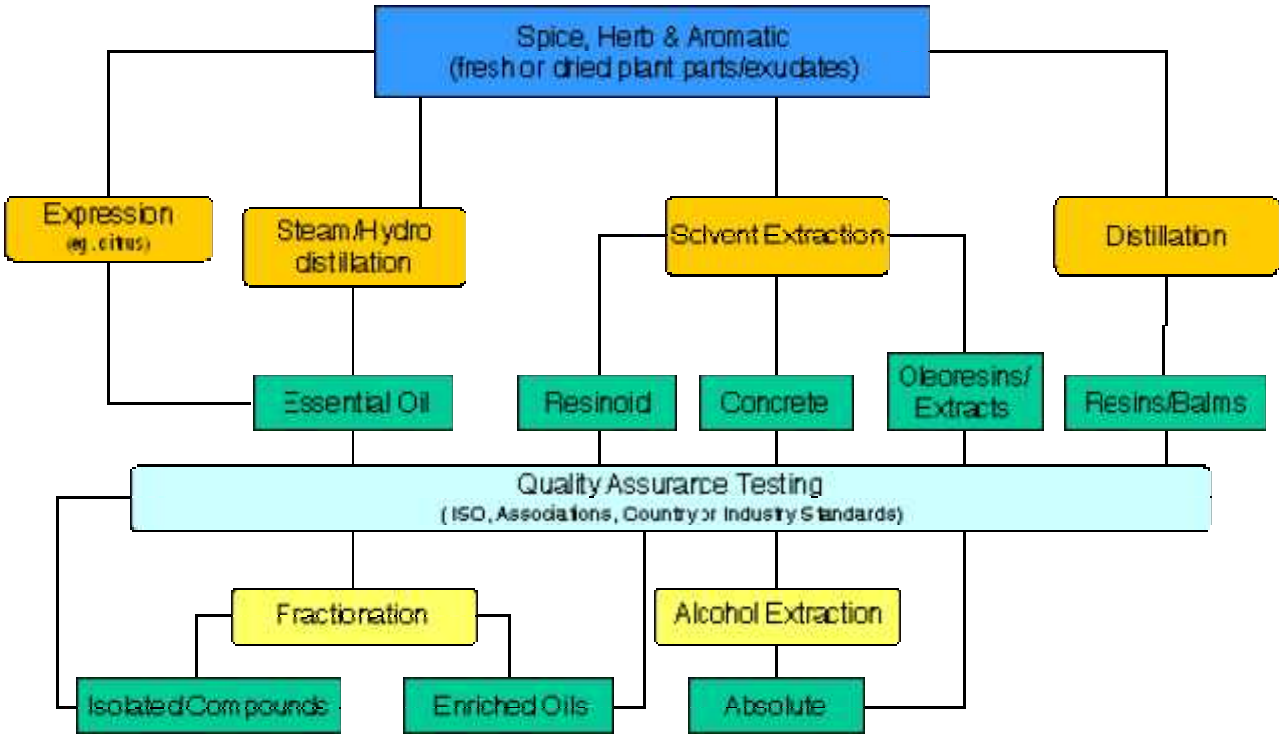


Figure 1: Standard extraction processes used to obtain essential oil

1.10. Key Control Parameters

Each extraction method must be appropriately controlled for the most optimum quality per unit cost achievable. These parameters include:

1.10.1. Temperature

The fragile aromatic molecules of an essential oil are easily destroyed or altered by high temperatures; and so, the Extraction Method must use low-temperatures. High temperatures seem to cause harshness in the oil. Even the oil's pH and the electropositive and electronegative balance are greatly affected. As examples, extraction of lavender oil must be < 245 °F while cypress should be extracted at 245 °F. Vegetable oils are also adversely affected by exposure to excessive heat.

1.10.2. Pressure

Likewise, high pressure during distillation can also damage the delicate volatile aromatic molecules resulting in a loss of critical elements or transforming them causing harshness and other undesirable results. For example, pressure during lavender extraction should not exceed 3 psi, and cypress at about 5 psi.

1.10.3. Exposure Time

The length of time it takes to process botanical material through the chosen extraction method can be critical to the result. As examples, lavender oil requires about 1-1/2 hours for distillation. Cypress oil requires 24 hours of distillation to extract all of its active ingredients. However, if distillation is shortened by only two hours, the resulting cypress oil will be missing 18-to-20 of its constituent chemical elements!

1.10.4. Equipment and accessories

The equipment and accessories used for any extraction method should be chosen carefully and be fully compatible with the requirements of the resulting oil. Distillation pots, siphon tubing, filtration screens and all equipment that is to come into direct contact with the botanical material or oil (in any part of the extraction process), should not be made of chemically-reactive metals (copper and aluminum) nor any material that might contaminate the oil. Therapeutic-grade or other food-grade oils shall only be handled using food-grade stainless-steel cooking chambers and carrying vessels.

1.10.5. Batch size

Some essential and plant oils require smaller batches of plant material in relatively small distillation units to facilitate extraction of higher-quality oils or when an oil requires more delicate, careful handling to prevent loss of critical aromatic and/or therapeutic elements. By contrast, commercial-grade essential and plant oils are produced more quickly in larger units and at lower cost, but the resulting oil quality will be compromised. However, such oils are perfectly suitable for many uses such as mass-produced food and beverages, soap manufacturing, and similar uses.

1.11. Extraction Methods

The following is a list of the extraction methods, in alphabetical order, currently in employ around the world.

1.11.1. Distillation

The vast majority of true essential oils are produced by distillation. Distillation converts the volatile liquid (the essential oils) into a vapor and then condenses back into a liquid.

It is the most popular and cost-effective Extraction Method in use today. However, due to use of heat in this Method, it may not be used on very fragile plant material, because major therapeutic characteristics would be adversely affected, or where the Method is employed with great difficulty.

1.11.2. Cohobation

Cohobation, sometimes referred to as re-distillation or folding, is a process whereby previously distilled oil (Fig 3) is added back to the remaining botanical material producing an increasingly more concentrated, "purified" form. "Purified" means that some of the oil's chemical constituents (anywhere from 200 to 800 in a given botanical material) are removed with each distillation. Thus, this process can be used to remove certain non-skin-safe chemicals from the oil. The resulting product is also more concentrated, requiring less for a given effect. Cohobated oils cannot be of a therapeutic grade. Some essential oils require cohobation. For example, when Rose oil is extracted during Water Distillation, the one main constituent, Phenyl Ethyl Alcohol, cannot be extracted as this Method dissolves it into the water of the distillation still. The requisite missing constituent is captured with redistillation of this still water, the exact missing proportion of which is added back to the previously extracted oil. The resultant "complete" oil is called Rose Otto oil.

1.11.3. Hydro-diffusion

Hydro-diffusion differs from normal steam distillation (see Figure 3) only in that the steam is introduced from the top, rather than bottom, of the botanical material. Consequently, the steam condenses in a bottom chamber where the resultant oil benefits from less steam exposure for a shorter duration and, thus, a higher yield.

1.11.4. Hydro-distillation

In Hydro-distillation, the botanical materials are immersed in water and brought to a boil. (Fig 2).

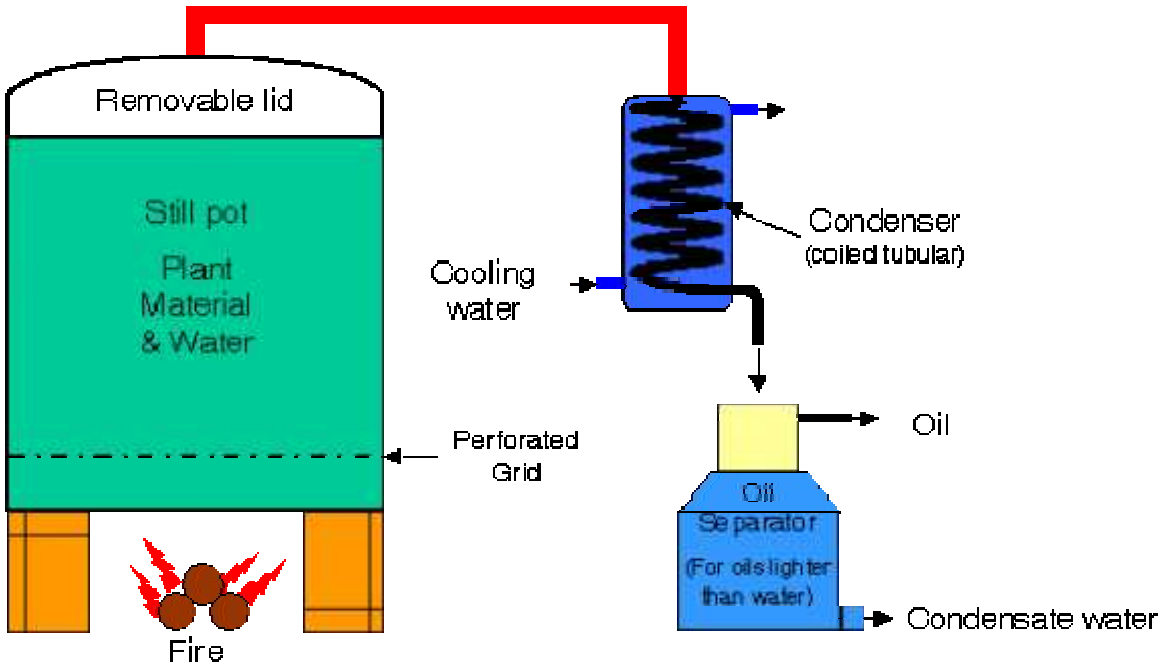


Figure 2. Hydro-distillation Diagram.

This Method provides limited overheating protection since the surrounding water acts as a heatsink to reduce the maximum temperature. Great care is also taken in the processing to exactly control temperature and length of exposure to preclude damage to the oils' character.

Hydro-distillation is used when the plant material has been dried and will not be damaged by boiling. It is also used for powdered materials such as powdered almond, and flowers, such as orange and rose, that need to float freely as they tend to lump together when just steam is passed through them.

Botanical material containing high amounts of esters are precluded from this Method since extended exposure to heat will start to break down the esters into its constituent alcohols and carboxylic acids.

Hydro-distillation can also be employed under vacuum which reduces the temperature to < 100 °C. This is beneficial in protecting both botanical materials and oils; e.g., Neroli oil, which is sensitive to heat, can be successfully extracted using this method.

The water from this process is also used and marketed as “floral waters” (also called “hydrosol” or “sweet water”), items such as rosewater, lavender water and orange water.

1.11.5. Rectification

Rectification is the process by which essential oil impurities are removed via redistillation, either by steam or vacuum.

1.11.6. Distillation

Essential oils are commonly extracted by steam distillation. The basic still design is shown in Fig 3.

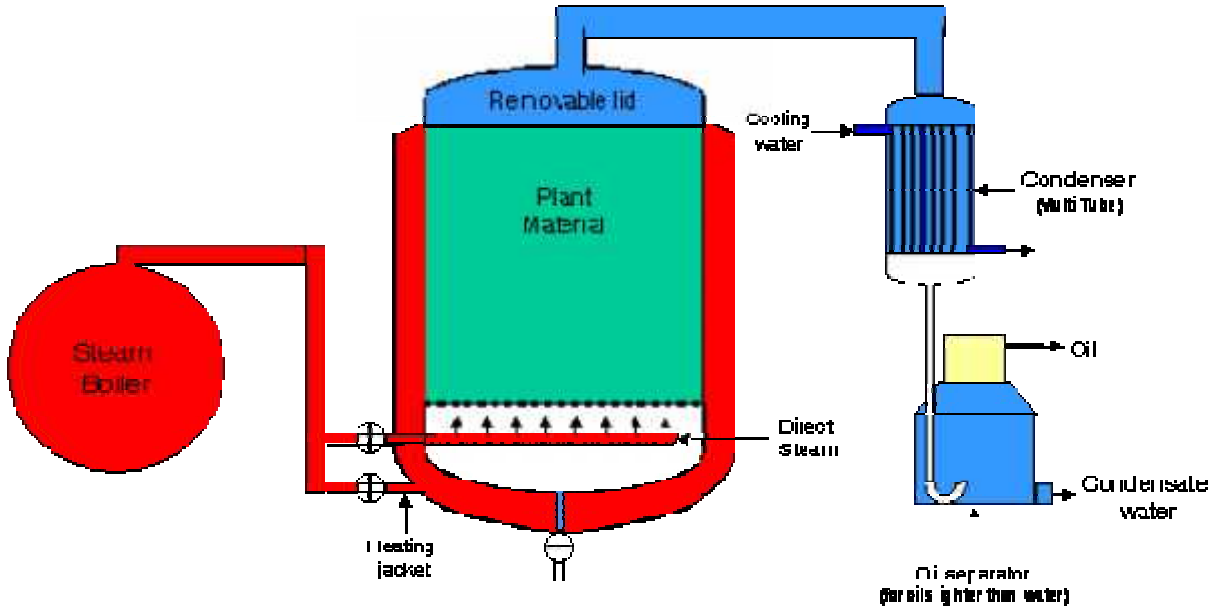


Figure 3. Typical Steam Distillation Process

Steam is piped from a boiler of the still vessel which contains the plant materials. The volatile oils in the plant material are released and carried upwards the steam and pass from the top of the still vessel through a condenser. Cold water flows continuously through a system of internal tubing in the condenser, cooling the steam/oil mix to a liquid. The water and oil flow into a separator (Florentine flask) here the oil, generally having a specific gravity less than water, floats to the surface and is collected. The still design, capacity, water purity levels and flow rates, operating temperatures and length of distillation are important factors that can affect the composition and quality of the oil. Technical assistance to optimize extraction efficiency is necessary (Guenther, 1952).

1.11.7. Expression

Most citrus peel oils are expressed mechanically or cold-pressed. Due to the large quantities of oil in citrus peel and the relatively low cost to grow and harvest the raw materials, citrus-fruit oils are cheaper than most other essential oils. Lemon or sweet orange oils that are obtained as by-products of the citrus industry are even cheaper. Prior to the discovery of distillation, essential oils (EO) were extracted by pressing.

1.11.8. Solvent extraction

Most flowers contain too little volatile oil to undergo expression and their chemical components are too delicate and easily denatured by the high heat used in steam distillation. Instead a solvent such as hexane or supercritical carbon dioxide is used to extract the oils. Extracts from hexane and other hydrophobic solvent are called concretes which are a mixture of essential oil, waxes, resins and other lipophilic (Oil soluble) plant material (Wanda, 2001).

Although highly fragrant, concretes contain large quantities of non-fragrant waxes and resins. As such another solvent often ethyl alcohol, which only dissolves the fragrant low-molecular weight compounds is used to extract the fragrant oil from the concrete. The alcohol is removed by a second distillation, leaving behind the absolute.

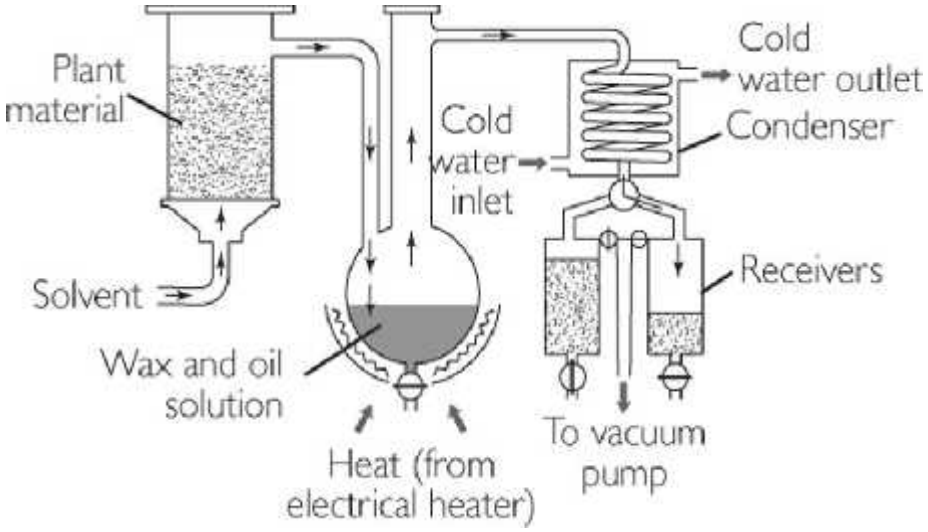


Figure 4: Typical Solvent Extraction Process

1.12. Classification of Phytochemical

Most of the antimicrobial activity in essential oils derived from spices and culinary herbs appears to derive from phenol compounds while other constituents are believed to contribute little to the antimicrobial effect. Plants synthesize a bewildering variety of phytochemicals but most are derivatives of a few biochemical motifs (Nychas, *et al*., 2003). Major classes of antimicrobials are listed in Table 1.2.

Table 1.2: Major classes of antimicrobial compounds from plants

Class	Subclass	Examples
Phenolics	Simple phenol	Catechol
		Epicatechin
	Phenolic	Cinnamic acid
	Quinones	Hypericine
	Flavonoids	Chrysin
	Flavones	Abyssinone
	Tannins	Ellagitannin
	Coumarins	Warfarine
Alkaloids		Berberine, piperine
Lectins and polypeptides		Mannose-specific agglutinin

1.13. Mode of action and inhibitory mechanisms

Microbial species/strain, stressed microorganisms, type and amount of antimicrobial compounds, number of microorganism present in food, presence of other antioxidant compounds, presence of other antimicrobial compounds, food additives, food components and carriers of phenolic antioxidants, temperature and other factors determine the antimicrobial activity of these antioxidant phenolic compounds.

Essential oils or their active compounds containing hydroxyl group (-OH) are highly antimicrobial (Farag, *et al.,* 1989b). Besides the presence of the hydroxyl group in the phenolic structure its position in the phenolic ring strongly influences the microbial effectiveness of components. The alkyl substitution into phenolic compound increases the antimicrobial activity (Pelczear, *et al*., 1988). Pauli and Knobloch (1987) reported that O- and P-alkyl substituted phenols from spice essential oils exhibited strong antifungal activity against 5 *Aspergillus* spp. , 4 *Penicillium* spp. and 2 *Fusarium* spp. Phenolic compounds in essential oils impair enzymatic mechanisms for energy production and cellular component synthesis (Conner and Beuchat, 1984b). It has been theorized that phenolic compounds are involved in multiple mode of action for their antimicrobial activity. These compounds interact with the cell membrane causing

leakage of cellular components, change fatty acid and phospholipid constituent, impair energy metabolism, alter nutrient uptake and electron transport and influence genetic material synthesis (Nychas, 1995). Phenolic compounds inhibit the cytoplasmic membrane-bound ATPase activity in *Staphylococcus aureus* (Ruco-munoz, *et al*., 1987). Essential oils of spices damage the structural and metabolic enzymes and inhibit repair of heat-injured yeast (Conner and Beuchat, 1984b). The inhibitory effect of the aldehydes, cinnamaldehyde, perillaldehyde, citral and citronrllal reduced in the presence of cysteine or glutathione indicating that the antifungal inhibition is mainly due to the reaction of aldehydes with -SH groups from fungi.

Antimicrobial activity of essential oil against microorganisms involves different modes of action depending on major components of the oils. Bioactive compounds interact with phospholipid bilayer of the cell membrane causing leakage of cellular materials (Kim, *et al*., 1995a; Juven, *et al*., 1994), inactivation of metabolic enzymes (Farag, *et al*., 1989b) or damage to DNA. Wendakoon and Sakaguchi (1995) reported that cinnamaldehyde and eugenol components of the spices were found to be strong inhibitor (Wendakoon and Sakaguchi, 1995). Essential oils damaged biological membrane interfering with membrane integrated enzymes (Knobloch, *et al*., 1989). Their site of action at the phospholipid bilayers of the cells inhibits the electron transport, protein translocation, phosphorylation and other enzymatic reaction. Cell wall lipopolysaccharides of Gram-negative bacteria inhibit the penetration of antimicrobial agents and their attachment to DNA that cause the death of cells. However, this trend is not always true since *Bacillus thermosphacta* was as resistant as *Serratia liquefaciens* (Ouattara, *et al*., 1997) and *Listeria monocytogenes* was more resistant to 11 essential oils than *Escherichia coli* 0157:H7, *Salmonella typhimurium* and *Vibrio vulnificus* (Kim, *et al.,* 1995a).

1.14. Factors influencing antimicrobial activity

Several factors may contribute to disparate observations on the antimicrobial potential of essential oils. Antibacterial activity depends on the following factors, such as:

1.14.1. Type, Concentration and Structure of the essential oils

Not all herbs and spices contain the same amount and composition of active ingredients. There is a fairly wide variation in the amount of important components in both fresh and commercial supplements. Chemical composition of essential oils depends on climate, seasonal and geographic conditions and harvesting period (Arras, *et al*., 1992) and distillation technique and distillation time. The volatility and poor solubility of most essential oils are problematic particularly with methods that rely on diffusion or dilution of the test substance in a microbiological medium.

1.14.2. The processing and the storage conditions of essential oil

In addition difference in composition related to variety, agronomic practice and processing are also likely to influence antimicrobial properties since these factors contribute to the both the profile and relative ingredients. The still design, capacity, water purity levels and flow rates, operating temperatures and length of distillation are important factors that can affect the composition and quality of the oil (Zaika, 1988). Antimicrobial activity of herbs and spices also depends on the extraction procedure of essential oil, extraction time and the physical and chemical parameters of extraction.

1.14.3. Type and concentration of the target microorganism

There are discrepancies about the spectrum of activity and potency of individual plant extracts against individual microorganisms such as Gram-positive bacteria were more sensitive to essential oils than Gram-negative bacteria (Smith, *et al.,* 1998).

1.15. Cinnamaldehyde

Cinnamic aldehyde or more precisely trans-cinnamldehyde, the only naturally-occurring form is the chemical compound that gives cinnamon its flavor and odor.

1.15.1. Natural occurrence

Cinnamaldehyde occurs naturally in the bark of cinnamon trees and species of the genus *Cinnamomum* as *Cinnamomum camphora* and *Cinnamomum* cassia. These trees are the natural sources of cinnamon and the essential oil of cinnamon bark is about 90% cinnamaldehyde. *Cinnamomum* spp. belongs to the family Lauraceae and frequently found in Srilanka and India (Iqbal, 1993 and Bell, 2009).

1.15.2. Harvesting and processing

Cinnamomum usually coppices well and commercial production of the bark spices entails cutting the stems low down after an initial establishment period of the bark spices the bushy re-growth stems at regular intervals thereafter. In Srilanka, a first harvest may be obtained after 3-4 years, although both quality and yield improve with subsequent cutting. The stems are cut during the rainy season to facilitate peeling of the bark. Details of harvesting practice differ slightly from country to country but the basic principles are the same. Strips of the bark are then formed into the familiar compound quills (cinnamon) or hollow quills (cassia) of the spices.

In Srilanka, cinnamon bark oil is produced by distillation of chips and variable amounts of featherings (pieces of inner bark from twings and twisted shoots) and quillings (Broken fragments of quills). In many cases the older form of hydro-distillation is used in which chips and water are placed together in the distillation vessel which is heated by direct fire. The oil distils over in two fractions, one lighter and one heavier than water and a form of cohobation is used to recover residual oil from the distillation waters. More modern methods involve steam distillation. The leaves left after trimming the cut stems, as well as those obtained from pruning operations, provide the raw material for production of cinnamon leaf oil. They are usually allowed to dry for a few days before distillation. Traditional stills in Srilanka are large wooden vessels capable of holding up to 200kg of leaves, on top of which is fitted a cooper still head. Steam is introduced from a separate a wood- fired boiler. In some cases all metal vessels are used and water steam distillation is employed.

1.15.3. Structure and physical properties

Cinnamaldehyde is a yellow oily liquid more viscous than water, smells strongly of cinnamon. Concentrated cinnamaldehyde is a skin irritant and the chemical is toxic in large doses but no agencies suspect the compound is a carcinogen or poses a long-term health hazard. Most cinnamaldehyde is excreted in urine as cinnamic acid, an oxidized form of cinnamaldehyde.

Both aromatic and an aldehyde, cinnamaldehyde has a mono-substituted benzene ring. A conjugated double bond (alkene) makes geometry of the compound planar. Cinnamaldehyde is technically trans-cinnamaldehyde because the terminal carbonyl is on the opposite side of the aromatic ring over the rigid double bond. A technique to synthesize cinnamaldehyde was published in 1884. Several methods of synthesis are now known, but cinnamaldehyde is most economically obtained from the steam distillation of the oil of cinnamon bark. The compound can be prepared from related compounds like alcohol (the alcohol form of cinnamaldehyde) but the first synthesis from unrelated compounds was the aldol condensation of benzaldehyde and acetaldehyde. Physicochemical properties of cinnamaldehyde are listed in Table 1.3.

Table1.3. Physicochemical properties of cinnamaldehyde

Properties	
Molecular formula	C_9H_8O
Molar mass	132.16g/mol
Appearance	Yellow oil
Density	1.05g/ml
Melting point	-7.5 C
Boiling point	248 C
Solubility in water	Slightly soluble
Refractive index	1.618 ~1.623

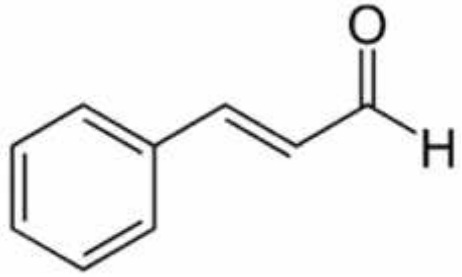


Figure 5: Structure of Cinnamaldehyde

1.15.4. Mode of action

Cinnamaldehyde acts on both Gram-positive and Gram-negative bacteria, including organisms that are food safety concern. Cinnamaldehyde has been reported to inhibit the growth of *Clostridium botulinium* (Bowles, 1993), *Staphylococcus aureus*, *Escherichia coli* 0157:H7 and *Salmonella* enteric serovar *typhimurium* (Helander, 1998). Cinnamaldehyde activity involves inhibition of cell wall synthesis (Kwon, *et al*., 2003) or inhibition of biosynthetic enzyme (Wendakoon, *et al*., 1995) is unlikely because of the rapidity of ATP inhibition or depletion. Interaction with the cell membrane causes disruption sufficient to disperse the proton motive force by leakage of small ions without leakage of larger cell components such as ATP.

1.15.5. Application

1.15.5.1. As a flavorant

The most obvious application for cinnamaldehyde is as flavoring in chewing gum, ice cream, candy, and beverages; use levels range from 9 to 4900 parts per million (ppm) (that is, less than 0.5%). It is also used in some perfumes of natural, sweet, or fruity scents. Almond, apricot, butterscotch, and other aromas may partially employ the compound for their pleasant smells. Cinnamaldehyde can be used as a food adulterant; powdered beechnut husk aromatized with cinnamaldehyde can be marketed as powdered cinnamon. (Fahlbusch, Hammerschmid, Panten, Pickenhagen, Bauer, Garb, and Surburg, 2002).

1.15.5.2. As an agrichemical

Cinnamaldehyde is also used as a fungicide (Pan Pesticide database, 2007). Proven effective on over 40 different crops, cinnamaldehyde is typically applied to the root systems of plants. Its low toxicity and well-known properties make it ideal for agriculture. Cinnamaldehyde is an effective insecticide, and its scent is also known to repel animals, such as cats and dogs (Pan Pesticide database, 2007). It has recently been recognized as a very effective insecticide for mosquito larvae (Muckentod, 2004). As little as 29 ppm of cinnamaldehyde kills half of *Aedes aegypti* mosquito larvae in 24 hours (Cheng, Liu and Tsai, 2004).

1.15.5.3. As an antimicrobial

Another use for cinnamaldehyde is as an antimicrobial. Researchers from the University of Illinois at Chicago (who were funded by the Wm. Wrigley Jr. Company) have found that cinnamic aldehyde, when used in Big Red, prevented oral bacterial growth by more than 50% (Science daily, 2009). It is especially effective against bacteria living at the back of the tongue, reducing anaerobic bacteria populations by about 43%. Cinnamaldehyde is also effective against bacteria *Listeria monocytogenes* in ground chicken meat (Hoque*, et al*., 2008).

1.15.5.4. As an anticancer agent

Recent research documents anticancer activity of cinnamaldehyde/cinnamic aldehyde observed in cell culture and animal models of the disease. Proliferation, invasion, and tumor growth were inhibited in a murine A375 model of human melanoma, though only at high doses not achievable through dietary intake (Yossa, et *al*., 2014).

1.15.5.5. Miscellaneous uses

Cinnamaldehyde is also known as a corrosion inhibitor for steel and other ferrous alloys in corrosive fluids. It can be used in combination with additional components such as dispersing agents, solvents and other surfactants. Its high refractive index of 1.6220 makes it a fairly safe and useful fluid for examining gemstone rough for inclusions.

1.16. Background of the study

In Bangladesh today, there still many people do not have adequate access to basic needs such as safe foods, fresh water, proper education, appropriate health services and clean environment among others. This is a major concern being addressed by the government at all amidst the rapidly growing population on one hand and a deteriorating environment on the other hand. For most of the developing countries especially for Bangladesh, the main issue of public health is concerned with the safe foods. Safe food is still the acute need basic health care, which is sadly lacking even at the most elementary level. This is true in both the rapidly growing cities and in the rural areas in Bangladesh. People have been suffering from various food borne diseases irrespective of all age groups, rich and common people of Bangladesh. The reason behind this is the unhygienic preparation of food and improper distribution to the consumer. Manufacturers on one hand do not categorize and identify the critical points of food chain for contamination by food borne pathogens; on the other hand, they are using chemical preservatives in processed foods at high concentration, which are sometimes health hazards. Some chemical preservatives are also used in homemade foods for keeping foods microbial contamination free, which are expensive and sometimes health hazards. Moreover, common food manufactures or people cannot use these chemical preservative, because of cost effective. Common people after infected by food borne pathogens also cannot access health care services. The World Health Organization (WHO) indicates that more than half of the world's population does not have access to adequate health care services. This is because poor people neither have to access to nor could afford the present health care services. Therefore, innovative alternative approaches are needed to address this problem.

Food borne disease are worldwide problem of great magnitude in terms both of the human suffering caused and the associated economic costs causing 70 of the 1.5 billion episodes of diarrhea that occur in the world annually.

1.17. Rationale

Herbs that have antimicrobial activities kill pathogenic bacteria and do not harm any beneficial bacteria. Many herbs help to protect viral infections as well as with bacterial infections. Whereas, pharmaceutical antibiotics will not protect a viral infection. Therefore, growing attention has been gives to natural antimicrobial compounds, which are more readily accepted by consumers. Thus plants make an important contribution to health care. This is due to the recognition of the value of traditional medical systems worldwide, particularly of Asian origin. Medicinal plants are distributed worldwide, but they are most abundant in tropical countries. Bangladesh is a green country with verities of plants for antimicrobial excellent activity against *Staphylococcus aureus* and *Shigella dysenterae,* the results will be published elsewhere. But no research was done on application of plant antimicrobials in foods for protection of food borne pathogens so far in Bangladesh. There is a bright possibility to work with medicinal herbs and spices to find antimicrobials for using in foods especially in RTE foods for controlling food borne pathogens. This is a new dimension of work in Bangladesh, which will open a door for few field of study. This is to be undertaken to develop antibiotic substitutes for the reduction of pathogens in foods or food items in Bangladesh.

1.18. Significance of the study in solving problems in the host country

Herbal plants for its antimicrobials properties offer alternative solution with tremendous opportunities. Herbs and spices not only provide access and affordable natural antimicrobials to poor people or common food manufacturers but are also cheap. The common food manufacturers can use these as preservatives in their foods. Homemaker can also use plant antimicrobials in their homemade food keeping food away from contamination of food borne pathogens and spoilage organisms herbs that have antimicrobial activities kill pathogenic bacteria and do not harm any bacterial infections, whereas pharmaceutical antibiotics will not protect a viral infection. Therefore, growing attention has been given to natural antimicrobial compounds, which are more readily accepted by consumers.

As stated earlier, Bangladesh is a green country with varieties of plants such as herbs, shrubs and trees including spices. But no research was carried out on the application of plant antimicrobials in foods for protection of food borne pathogens so far in Bangladesh. There is a bright possibility to work herbs and spices to find antimicrobials for using in foods. These are a new dimension of work in Bangladesh which will open a door for new field of study. Therefore findings will motivate longing producers to produce plant antimicrobials for controlling food borne pathogens and spoilage causing microorganisms. This can motivate farmers to grow herbs and spices for which they can earn money. This will create employment opportunities in the country and also earn foreign currency for the country that undoubtedly would have significant contribution to the national economy.

1.19. Objective of the study

1.19.1. General objective

Food safety becomes a key concern for the food-processing industry due to contamination of food by unsanitary practices. The excessive use of chemical preservatives are suspect because of their potential carcinogenic and teratogenic attributes has resulted in increasing pressure on food manufacturers to either completely remove chemical preservatives from their food products or to adopt more “natural” alternatives for the maintenance or extension of a product’s shelf life and quality. So, in the present study an attempt has been taken for designing of a complete protocol for occurrence of emerging food borne pathogens in common fast foods and inactivation of pathogens with essential oil.

1.19.2. Specific objectives

- Isolation of food borne pathogens from common fast food samples in Bangladesh.
- Identify the pathogens by conventional microbiological method.
- Screening of the essential oil for antibacterial activity against food borne pathogens and spoiling bacteria.
- Determination of the Minimum Inhibitory Concentration (MIC) and Minimum Bacterial Concentration (MBC).
- Optimization of pH and temperature for better antibacterial activities of essential oil
- Application of cinnamaldehyde in ground chicken meat to determine its ability in inhibiting *Escherichia coli, Bacillus cereus* and *Staphylococcus aureus.*

Chapter Two

Materials and Methods

2. Materials and Methods

2.1. Sampling site

Food samples were collected from different areas of Dhaka. Samples were collected from different food shop and street food of Newmarket, Gulshan, Mirpur and Dhanmondi.

2.2. Sample collection

For the purpose of sampling some clean dry, sterile zip lock bags were taken to the sampling site. About 100g of food sample was collected for each test and the samples were kept in the bags and tagged with labels, indicating the place, date and time of sampling of fast foods. Measures were taken to avoid contamination as far as possible. The samples were transferred to the laboratory as soon as possible.

2.3. Essential oil

Essential oils that were used in this study were cinnamaldehyde, carvacrol and euginol. The same lots of each oil were used throughout the experiments. Cinnamaldehyde was purchased from Nacalai Tesque Co (Kyoto, Japan) and carvacrol and euginol from Wako Pure Chemical Industries Limited (Osaka, Japan).The high quality of these chemicals (purity>95%) was confirmed by gas chromatographic (GC) analysis.

2.4. Reference strain

A total of 3 strains or species of frequently reported food borne pathogens and food spoilage bacteria were used in this study (Table 2.4.1). The test organisms were obtained from department of Microbiology. The long time stock cultures of the test organism in 20% glycerol in cryogenic vials were kept at 70°C. Working cultures were kept at 4°C on Tryptone soy agar slants and were periodically transferred to fresh slants.

Table 2.4.1. Test organisms used in this study

Organisms	No. of type culture	Source
Staphylococcus aureus	ATCC 25923	DU MICROBIOLOGY
Escherichia coli	ATCC 25922	DU MICROBIOLOGY
Bacillus cereus	ATCC 11778	DU MICROBIOLOGY

2.5. Media

Three types of media were used for the determination of antimicrobial activity of the essential oil.

2.5.1. Mueller-Hinton Agar (MHA)

- Mueller-Hinton agar was used as a basal medium. Mueller-Hinton agar was considered to be the best for routine susceptibility testing for the following reasons:
- It shows acceptable batch to batch reproducibility for susceptibility testing.
- It is low in sulphonamide, trimethoprim and tetracycline inhibitors.
- It gives satisfactory growth of most non fastidious pathogens.
- A large body of data and experience has been collected concerning susceptibility tests performed with this medium.
- Although Mueller-Hinton agar is reliable generally for susceptibility testing, results obtained with some batches may, on occasion, vary significantly. Dehydrated Mueller-Hinton agar was used.

2.5.2. Tryptone Soya Agar (TSA)

This media was used for the preparation of working culture. Working cultures were kept at 4°C on Tryptone soya agar (TSA) slants and were periodically transferred to fresh slants.

2.5.3. Mueller- Hinton Broth (MHB)

Mueller- Hinton broth was prepared for the inocula preparation. MHB contained all the components except the agar.

2.6. Culture Media

For isolation of *Escherichia coli, Staphylococcus aureus* and *Bacillus cereus* from food samples 3 types of selective media was used.

1. Sorbitol MacConkey agar
2. Mannitol salt agar
3. *Bacillus cereus* selective agar base supplemented with egg yolk and Polymyxin B

2.7. Preparation of food suspension

10 gm of the food sample was taken into a 250 ml conical flask containing 100 ml of sterilized physiological saline and homogenized with a homogenizer (NISSEI, Japan) for one minute. The resulting suspension was diluted in sterile normal saline (physiological saline) following the tenfold serial dilution technique (Cappuccino and Sherman, 1999).

2.8. Inoculation and incubation

0.1ml of sample suspension from each dilution (original to 10^{-5}) was taken onto the selective medium with a sterile micropipette tips and was spread uniformly over the agar surface by a sterile spreader. In this way three sets of plates for each dilution of each sample were made. Three sets of plates were incubated at 37°C for 24 hours.

2.9. Isolation of colonies

The plates with discrete bacterial colonies were selected for isolation. Selected colonies were marked and their colonial characteristics were recorded. The selected colonies were streaked on fresh agar plates and then incubated at 37°C for 24 hour.

2.10. Maintenance and preservation of the isolates

The purified bacterial isolates were maintained throughout the study on TSA slant. The culture tubes were wrapped with polythene bags and kept a refrigerator at 4° C for the purpose of preservation for short time. These isolates were transferred to fresh medium periodically.

For long time preservation the isolates were kept at -20°C in 20% glycerol broth (Hopwood, *et al*., 1985).

2.11. Standard antibiotics

Antibiotic and its disc potency used was Imipenem (10µg) manufactured by Oxoid Ltd.

2.12. Taxonomic study

For this purpose an array of microscopic and biochemical tests were performed that served for the identification of the isolates.

2.13. Colonial morphology

On plating medium the growth of bacterial colonies were morphologically studied as their form, elevation and margin.

2.14. Gram staining

Gram staining of the isolates was carried out following the method described by Hucker and Conn (1923). The result was recorded as Gram Positive and Gram negative.

2.15. Biochemical studies of the isolated bacterial species

The following tests were carried out to determine the biochemical activities of the suspected isolates of *Escherichia coli, Bacillus cereus* and *Staphylococcus aureus.*

2.15.1. IMViC test (Cappuccino and Sherman, 1999)

Using sterile technique, each isolates was inoculated into appropriately labeled peptone water tube, MR-VP broth and Simmons citrate agar slant. All the tubes were incubated at 37°C for 24 hours. Then the corresponding reagents were added to the tube; results were recorded.

2.15.2. Indole production test

To determine indole production ability, 10 drops of Kovac's reagent were added in each peptone water tube and the tube was shaken vigorously for 1 min. A red color in the reagent layer indicates positive reaction.

2.15.3. Methyl red test

Approximately one third of each MR-VP broth culture was transferred into an empty test tube and five drops of methyl red indicator was added to each culture and read immediately. Bright red color indicate positive MR test while the negative test showed yellow color.

2.15.4. Voges-Proskauer test

10 drops of Barritt's reagent A and then Barritt's reagent B were added to each MR-VP broth culture. Vigorous shaking was done after addition of each reagent to aerate the culture. Bright orange red color indicates positive reaction.

2.15.5. Citrate utilization test

Simmon's citrate agar slants were examined for change in color from greenish to blue. Presence of blue color indicates citrate utilization.

2.15.6. Nitrate reduction test (Collins and Lyne, 1984)

This test was performed to demonstrate if the organisms were capable of reducing nitrate to nitrite. Formation of nitrite indicated that the species is capable of producing the enzyme Nitrate reductase.The following three reagents were used for this test

Reagent A: Sulfanilic acid- acetic acid solution

Sulfanilic acid	8.0 g
5 N Acetic acid	1.0 L (1 part chemically pure acetic acid to 2.5 part distilled water)

Sulfanillic acid was dissolved in acid and stored in brown glass bottle.

Reagent B: Dimethyl α-naphthalamin solution

Dimethyl α-naphthalamin	6.0 ml
5 N Acetic acid	1.0 L

The reagent was stored in brown bottle.

Reagent C: Zinc dust

The tube of nitrate broth in duplicates were inoculated with bacterial cultures and incubated at 37°C for 24 hr. After incubation, 1 ml of reagent A was added to the incubated tube and the tube was shaken. Then 1 ml of reagent B was added to the incubated tube and the tube was shaken. Formation of a distinct red or pink color indicated the reduction of nitrate to nitrite. An absence of pink or red color in the broth did not necessarily mean that the organism was incapable of reducing nitrate to nitrite. So the broth was tested further. A small pinch of zinc dust was added to each tube to reduce any remaining nitrate. A characteristic pink or red color would be produced if there were any remaining nitrate in the broth.

2.15.7. Test for gelatin hydrolysis (Collins and Lyne, 1984)

Nutrient gelatin deep tubes were inoculated with the test organism and kept at 37°C for 24 hr. Following incubation the cultures were placed under refrigeration at 4°C for 30 min. Culture that remained liquid produced gelatinase and demonstrated gelatin hydrolysis. Culture that solidified on refrigeration lacked gelatinase and gave negative reaction.

2.15.8. Test for starch hydrolysis (Collins and Lyne, 1984)

Starch agar plates were inoculated with the test organisms by streaking and incubated at 37°C for 24 hr. After incubation the plate culture were flooded with Gram iodine solution. Clear zone around the streaking line indicated positive starch hydrolysis. Presence of the blue color surrounding the growth indicated the negative starch hydrolysis.

2.15.9. Urease test

Urea broth tubes were inoculated with test organisms and incubated at 37°C for 24 hr. After incubation the tubes were examined for color change. Formation of Bright pink color from orange color broth indicated positive urease test and absence of pink color in the broth means negative reaction.

2.15.10. Catalase test (Collins and Lyne, 1984)

The enzyme catalase capable of decomposing hydrogen per oxide into water and molecular oxygen. For this test, Nutrient broth tubes were inoculated and incubated at 37°C for 24 hr. After incubation a drop of culture broth from each tube was taken on different clean dry slides and a drop of 3% hydrogen peroxide was added to each of them. Production of bubbles indicated the positive result.

H_2O_2---------- H_2O+O_2

2.15.11. Coagulase test

For this test, Nutrient agar slants were inoculated with the test organism and incubated at 37°C for 24 hr. The slide was divided into two sections with grease pencil; one should be labeled as “test” and the other as “control .A small drop of distilled water was placed on each area and colonies of bacteria were emulsified on each drop to make a smooth suspension. The test suspension was treated with a drop of citrated plasma. Clumping within 5-10 seconds was taken as a positive reaction.

2.15.12. Oxidase test (Gordon and McLeod, 1928)

A filter paper was soaked with the substrate tetramethyl-p-phenylenediamine dihydrochloride. Then the colony to be tested was picked with platinum loop and made a smear on the filter paper. Within 10-30 seconds inoculated area of the paper was changed to deep blue or purple color which means positive reaction. No color changed means negative reaction.

2.15.13. Fermentation tests

2.15.13.1. General fermentation test (Collins and Lyne, 1984)

Fermentation test is of considerable significance in the identification and classification of microorganisms. They differ in their ability to ferment different carbohydrates. Some of them produced both acid and gas in fermentation medium. There are still others that cannot ferment carbohydrate at all. In this study, fermentation test of the sugars e.g. Glucose, Sorbitol, Sucrose, Lactose, Fruc-tose, Galactose, Mannose, Xylose, Arabinose, Rhamnose, Maltose, Raffinose and Starch was done. Fermentation tubes with these compounds were made using phenyl red as an indicator. One Durham tube was introduced in each of the fermentation tubes. The tubes were inoculated in duplicates and allowed to incubate for 24 hr at 37°C.The change of color of the indicator to yellow showed the production of acid. Pink color of the indicator indicated alkalinity. If carbon dioxide would be formed during fermentation, it would be collected in Durham tube.

2.15.14. Categorization and identification of suspected isolates

On the basis of different microscopic observations, cultural and biochemical properties, the isolates were categorized as the isolate no. Ec-01 to Ec -19; Sa-01 to Sa-23 and Bc-01 to Bc-12.This isolates were identified following the criteria of the Bergey's manual of systematic Bacteriology, vol-4, 9th edition (John, 1994) .

2.2. Methods

2.2.1. Screening of the essential oil

The antimicrobial activities of the essential oils were evaluated using disc-diffusion assay (Bauer, *et al*., 1996) against food borne pathogens and spoilage organisms.

2.2.2. Preparation of Mueller-Hinton Agar

Mueller-Hinton agar was prepared by the following steps:
Mueller-Hinton agar was prepared from a commercially available dehydrated base according to the manufactures instructions.
Immediately after autoclaving, it was allowed to cool in a 45 to 50°C water bath.
The freshly prepared and cooled medium was poured into sterile glass flat bottomed Petri plate on a level, horizontal surface to give a uniform depth of approximately 4mm.
The agar medium was allowed to cool to room temperature and the plate was used on the same day or was stored in a refrigerator (4°C).

2.2.3. Preparation of essential oil stock solutions

Essential oil was received as liquid form. The crude samples contain 100% cinnameldehyde, carvacrol and euginol. Liquid essential oil was accurately measured with a measuring cylinder and diluted in the 95% ethanol to yield 10% concentration. The stock was made aliquot in 5 ml volumes and frozen at -20°C.

2.2.4. Impregnation of filter paper discs

Discs (8mm in diameter) made of Whatman filter paper no.1 (ADVANTEC; Toyo Roshi Kaisha Ltd Japan) were impregnated with 50µl of each 3% cinnamaldehyde, carvacrol and euginol and were then dried at 40°C for 1 hour in hot air oven (Barnstead Labline, USA) and were stored at 4°C until use. Negative control (without the essential oil) was prepared in ethanol.

2.2.5. Preparation of inocula

One loopful of each test organism from cryogenic vial was transferred into 9 ml of sterile Tryptone Soya Broth (TSB) and grown at 37°C for 24 hours. After that 1 ml of the TSB culture was again transferred into 9 ml of sterile TSB and grown at the same conditions. One loopful of the TSB culture was then streaked into the TSA plate and grown at 37°C for 24 hours.

The inocula of the test organisms were prepared by transferring 3 to 4 colonies of the cultures on TSA into 9 ml of sterile Mueller-Hinton Broth (Difco) and incubated at 37°C for 5 to 6 hour, if necessary 12 to 18 hour growth was considered. The MHB culture was compared with McFarland 0.5 (Jorgensen, *et al*., 1999) turbidity standards (10^7 CFU/ml). This resulted in a suspension containing approximately 1 to 2×10^7 CFU/ml for each inoculum.

2.2.6. Preparations of the McFarland standard:

0.05 ml (50µl) of 0.048 M $BaCl_2$ (1.17% w/v $BaCl_2.2\ H_2O$) was added to 9.95 ml of 0.18 M H_2SO_4 (1% v/v) in a test tube with constant stirring. The tube was then sealed tightly stored in dark at room temperature to prevent loss of evaporation. The standard may be stored for up to 6 months, after which time it should be discarded.

2.2.7. Inoculation of test plates:

1. Within 15 minutes after adjusting the turbidity of the inoculums suspension, a sterile cotton swab was dipped into the adjusted suspension. The swab was rotated several times and pressed firmly on the inside wall of the tube above the fluid level. This removed excess inoculums from the swab.
2. The dried surface of a Mueller-Hinton agar plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculums.

2.2.8. Application of discs to inoculated Agar plates

The essential oil impregnated discs were dispensed onto the surface of the inoculated agar plate appropriate spatial arrangement using an ethanol dipped and flamed forceps. Each disc was pressed down to ensure complete contact with the agar surface. The discs were placed so that they are no closer than 24 mm from centre to centre of the discs. For each plates 5 disc were placed. Plates were kept at refrigeration temperature for 30 minutes for better absorption, during this time microorganisms will not grow but absorption of extracts would take place. Discs for negative control were prepared using the same solvent without the essential oil. The plates are inverted and placed in an incubator at 37°C for 24 hours.

2.2.9. Evaluation of plates

Antibacterial activity was evaluated by measuring the zone of inhibition in mm (Including the 8 mm disc) with calipers near the agar surface and the results were recorded. A reading of 8 mm considers no zone of inhibition. The endpoint was taken as complete inhibition of growth as determined by the naked eye. Each essential oil was tested triplicates and assay in this experiment was repeated thrice.

2.3. Effect of temperature on antimicrobial activity of essential oil

The effects of temperature on antimicrobial activity of essential oil were determined by the methods as described by Lee, *et al*., (2004).

2.3.1. Preparation of essential oil

The test samples i.e. 3% of EO of cinnamaldehyde was prepared for antibacterial activity from the stock (10%) with the 95% ethanol and transferred into five vials. Negative control (without the essential oil) was prepared in ethanol. Then vials with the essential oil and the Negative control were incubated in water bath set as 25, 37, 50, 75 and 100°C respectively for 30 min. the water bath was pre-adjusted with those temperatures for 5 minutes before placing the samples. Then the heated EO at different temperatures were cooled and stored at 4°C until use.

2.3.2. Determination of antibacterial activity at different temperature

After the temperature treatment the antibacterial activity of the EO of cinnamaldehyde was carried out against the organisms. The antibacterial activity was assayed by the disc diffusion methods by Bauer, *et al.,* (1966) which was described above.

2.3.3. Effect of pH on antibacterial activity of essential oils

The Effect of pH on antibacterial activity of essential oil of cinnamaldehyde was assayed by using reported previously with slight modification (Shibata, *et al.,* 1995*,* Ohno, *et al.,* 2003). The buffer solutions used were 50mM Tris-HCL buffer (pH 9.0).

2.3.3.1. Preparation of pH buffer solution

For pH=5.00

19.21g of citric acid was dissolved in 1000 ml of sterile distilled water to make 0.1M solution of citric acid and 53.65g of di-Sodium hydrogen phosphate ($Na_2HPO_4.7H_2O$) in 1 liter volume of sterile distilled water to make 0.2M solution. Then 24.3 ml of 0.1M solution of citric acid was added to and 25.7ml of 0.2M solution di-Sodium hydrogen phosphate and finally diluted to 100ml (Walpole, 1914). The concentration of the resulted citric buffer was 0.1M which was then converted to 50 µl with the sterile de-ionized water.

For pH=7.00

27.8g of monobasic sodium hydrogen phosphate was dissolved in 1000ml of sterile distilled water to make 02.M solution and 53.65g of disodium hydrogen phosphate ($Na_2HPO_4.7H_2O$) in 1 liter volume of sterile distilled water to make 0.2M solution. Then 39.0ml of 0.2M solution of monobasic sodium hydrogen phosphate was added to and 61ml of 0.2M solution of di-sodium hydrogen phosphate and finally diluted to 200ml (Plumet, 1914). The concentration of the resulted phosphate buffer was 0.2M which was then converted to 50 µl with the sterile de-ionized water.

For pH=9.00

24.2g of Tris (hydroxyl methyl) amino methane was dissolved in 1000ml of sterile distilled water to make 02.M solution. Then 50.0ml of 0.2M solution of Tris (hydroxyl methyl) amino methane was added to .05ml of 0.2M HCl and finally diluted to 200ml (Michaelis, 1930). The concentration of the resulted Tris buffer was 0.2M which was then converted to 50 µl with the sterile de-ionized water.

All the buffers were sterilized through 0.45 µm of membrane filter, stored at 4°C in a sterile bottle.

2.3.3.2. Determination of pH of the essential oil

The pH of the essential oil was determined with sterile pH paper strip. At 10% concentration pH of the cinnamaldehyde was 5.

2.3.3.3. Preparation of essential oil

From the stock (10%) solution 3% of EO of cinnamaldehyde was prepared of pH 5, 7 and 9 by adding measured amount in buffer. Negative control (without the essential oil) was prepared in de-ionized water. Three negative controls were prepared in de-ionized water.

2.3.3.4. Impregnation of filter paper discs

Discs (8 mm in diameter) made of Whatman filter paper no. 1 (ADVANTEC; Toyo Roshi Kaisha, Ltd. Japan) were Impregnated with 50 µl of 3% cinnamaldehyde of each pH and were then dried at 40°C for 1 hour in hot air oven (Labline USA) and were stored at 4°C until use.

2.3.3.5. Preparation MHA

Mueller-Hinton agar was prepared from a commercially available dehydrated base according to the manufacturer's instructions and the pH of the media was adjusted as 7.0 by adding NaOH or HCl. Immediately after autoclaving, it was allowed to cool in a water bath set at 50°C. The freshly prepared and cooled medium was poured into glass flat-bottomed Petri plate on a distinctive level, horizontal surface to give a uniform depth of approximately 4mm. the agar medium was allowed to cool to room temperature and the plate was used on the same day, or was stored in a refrigerator (4°C) until use.

2.3.3.6. Application of disc to Inoculated Agar Plates

The prepared different buffered essential oil discs were dispensed onto the surface of the inoculated agar plate. Negative controls were prepared using the different buffer solution without the essential oil. In each plate three discs were of the same pH. The plates were kept at 4°C for 30 min for better absorption of the sample and then inverted and placed in an incubator set to 37°C for 24 hours.

2.3.3.7. Evaluation of the plate

Antibacterial activity was evaluated by measuring the zones of inhibition in mm (including the 8mm disc) for different p^H with calipers near the agar surface and the results were recorded. A reading of 8mm meant no zone of inhibition. The endpoint was taken as complete inhibition of growth as determined by the naked eye. Each essential oil was tested in triplicates and assay in this experiment was repeated thrice.

2.4. Determination of the Minimum Inhibitory Concentration (MIC)

The determination of the EO of cinnameldehyde, carvacrol and euginol was determined by tube dilutions techniques in Muller-Hinton broth according to Sengul, *et al*., (2005). The series of concentration used was 5, 2.5, 1.25, 0.0625 and 0.03125 and were prepared by using 95% ethanol. The MIC was done at 37°C.

2.5. Inoculation of vials

0.9 ml of the MHB was taken in each of the sterile and dry glass vials appropriately labeled with concentration of essential oil.1.0 ml of the respective essential oil concentration was dispensed into the respective vials and 100 µl of each test organisms were added to the vials with their names labeled to make sure that each of the organisms faced a different concentration of the essential oil.

So, the final reaction volume became 2 ml. A positive control was made with MHB and sterile distilled water plus culture of test organisms. A negative control was made with essential oil and MHB but no test organisms. All the prepared vials were then incubated at 37°C for 24 hours.

2.6. Reading of MIC and MBC

Because when oils were diluted in buffer and media became turbid for this growth was not visible in the MHB broth. For determination of MIC and MBC, 10µl inoculums from each test vials were transferred onto MHA plate by a micropipette. The plates were then incubated at 37°C for 24 hours. The MIC was considered as the lowest concentration of essential oil that inhibits growth of bacteria on plate.

The lowest concentration that killed 100% of the bacteria (no growth on plate) was recorded as Minimum Bactericidal Concentrations (MBC).

2.7. Determination of antibiotic sensitivity against *Escherichia coli*, *Bacillus cereus* and *Staphylococcus aureus*

Escherichia coli, *Bacillus cereus* and *Staphylococcus aureus* were tested for their ability to grow in the presence of different antibiotics at concentration selected to be of diagnostic value. The antibiotic discs were placed on the surface of Muller-Hinton agar plates inoculated with 0.1 ml of bacterial suspension. The plates were then incubated at 37°C for 24 hr. Reading was taken and the result was recorded. The following antibiotic discs (Oxoid Ltd) were used:

AMC 30=Amoxyclave 30 µg, ATM 30=Aztrenam 30 µg, AK 30=Amikacin 30 µg, CEC 30=Cefaclor 30 µg, CRO 30=Ceftriaxone 30 µg, CL 30=Cephalexin 30 µg, NA 30=Nalidixic acid 30 µg, F 30=Nitrofurantoin 30 µg, NET 30=Netilmycin 30 µg, MEM 10=Meropenem 10 µg, MEL 25=Mecillinam 25 µg, IPM 10=Imipenem 10 µg, SXT 25=Co trimoxazole 25 µg, CN 10=Gentamycin 10 µg, CIP 5=Ciprofloxacin 5 µg, CFM 5=Cefixime 5 µg, DO 30=Doxycline 30 µg, AZM 15=Azithromycin 15 µg, OB 5=Cloxacillin 5 µg, P 10=Penicillin 10 µg, C 30=Chloramphenicol 30 µg, E 15=Erythromycin 15 µg, TE 30=Tetracycline 30 µg, CTX 30=Cefotaxime 30 µg.

2.8. Use of cinnameldehyde to inactivate *Escherichia coli*, *Bacillus cereus* and *Staphylococcus aureus* in ground chicken meat

The procedure for inactivation of *Escherichia coli*, *Bacillus cereus* and *Staphylococcus aureus* in ground chicken meat was followed with slight modifications described by Hoque, *et al*.,(2008). A 500 gm ground chicken meat sample was sterilized with ethanol and then with 15 minutes UV treatment for complete reduction of the contamination or other microbes. Then 5 gm meat sample was collected to examine the sample. Rest chicken sample was inoculated with the organisms to obtain a final concentration of about 10^7 CFU/gm. Inoculated ground chicken samples were uniformly mixed for 1 min with sterilized spoon. Cinnamldehyde was then added with half of the chicken sample and mixed uniformly for 1 min. Then the treated and the control chicken samples were divided in small aliquots (5 gm each) and packed in sterilized Ziploc packs and stores at -20°C for 10 days. Microbiological analysis of control and treated sample was done on 0-10 days of post storage. Each sample was homogenized for 1 min in sterilized distilled water using homogenizer. From the mixture tenfold serial dilution (10^{-5}) was prepared and 0.1 ml of each diluent was spread plated on MHA (non-selective) and SMA, MSA and MM (selective for *Escherichia coli, Staphylococcus aureus* and *Bacillus cereus.* The plates were then incubated at 37°C for 24 hours and then presumptive colonies of the test organism were counted and recorded.

2.9. Statistical analysis

The inhibition zone was calculated as means± S.D (n=3 or 4).The significance among different data was evaluated by analysis of variance (ANOVA) using Microsoft excel program. Significant differences in the data were established by least significant difference at the 5% level of significance.

Chapter Three

Results

3. RESULTS

3.1. Collection of food sample

The food sample was collected from different parts of Dhaka city at different time. All parameters regarding collection of samples were shown in table 3.1

Table 3.1: Physical parameters of collected sample

Sample size	Name of the sample	Date of collection	Site of collection
5	Beef Burger	5/12/2011	New market,Gulshan
5	Beef patties	5/12/2011	New market
5	Egg chop	5/12/2011	Chandi Chalk
5	Chicken sandwich	5/12/2011	New market
5	Chicken patties	5/12/2011	New market
5	Chicken sorma	6/12/2011	Mirpur
5	Faluda	6/12/2011	Gulshan
5	Ice-cream	6/12/2011	New market
5	Pasteurized milk	7/12/2011	New market
5	Pudding	7/12/2011	Mirpur
5	Pastry	7/12/2011	Mirpur, New market

3.2. Suspected *Escherichia coli* (Based on selective medium)

Based on characteristic colony of selective medium, the suspected *Escherichia coli* colony prevalence was shown in Table 3.2.1.

Table 3.2.1: Prevalence of suspected *Escherichia coli* (considering the characteristic colony) in food samples

Name of the samples	Sample size	Range of counts/ ml or g	Average counts/ ml or g	Mean± SD
Beef burger	5	1.7×10^1-4.0×10^1	2.82×10^1	1.45 ± 0.14
Beef patties	5	2.9×10^3-5.3×10^3	4.10×10^3	3.61 ± 0.11
Chicken sandwich	5	2.6×10^3-13.5×10^4	1.92×10^4	4.28 ±0.34
Chicken sorma	5	1.13×10^4-5.5×10^4	3.23×10^4	4.50 ± 0.24
Ice cream	5	--------	-------	--------
Pasteurized milk	5	--------	-------	-------

3.3. Suspected *Staphylococcus aureus* (Based on selective medium)

Based on characteristic colony of selective medium, the suspected *Staphylococcus aureus* colony prevalence was shown in Table 3.3.1.

Table3.3.1.Prevalence of suspected *Staphylococcus aureus* (considering the characteristic colony) in food samples

Name of the samples	Categories of samples	Sample size	Range of counts/ml or g	Average counts/ml or g	Mean± SD
Milk based products	Raw milk	5	5.0×10^4-9.2×10^4	6.6×10^4	4.82 ± 0.1
	Ice cream	5	-----	------	------
	Pasteurized milk	5	------	-----	------
Egg-milk based product	Pudding	5	1.2×10^3-2.8×10^3	2.2×10^3	3.34 ± 0.15
Beef products	Beef burger	5	6.0×10^4-2.0×10^6	3.49×10^5	5.54 ± 0.58
Chicken based products	Chicken sandwich	5	8.0×10^3-1.3×10^5	1.41×10^4	4.14 ± 0.29
	Chicken sorma	5	1.0×10^4-8.0×10^6	2.12×10^5	6.32 ± 0.51
	Chicken patties	5	7.0×10^4-1.0×10^5	6.5×10^4	4.81 ± 0.13

3.4. Suspected *Bacillus cereus* (Based on selective medium)

Based on characteristic colony of selective medium, the suspected Bacillus cereus colony prevalence was shown in Table 3.4.1.

Table3.4.1. Prevalence of suspected *Bacillus cereus* (Based on selective medium) in milk based food samples

Name of the samples	Sample size	Range of counts/ml or g	Average counts/ml or g	Mean± SD
Egg chop	5	2.0×10^2-5.0×10^2	2.5×10^2	2.47±0.27
Faluda	5	1.0×10^3-5.0×10^3	3.19×10^3	2.44±0.31
Pastry	5	0.1×10^2-0.3×10^2	1.1×10^2	2.04 ± 0.08
Ice cream	5	------	------	------
Pasteurized milk	5	------	------	-------

3.5. Growth characteristics of the selected isolates of *Escherichia coli* on different solid media

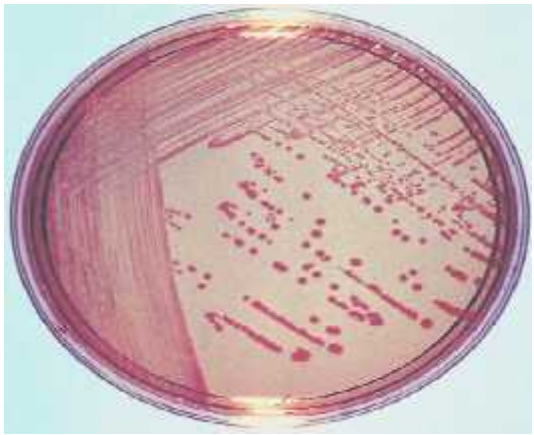
The selected isolates of *Escherichia coli* were grown on Nutrient agar, MacConkey agar, Eosine Methylene Blue (EMB), Sorbitol- MacConkey Agar (SMAC) and Blood Agar. Growth characteristics were observed and results shown in Table 3.5.1 and growth characteristics of the representative isolates are shown in Fig 6.

Table3.5.1. Cultural properties of *Escherichia coli*

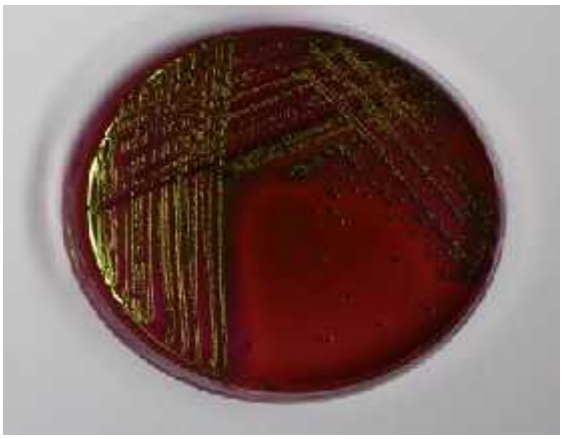
Cultural properties	
Media	**Growth characteristics**
Nutrient Agar (NA)	Colorless, transparent, moderate sized, non-viscous, convex colonies
MacConkey Agar	Pink, elevated, moderately non-viscous colonies
Eosine Methylene Blue (EMB)	Moderately sized colonies with green metallic sheen and black centered
Sorbitol-MacConkey Agar (SMAC)	Round, glossy, transparent, entire, convex small, pinpoint to pinhead colonies
Blood Agar	α-Hemolysin

(A)

(B)



(C)



(D)

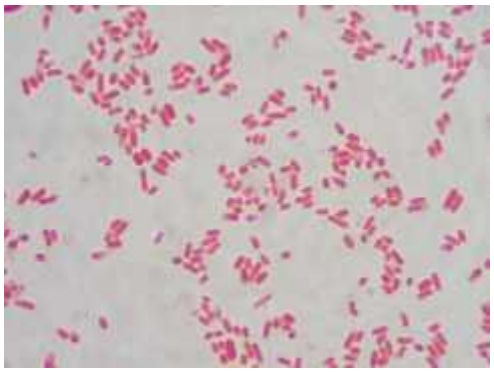
Fig 6: Growth characteristics of the selected isolates of *Escherichia coli* on different solid media (A) Nutrient agar plate (B) MacConkey agar plate (C) Sorbitol MacConkey agar plate (D) Eosin Methylene Blue agar plate

3.6. Staining properties of the selected isolates

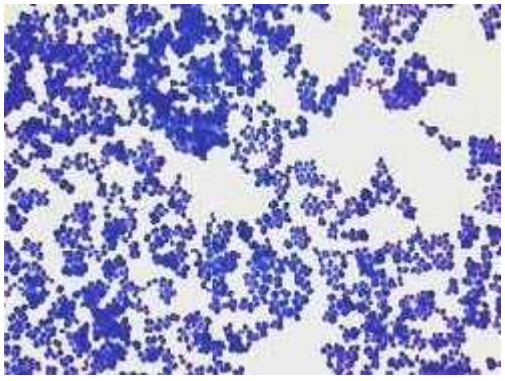
Gram stain of the selected isolates were carried out and observed under compound microscope (Model Olympus CH 20, BIMF 200 Japan). The results are shown in Table 3.6.1. and microscopic pictures of the representative isolates are shown in Fig 7.

Table3.6.1: Staining and morphological properties of the selected isolates

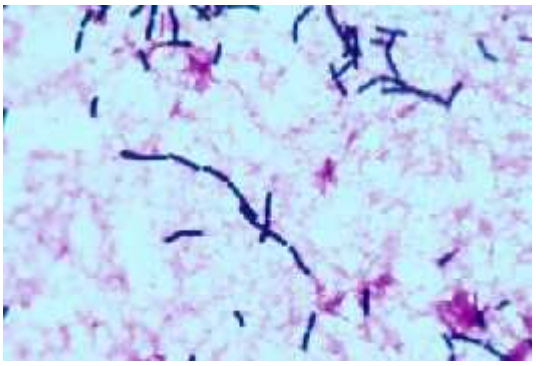
Isolate no.	Gram reaction /Morphological characteristics
Ec 01, 02, 03, 04, 05, 07, 11, 12, 13, 15, 16, 17, 18, 19	Gram negative Small rod shape, non- spore former
Sa 01, 02, 03, 04, 05, 06, 07, 08, 09, 10, 11, 12, 14, 16, 17, 22, 23	Gram positive Round, form cluster, non spore former
Bc 01, 02, 04, 05, 06, 07, 10, 11, 12	Gram positive Rod shape and spore former



(A)



(B)



(C)

Fig 7: Staining properties of the selected isolates under microscope (A) *E. coli* (B) *S. aureus* (C) *B. cereus*

3.7. Biochemical properties of the selected isolates

Selected isolates were studied for their biochemical properties. The results are shown in Table 3.7.1, 3.7.2, 3.7.3, 3.7.4, 3.7.5, 3.7.6 and 3.7.7.and Biochemical tests of the representative isolates are shown in Fig 8a & 8b.

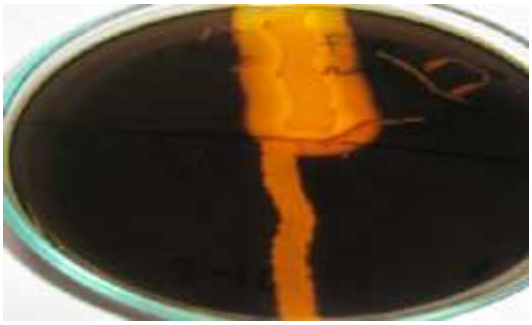
Table 3.7.1: Biochemical properties of the isolates (suspected as *Escherichia coli)*

Isolates	MR	VP	Indole	citrate	Urease	H_2S	Gelatin	Oxidase	Catalase
Ec-00	+(VE)	-(VE)	+(VE)	-(VE)	-(VE)	-(VE)	-(VE)	-(VE)	+(VE)
Ec-01	+(VE)	-(VE)	+(VE)	-(VE)	-(VE)	-(VE)	-(VE)	-(VE)	S+(VE)
Ec-02	+(VE)	-(VE)	+(VE)	-(VE)	-(VE)	-(VE)	-(VE)	-(VE)	+(VE)
Ec-03	+(VE)	-(VE)	+(VE)	-(VE)	-(VE)	-(VE)	-(VE)	-(VE)	+VE)
Ec-04	+(VE)	-(VE)	+(VE)	-(VE)	-(VE)	-(VE)	-(VE)	-(VE)	+(VE)
Ec-05	+(VE)	-(VE)	+(VE)	-(VE)	-(VE)	-(VE)	-(VE)	-(VE)	W+(VE)
Ec-07	+(VE)	-(VE)	+(VE)	-(VE)	-(VE)	-(VE)	-(VE)	-(VE)	S+(VE)
Ec-11	+(VE)	-(VE)	+(VE)	-(VE)	-(VE)	-(VE)	-(VE)	-(VE)	+(VE)
Ec-12	+(VE)	-(VE)	+(VE)	-(VE)	-(VE)	-(VE)	-(VE)	-(VE)	+(VE)
Ec-13	+(VE)	-(VE)	+(VE)	-(VE)	-(VE)	-(VE)	-(VE)	-(VE)	+(VE)
Ec-15	+(VE)	-(VE)	+(VE)	-(VE)	-(VE)	-(VE)	-(VE)	-(VE)	+(VE)
Ec-16	+(VE)	-(VE)	+(VE)	-(VE)	-(VE)	-(VE)	-(VE)	-(VE)	+(VE)
Ec-17	+(VE)	-(VE)	+(VE)	-(VE)	-(VE)	-(VE)	-(VE)	-(VE)	S+(VE)
Ec-18	+(VE)	-(VE)	+(VE)	-(VE)	-(VE)	-(VE)	-(VE)	-(VE)	+(VE)
Ec-19	+(VE)	-(VE)	+(VE)	-(VE)	-(VE)	-(VE)	-(VE)	-(VE)	W+(VE)

Ec-00= Control *E. coli* ATCC25922; S= Strong W=Weak. All results of biochemical tests for the isolates studied have been matched with the biochemical properties of *E. coli*.

(A)

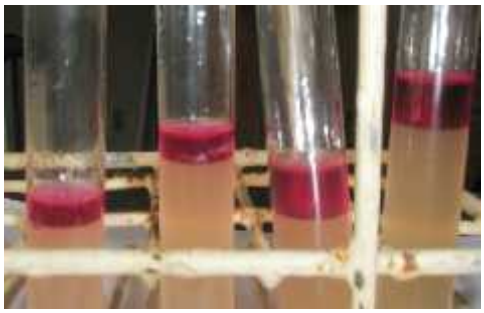
(B)



(C)

(D)

Fig 8 a - Biochemical test of the selected isolates (A) Citrate test (B) Methyl red test (C) Starch Hydrolysis (D) Oxidase test



(A)

(B)

(C)

(D)

Fig 8 b - Biochemical test of the selected isolates (A) Indole Test (B) Catalase Test (C) Gelatin Hydrolysis (D) Fermentation test

Table 3.7.2: Fermentation tests for isolates (suspected as *Escherichia coli)*

Isolates	Glucose utilization Acid +Gas	Sorbitol
Ec-00	+	+
Ec-01	+	+
Ec-02	+	+
Ec-03	+	+
Ec-04	+	+
Ec-05	+	+
Ec-07	+	+
Ec-11	+	+
Ec-12	+	+
Ec-13	+	+
Ec-15	+	+
Ec-16	+	+
Ec-17	+	+
Ec-18	+	+
Ec-19	+	+

Ec-00= Control *E. coli* ATCC25922. All isolates studied showed positive response to glucose utilization and sorbitol tests.

Table-3.7.3: Biochemical properties of the selected isolates (suspected as *Staphylococcus aureus*)

Org	Motility	MR	VP	Citrate	Catalase	Oxidase	Starch	Indole	Urease	Coagulae
Sa-00	NM	+(VE)	+(VE)	-(VE)	+(VE)	-(VE)	-(VE)	-(VE)	-(VE)	+(VE)
Sa-01	NM	+(VE)	+(VE)	-(VE)	+(VE)	-(VE)	-(VE)	-(VE)	-(VE)	+(VE)
Sa-02	NM	+(VE)	+(VE)	-(VE)	+(VE)	-(VE)	-(VE)	-(VE)	-(VE)	+(VE)
Sa-03	NM	+(VE)	+(VE)	-(VE)	+(VE)	-(VE)	-(VE)	-(VE)	-(VE)	+(VE)
Sa-04	NM	+(VE)	+(VE)	-(VE)	+(VE)	-(VE)	-(VE)	-(VE)	-(VE)	+(VE)
Sa-05	NM	+(VE)	+(VE)	-(VE)	+(VE)	-(VE)	-(VE)	-(VE)	-(VE)	+(VE)
Sa-06	NM	+(VE)	+(VE)	-(VE)	+(VE)	-(VE)	-(VE)	-(VE)	-(VE)	+(VE)
Sa-07	NM	+(VE)	+(VE)	-(VE)	+(VE)	-(VE)	-(VE)	-(VE)	-(VE)	+(VE)
Sa-08	NM	+(VE)	+(VE)	-(VE)	+(VE)	-(VE)	-(VE)	-(VE)	-(VE)	+(VE)
Sa-09	NM	+(VE)	+(VE)	-(VE)	+(VE)	-(VE)	-(VE)	-(VE)	-(VE)	+(VE)
Sa-10	NM	+(VE)	+(VE)	-(VE)	+(VE)	-(VE)	-(VE)	-(VE)	-(VE)	+(VE)
Sa-11	NM	+(VE)	+(VE)	-(VE)	+(VE)	-(VE)	-(VE)	-(VE)	-(VE)	+(VE)
Sa-12	NM	+(VE)	+(VE)	-(VE)	+(VE)	-(VE)	-(VE)	-(VE)	-(VE)	+(VE)
Sa-14	NM	+(VE)	+(VE)	-(VE)	+(VE)	-(VE)	-(VE)	-(VE)	-(VE)	+(VE)
Sa-16	NM	+(VE)	+(VE)	-(VE)	+(VE)	-(VE)	-(VE)	-(VE)	-(VE)	+(VE)
Sa-17	NM	+(VE)	+(VE)	-(VE)	+(VE)	-(VE)	-(VE)	-(VE)	-(VE)	+(VE)
Sa-22	NM	+(VE)	+(VE)	-(VE)	+(VE)	-(VE)	-(VE)	-(VE)	-(VE)	+(VE)
Sa-23	NM	+(VE)	+(VE)	-(VE)	+(VE)	-(VE)	-(VE)	-(VE)	-(VE)	+(VE)

Sa-00= Control *Staphylococcus aureus* ATCC25923. All results of biochemical tests for the isolates studied have been matched with the biochemical properties of *Staphylococcus aureus*.

Table-3.7.4. Fermentation test of the selected isolates (suspected as *Staphylococcus aureus)*

Isolate no	Lactose		Sucrose		H_2S production
	Acid	Gas	Acid	Gas	Acid
Sa-00	+	-	+	-	-
Sa-01	+	-	+	-	-
Sa-02	+	-	+	-	-
Sa-03	+	-	+	-	-
Sa-04	+	-	+	-	-
Sa-05	+	-	+	-	-
Sa-06	+	-	+	-	-
Sa-07	+	-	+	-	-
Sa-08	+	-	+	-	-
Sa-09	+	-	+	-	-
Sa-10	+	-	+	-	-
Sa-11	+	-	+	-	-
Sa-12	+	-	+	-	-
Sa-14	+	-	+	-	-
Sa-16	+	-	+	-	-
Sa-17	+	-	+	-	-
Sa-22	+	-	+	-	-
Sa-23	+	-	+	-	-

Sa-00= Control *Staphylococcus aureus* ATCC25923. All isolates suspected as *Staphylococcus aureus* were found to produce acid but no gas in Lactose, Sucrose and negative response to H_2S production.

Table-3.7.5.Biochemical properties of the selected isolates (suspected as *Bacillus cereus*)

Isolate	MR	VP	Indole	Nitrate	Glucose	Starch	Citrate	Catalase	Oxidase	Gelatin
Bc-00	-(VE)	+(VE)	-(VE)	+(VE)	+(VE)	+(VE)	+(VE)	+(VE)	-(VE)	+(VE)
Bc-01	-(VE)	+(VE)	-(VE)	+(VE)	+(VE)	+(VE)	+(VE)	+(VE)	-(VE)	+(VE)
Bc-02	-(VE)	+(VE)	-(VE)	+(VE)	+(VE)	+(VE)	+(VE)	+(VE)	-(VE)	+(VE)
Bc-04	-(VE)	+(VE)	-(VE)	+(VE)	+(VE)	+(VE)	+(VE)	+(VE)	-(VE)	w+(VE)
Bc-05	-(VE)	+(VE)	-(VE)	+(VE)	+(VE)	+(VE)	+(VE)	+(VE)	-(VE)	+(VE)
Bc-06	-(VE)	+(VE)	-(VE)	+(VE)	+(VE)	+(VE)	+(VE)	+(VE)	-(VE)	+(VE)
Bc-07	-(VE)	+(VE)	-(VE)	+(VE)	+(VE)	+(VE)	+(VE)	w+(VE)	-(VE)	+ (VE)
Bc-08	-(VE)	+(VE)	-(VE)	+(VE)	+(VE)	+(VE)	+(VE)	+(VE)	-(VE)	+(VE)
Bc-09	-(VE)	+(VE)	-(VE)	+(VE)	+(VE)	+(VE)	+(VE)	+(VE)	-(VE)	w+(VE)
Bc-10	-(VE)	+(VE)	-(VE)	+(VE)	+(VE)	+(VE)	+(VE)	+(VE)	-(VE)	+(VE)
Bc-11	-(VE)	+(VE)	-(VE)	+(VE)	+(VE)	+(VE)	+(VE)	w+(VE)	-(VE)	+(VE)
Bc-12	-(VE)	+(VE)	-(VE)	+(VE)	+(VE)	+(VE)	+(VE)	+(VE)	-(VE)	+(VE)

Bc00= Control *Bacillus cereus* ATCC11778

All results of biochemical tests for the isolates studied have been matched with the biochemical properties of *Bacillus cereus.*

Table-3.7.6. Fermentation tests of the isolates (suspected as *Bacillus cereus)*

Isolate No.	Monosaccharides						
	Pentose sugars*			Hexose sugars*			
	Arabi-nose	Rham-nose	Glucose	Fruc-tose	Galac-tose	Man-nose	Xylose
Bc-00	-	-	+	+	-	+	-
Bc-01	-	-	+	+	-	+	-
Bc-02	-	-	+	+	-	-	-
Bc-04	-	-	+	+	-	-	-
Bc-05	-	-	+	+	-	+	-
Bc-06	-	-	+	+	-	+	-
Bc-07	-	-	+	+	-	-	-
Bc-08	-	-	+	+	-	+w	-
Bc-09	-	-	+	+	-	+w	-
Bc-10	-	-	+	+	-	+	-
Bc-11	-	-	+	+	-	+w	-
Bc-12	-	-	+	+	-	+	-

*, No gas production; Bc00= Control *Bacillus cereus* ATCC11778

All isolates studied showed negative response to arabinose, rhamnose, galactose and xylose and positive response to glucose and fructose but variable response to mannose.

Table-3.7.7. Fermentation tests of the isolates (suspected as *Bacillus cereus)*

Isolate No.	Disaccharides*			Polysaccharides*		Sugar alcohol*
	Lactose	Maltose	Sucrose	Raffinose	Starch	(Mannitol)
Bc-00	-	+	+	-	+	-
Bc-01	-	+	+	-	+	-
Bc-02	_	+	+	-	+w	-
Bc-04	-	+	_	-	+	-
Bc-05	-	+	+	-	+	-
Bc-06	-	+	+	-	+	-
Bc-07	-	+	-	-	+	-
Bc-08	-	+	+	-	+	-
Bc-09	+	+	-	-	+w	-
Bc-10	-	+	-	-	+	-
Bc-11	+	+	+	-	+w	-
Bc-12	-	+	-	-	+	-

*, No gas production; Bc00= Control *Bacillus cereus* ATCC11778

All isolates studied showed positive response to maltose and starch, and negative response to raffinose and mannitol but variable response to lactose and sucrose.

3.8. Antibiotic sensitivity pattern of the selected isolates

The strains were tested for their ability to grow in the presence of the antibiotics at specific connections. The zone of inhibition was observed and reading was taken after 24 hours. Readable results were recorded in Table 3.8.1, 3.8.2. and 3.8.3.From the result, it was seen that the strains vary from each other in their antibiotic resistance capacity.

Table-3.8.1. Antibiotic sensitivity of *E. coli*

Name of the organism	AMC 30	ATM 30	AK 30	CEC 30	CFM 5	CRO 30	CL 30	CIP 5	CN 10	SXT 25	IPM 10	MEL 25	NA 30	F 30	MEM 10	NET 30
Ec-17	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
Ec-18	R	R	S	S	R	R	S	S	S	R	S	R	R	S	S	S
Ec-02	I	R	S	S	R	R	S	R	S	S	S	R	R	S	S	S
Ec-07	R	R	S	R	R	R	R	S	S	R	S	S	R	S	S	S
Ec-13	R	S	S	S	S	S	S	I	S	S	S	S	R	S	S	S
Ec-11	R	R	S	S	I	S	S	I	S	I	S	R	R	R	S	S
Ec-19	R	I	S	R	R	R	R	R	S	R	S	S	R	S	S	S
Ec-16	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
Ec-01	R	R	S	R	R	R	R	S	S	R	S	S	S	S	S	S
Ec-05	R	R	S	S	R	S	S	R	S	R	S	S	R	S	S	S
Ec-03	R	S	S	S	R	R	R	S	S	R	S	R	R	S	S	S
Ec-04	R	R	S	S	I	S	S	R	S	I	S	R	R	S	S	S
Ec-12	R	R	S	I	R	I	S	I	S	S	S	I	R	I	S	S
Ec-15	I	R	S	R	R	S	I	R	S	R	S	S	R	R	S	S

Table-3.8.2. Antibiotic sensitivity of *S. aureus*

Name of the organism	AMC 30	AK 30	SXT 25	CRO 30	CEC 30	F 30	ATM 30	DO 30	AZM 15	CFM 5	CIP 5	OB 5	P 10	CL 30
Sa-08	R	S	R	R	S	S	R	I	S	R	S	R	R	S
Sa-06	R	S	R	R	S	S	R	S	S	R	S	R	R	R
Sa-12	R	S	R	R	I	S	R	I	I	R	S	R	R	I
Sa-16	R	S	R	R	I	S	R	S	S	R	S	R	R	I
Sa-04	R	S	I	R	R	S	R	S	S	R	S	R	R	S
Sa-23	R	S	R	R	S	S	R	S	S	R	S	R	R	S
Sa-14	R	S	R	R	I	S	R	S	S	R	S	R	R	S
Sa--22	R	S	R	R	S	S	R	S	S	R	S	S	R	R
Sa-07	R	S	R	R	I	S	R	S	R	R	S	R	R	S
Sa-01	R	S	I	R	S	S	R	S	S	R	S	R	R	S
Sa-03	R	S	R	R	R	S	R	I	S	R	S	R	R	S
Sa-02	R	S	R	R	S	S	R	S	I	R	S	R	R	S
Sa-11	R	S	R	R	S	S	R	I	S	R	S	R	R	S
Sa-09	R	S	R	R	I	I	R	I	S	R	S	R	R	S
Sa-05	R	S	R	R	S	S	R	S	S	R	S	R	R	I
Sa-10	R	S	R	R	I	S	R	I	S	R	S	R	R	R
Sa-17	R	S	R	R	I	I	R	S	S	R	S	I	R	S

Table-3.8.3. Antibiotic sensitivity of *B. cereus*

Name of the organism	C 30	SXT 25	E 15	IPM 10	CIP 5	CFM 5	TE 30	P 10	AK 30	CRO 30	CL 30	NET 30	CN 10	CEC 30	CTX 30
Bc-07	S	S	S	S	S	R	R	R	S	R	S	S	S	R	R
Bc-04	S	R	S	S	S	R	S	R	S	R	S	S	S	S	R
Bc-02	I	R	S	S	R	R	I	R	S	R	S	S	S	R	R
Bc-10	R	R	S	S	I	R	S	R	S	R	S	S	S	R	R
Bc-09	S	R	S	S	S	R	R	R	S	R	S	S	S	I	R
Bc-08	S	R	S	S	S	R	S	R	S	R	S	S	S	R	R
Bc-11	R	R	S	S	S	R	S	R	S	R	S	S	S	R	R
Bc-06	S	R	S	S	S	R	S	R	S	R	I	S	S	R	R
Bc-12	R	R	S	S	S	R	S	R	S	R	S	S	S	R	R
Bc-01	S	S	S	S	S	R	R	R	S	R	S	S	S	I	R
Bc-05	S	R	S	S	I	R	S	R	S	R	I	S	S	R	R

3.9. Screening of the essential oil

Results for screening of antibacterial activity of cinnamaldehyde, euginol and carvacrol are summarized in table 3.9.1, 3.9.2 and 3.9.3.Cinnmaldehyde showed good inhibitory activity against the three food borne pathogens with zones of inhibition ranging from 28 to 39 mm. Cinnamaldehyde showed maximum inhibition for *S. aureus* (39 mm) and minimum inhibition for *E. coli* (28.5 mm) with zones of inhibition larger than those observed against the antibiotic Imipenem (10µg). Euginol showed maximum inhibition for *S. aureus* (27 mm) and minimum inhibition for *E. coli* (15.5 mm) and carvacrol showed maximum inhibition for *S. aureus* (29 mm) and minimum inhibition for *E. coli* (18 mm). The anti bacterial activity of 3% cinnamaldehyde, euginol and carvacrol against representative bacteria are shown in Fig 9.

Table-3.9.1: The antimicrobial activities of cinnamaldehyde

Organism	Zone of inhibition(in mm)	
	CNN 3%	Standards(IPM) (10µg)
Ec 01	28.5 ± 0.70	15.5 ± 0.5
Ec 17	29.0 ± 1.41	19.0 ± 1.0
Ec 07	29.0 ± 1.41	15.5 ± 1.5
Bc 01	30.5 ± 0.5	26.0 ± 2.0
Bc 06	33.0 ± 1.0	22.5 ± 2.5
Bc 10	34.5 ± 0.5	22.5 ± 1.5
Bc 02	31.0 ± 1.0	22.5 ± 2.5
Bc 12	33.0 ± 1.0	21.5 ± 2.5
Sa 05	38.0 ± 2.0	17.0 ± 1.0
Sa 08	38.0 ± 2.0	27.5 ± 2.5
Sa 12	37.0 ± 1.0	32.0 ± 2.0
Sa 09	34.0 ± 2.0	26.5 ± 1.5
Sa 22	39.0 ± 1.0	25.5 ± 1.5

Mean diameter of zone of inhibition in mm including the diameter of the disc (8 mm) CNN stands for cinnamaldehyde respectively Mean±S.D (n=2) IPM Imipenem

Table-3.9.2: The antimicrobial activities of euginol

Organism	Zone of inhibition(in mm)	
	EU 3%	Standards(IPM) (10µg)
Ec 01	18.0 ± 2.0	15.5 ± 0.5
Ec 17	15.5 ± 0.5	19.0 ± 1.0
Ec 07	17.5 ± 1.5	15.5 ± 1.5
Bc 01	23.5 ± 0.5	26.0 ± 2.0
Bc 06	19.5 ± 0.5	22.5 ± 2.5
Bc 10	20.5 ± 0.5	22.5 ± 1.5
Bc 02	21.0 ± 1.0	22.5 ± 2.5
Bc 12	19.5 ± 0.5	21.5 ± 2.5
Sa 05	19.0 ± 1.0	17.0 ± 1.0
Sa 08	23.5 ± 0.5	27.5 ± 2.5
Sa 12	22.5 ± 0.5	32.0 ± 2.0
Sa 09	24.5 ± 1.5	26.5 ± 1.5
Sa 22	27.0 ± 1.0	25.5 ± 1.5

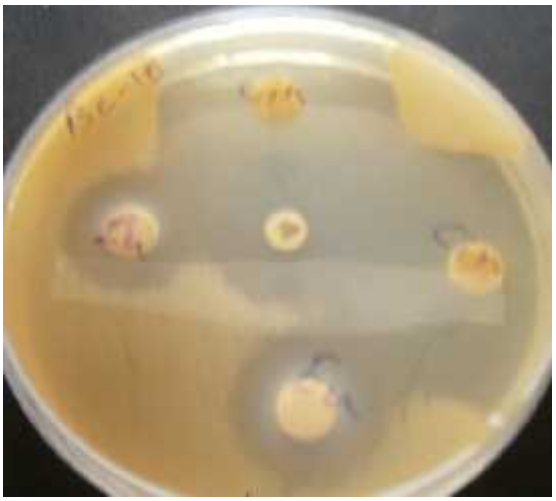
Mean diameter of zone of inhibition in mm including the diameter of the disc (8 mm) EU stands for euginol respectively Mean±S.D (n=2) IPM Imipenem

Table-3.9.3: The antimicrobial activities of carvacrol

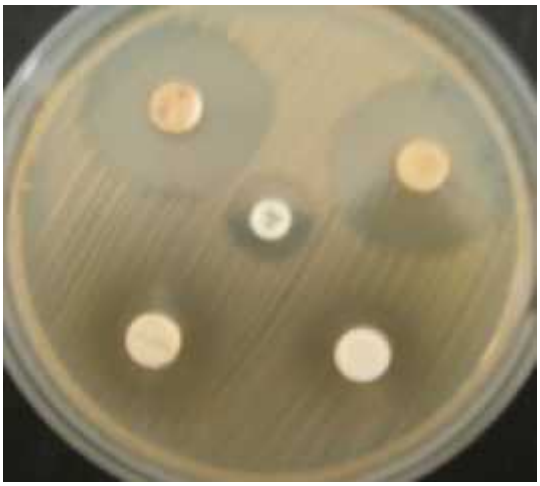
Organism	Zone of inhibition(in mm)	
	CAR 3%	Standards(IPM) (10µg)
Ec 01	21.0 ± 1.0	15.5 ± 0.5
Ec 17	24.0 ± 1.0	19.0 ± 1.0
Ec 07	24.0 ± 2.0	15.5 ± 1.5
Bc 01	27.0 ± 1.0	26.0 ± 2.0
Bc 06	23.0 ± 1.5	22.5 ± 2.5
Bc 10	18.5 ± 1.5	22.5 ± 1.5
Bc 02	20.0 ± 2.0	22.5 ± 2.5
Bc 12	26.5 ± 1.5	21.5 ± 2.5
Sa 05	25.0 ± 1.0	17.0 ± 1.0
Sa 08	18.0 ± 2.0	27.5 ± 2.5
Sa 12	29.0 ± 1.0	32.0 ± 2.0
Sa 09	22.0 ± 2.0	26.5 ± 1.5
Sa 22	25.5 ± 1.5	25.5 ± 1.5

Mean diameter of zone of inhibition in mm including the diameter of the disc (8 mm)

CAR stands for Carvacrol respectively Mean±S.D (n=2) IPM Imipenem



(A)



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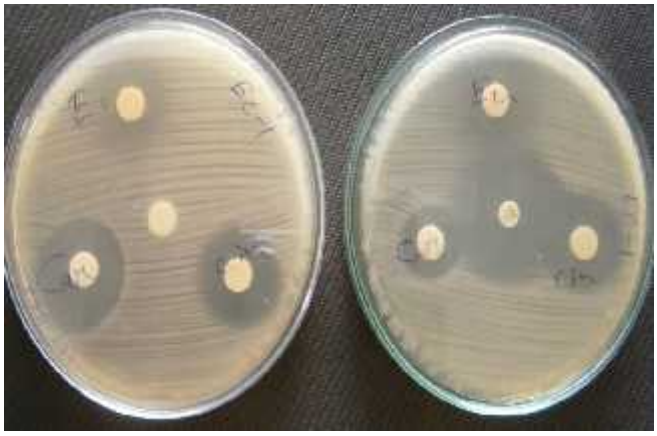
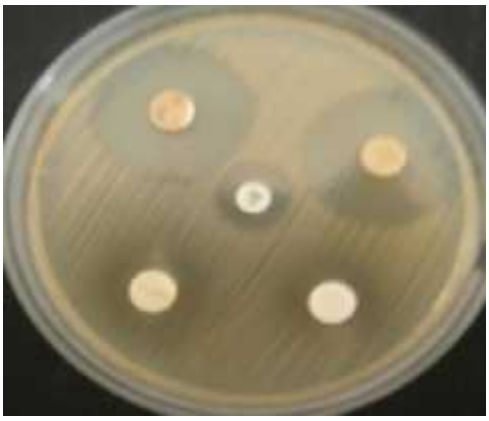


(C)



(D)

Fig 9: The antibacterial activity of 3% cinnamaldehyde, euginol and carvacrol against (A) *Bacillus cereus* (B) *Escherichia coli* (C) *Escherichia coli* (D) *Staphylococcus aureus*

3.10. Minimum Inhibitory Concentration of cinnamaldehyde, carvacrol and euginol

MICs of cinnamaldehyde carvacol and euginol determined broth dilution method at 37 °C. MIC values of cinnamaldehyde, carvacrol and euginol against the suspected bacteria ranged from 5-0.03125% which are shown in Table 3.10.1, 3.10.2, 3.10.3, 3.10.4, 3.10.5 and 3.10.6. MIC of cinnamaldehyde carvacol and euginol are shown in Fig 10, 11 and 12.

Table 3.10.1: Minimum Inhibitory Concentrations (MICs) of cinnamaldehyde (%v/v)

Organism	10%	5%	2.5%	1.25%	0.0625%	0.03125%	Control
Ec 01	-	-	+	+	+	+	+
Ec 07	-	-	+	+	+	+	+
Ec 17	-	-	+	+	+	+	+
Sa 09	-	-	+	+	+	+	+
Sa 05	-	+	+	+	+	+	+
Sa 08	+	+	+	+	+	+	+
Sa 22	+	+	+	+	+	+	+
Sa 12	+	+	+	+	+	+	+
Bc 01	-	-	+	+	+	+	+
Bc 02	-	-	+	+	+	+	+
Bc 06	-	-	+	+	+	+	+
Bc 10	-	-	+	+	+	+	+
Bc 12	-	-	+	+	+	+	+

-Represents negative activity

+ Represents positive activity

Table-3.10.2: MIC and MBC value of cinnamaldehyde (%v/v)

Organism	MIC	MBC
Ec 01	2.5%	5%
Ec 07	2.5%	5%
Ec 17	2.5%	5%
Sa 09	2.5%	5%
Sa 05	5%	10%
Sa 08	10%	15%
Sa 22	10%	15%
Sa 12	10%	15%
Bc 01	2.5%	5%
Bc 02	2.5%	5%
Bc 06	2.5%	5%
Bc 10	2.5%	5%
Bc 12	2.5%	5%

(A)

(B)

(C)

Fig 10: Minimum Inhibitory Concentration of cinnamaldehyde against (A) *E. coli* (B) *S. aureus* (C) *B. cereus*

Table 3.10.3: Minimum Inhibitory Concentrations (MICs) of carvacrol (%v/v)

Organism	10%	5%	2.5%	1.25%	0.0625%	0.03125%	Control
Ec 01	-	-	+	+	+	+	+
Ec 07	-	-	+	+	+	+	+
Ec 17	-	-	+	+	+	+	+
Sa 09	-	-	+	+	+	+	+
Sa 05	-	+	+	+	+	+	+
Sa 08	-	+	+	+	+	+	+
Sa 22	-	+	+	+	+	+	+
Sa 12	+	+	+	+	+	+	+
Bc 01	-	+	+	+	+	+	+
Bc 02	-	-	+	+	+	+	+
Bc 06	-	+	+	+	+	+	+
Bc 10	-	+	+	+	+	+	+
Bc 12	-	-	+	+	+	+	+

-Represents negative activity

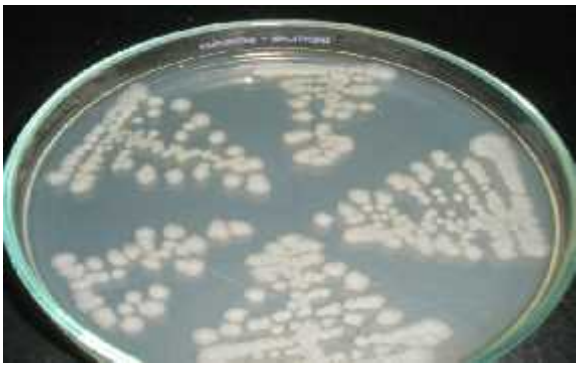
+ Represents positive activity

Table-3.10.4. MIC and MBC value of carvacrol (%v/v)

Organism	MIC	MBC
Ec 01	2.5%	5%
Ec 07	2.5%	5%
Ec 17	2.5%	5%
Sa 09	2.5%	5%
Sa 05	5%	10%
Sa 08	5%	10%
Sa 22	5%	10%
Sa 12	10%	15%
Bc 01	5%	10%
Bc 02	2.5%	5%
Bc 06	5%	10%
Bc 10	5%	10%
Bc 12	2.5%	5%

(A)

(B)



(C)

Fig 11: Minimum Inhibitory Concentration of carvacrol against (A)*E. coli* (B) *S. aureus* (C) *B. cereus*

Table3.10.5.Minimum Inhibitory Concentrations (MICs) of euginol

Organism	10	5%	2.5%	1.25%	0.0625%	0.03125%	Control
Ec 01	-	-	+	+	+	+	+
Ec 07	-	-	+	+	+	+	+
Ec 17	-	-	+	+	+	+	+
Sa 09	-	-	+	+	+	+	+
Sa 05	-	+	+	+	+	+	+
Sa 08	+	+	+	+	+	+	+
Sa 22	-	+	+	+	+	+	+
Sa 12	-	+	+	+	+	+	+
Bc 01	+	+	+	+	+	+	+
Bc 02	-	+	+	+	+	+	+
Bc 06	-	+	+	+	+	+	+
Bc 10	-	-	+	+	+	+	+
Bc 12	-	-	+	+	+	+	+

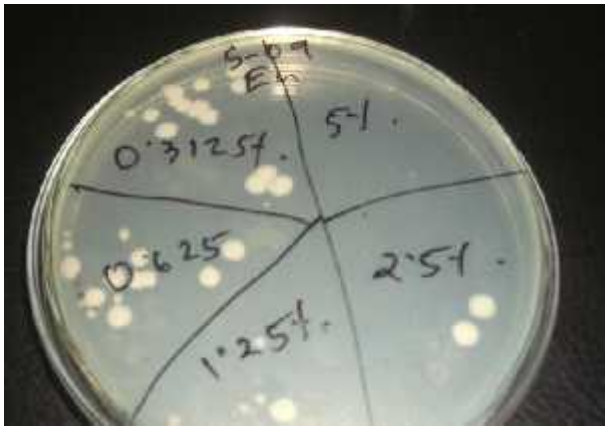
-Represents negative activity

+ Represents positive activity

Table-3.10.6: MIC and MBC value of euginol (%v/v)

Organism	MIC	MBC
Ec 01	2.5%	5%
Ec 07	2.5%	5%
Ec 17	2.5%	5%
Sa 09	2.5%	5%
Sa 05	5%	10%
Sa 08	10%	15%
Sa 22	5%	10%
Sa 12	5%	10%
Bc 01	10%	15%
Bc 02	5%	10%
Bc 06	5%	10%
Bc 10	2.5%	5%
Bc 12	2.5%	5%

(A)



(B)

(C)

Fig 12: Minimum Inhibitory Concentration of euginol against (A) *E. coli (B) S. aureus* (C) *B. cereus*

3.11. Effect of temperature on antibacterial activity of essential oil

The effects of the temperatures on the antibacterial activity of cinnamaldehyde was found at all temperatures employed (25°, 37°, 50°,75°, and 100° C) suggesting that the activity of cinnamaldehyde was not inactivated at high temperature even at 100°C for 30 min treatment. Moreover, the antibacterial activities were found to increase with the increasing temperature. Highest activity was found at 100° C and lowest activity was found at 50° C for the test bacteria. The result of the effect of temperatures is shown in Table- 3.11.1.

Table-3.11.1. Effect of temperatures on antibacterial activity of cinnamaldehyde

Isolates	Zones of inhibition (in mm)				
	Temperatures in °C				
	25°C	37°C	50°C	75°C	100°C
Ec 00	22.0 ± 0.1	25.0 ± 0.2	22.0 ±0.4	23.5 ± 0.5	20.0± 0.1
Ec 01	26.5 ± 0.2	29.4 ± 0.8	24.8 ± 0.58	26.0 ± 0.3	31.0 ± 0.1
Ec 07	25.0 ± 0.8	27.0 ± 1.0	23.2 ± 0.5	27.0 ± 0.1	28.0 ± 0.3
Ec 17	22.0 ± 0.3	25.0 ± 0.7	22.2 ± 0.64	24.3 ± 0.2	26.0 ± 0.1
Sa 00	22.8 ± 0.2	13.8 ± 0.2	11.0 ± 0.1	23.0 ± 0.1	24.5 ± 0.7
Sa 09	21.5 ± 0.4	24.0 ± 1.1	19.5 ± 0.6	24.8 ± 0.2	26.0 ± 0.1
Sa 05	11.9 ± 1.1	13.8 ± 0.2	11.0 ± 0.1	14.5 ± 0.2	16.0 ± 0.6
Sa 08	24.2 ± 0.4	28.5 ± 0.7	22.3 ± 0.2	27.5 ± 0.4	29.5 ± 0.1
Sa 22	20.5 ± 0.4	22.0 ± 1.1	18.5 ± 0.6	22.8 ± 0.2	23.0 ± 0.1
Sa 12	21.2 ± 0.3	24.5 ± 0.7	21.3 ± 0.2	23.5 ± 0.4	26.5 ± 0.1
Bc 00	19.5 ± 0.2	22.5 ± 0.2	20.2 ± 0.5	22.0 ± 1.1	25.0 ± 0.3
Bc 01	22.0 ± 0.3	21.0 ± 0.7	20.2 ± 0.64	21.3 ± 0.2	25.0 ± 0.1
Bc 02	23.0 ± 0.8	26.0 ± 1.0	20.2 ± 0.5	24.0 ± 0.1	25.0 ± 0.3
Bc 06	19.5 ± 0.2	22.0 ± 1.1	23.5 ± 0.6	26.8 ± 0.2	24.0 ± 0.1
Bc 10	22.0 ± 0.3	21.0 ± 0.7	25.2 ± 0.64	22.5 ± 0.2	26.0 ± 0.2
Bc 12	22.5 ± 0.3	22.0 ± 1.1	16.5 ± 0.3	26.8 ± 0.1	23.0 ± 0.1

All the values are mean± standard deviation of three determinations; Mean diameter of zone of inhibition in mm including the diameter of the disc 8 mm Mean±S.D mm(n=3); P< 0.05 concentration=3%

3.12. Effect pH on antibacterial activity of cinnamaldehyde

The antibacterial activity was not affected at pH 5.0, though a significant decrease of inhibitory was found. The pH 7 and 9 favored the antimicrobial activities of cinnamaldehyde against most of the isolates tested, where the highest activities were found at pH 7.0. The results of the cinnamaldehyde are shown in table 3.12.1.

Table-3.12.1. Effect of pH on antimicrobial activity of cinnamaldehyde

Isolates	Zone of inhibition		
	pH 5.0	pH 7.0	Ph 9.0
Ec 00	22.5 ± 0.5	24.0 ± 0.63	24.8 ± 1.0
Ec 01	19.3 ± 0.9	21.5 ± 0.8	26.2 ± 0.6
Ec 07	15.8 ± 0.5	19.5 ± 1.0	22.0 ± 0.1
Ec 17	21.2 ± 0.7	28.3 ± 0.9	27.8 ± 0.5
Sa 00	25.0 ± 1.0	29.6 ± 0.57	28.0 ± 0.1
Sa 09	20.0 ± 0.5	24.0 ± 0.2	26.2 ± 0.6
Sa 05	16.5 ± 0.3	18.7 ± 0.6	21.6 ± 0.9
Sa 08	19.5 ± 0.8	21.3 ± 0.9	25.2 ± 0.6
Sa 22	23.3 ± 0.9	19.5 ± 0.8	26.2 ± 0.6
Sa 12	21.2 ± 0.7	28.3 ± 0.9	27.8 ± 0.5
Bc 00	20.0 ± 0.6.	23.3 ± 0.57	26.8 ± 0.6
Bc 01	19.0 ± 0.2	25.0 ± 0.7	26.0 ± 0.5
Bc 02	20.0 ± 0.5	24.0 ± 0.2	26.2 ± 0.6
Bc 06	19.3 ± 0.9	21.5 ± 0.8	23.2 ± 0.6
Bc 10	23.5 ± 0.3	25.7 ± 0.5	24.5 ± 0.3
Bc 12	19.8 ± 0.6	27.0 ± 0.1	23.0 ± 0.4

All the values are mean± standard deviation of three determinations; Mean diameter of zone of inhibition in mm including the diameter of the disc 8 mm Mean±S.D mm(n=3); P< 0.05 concentration=3%

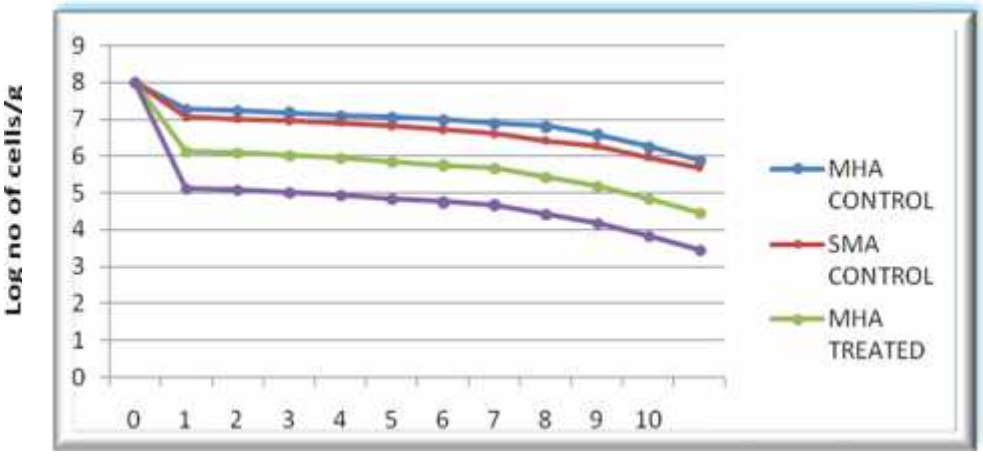
3.13. Inactivation of common food borne pathogens (*E. coli, S. aureus and B. cereus*) in ground chicken meat with essential oil

Based on MIC level of cinnamaldehyde, carvacrol and euginol (Tables 3.10.2, 3.10.4, 3.10.6) better MIC results were found in case of cinnamaldehyde, for which cinnamaldehyde was selected for further study.

Ground chicken meat was inoculated with each of 10^7 CFU/ml of *E. coli, S. aureus and B. cereus* and the inoculated meat samples were challenged by MIC level, 3% MIC level and 5% MIC level of cinnamaldehyde.

The results for *E. coli* are shown in Figure 13a, b, c; 14 a, b, c and 15 a, b, c respectively. From the figure 13 a, b and c it was apparent that only a few log reduction of *E. coli* 01 was found in selective medium in case of MIC level of cinnamaldehyde, whereas, in case of 3% and 5% cinnamaldehyde within 24 hours 7 log reduction of *E. coli* 01 was found but in non- selective medium (MHA) two or one log survival of *E. coli* 01 was found (Figure 13 b and c) because of resuscitation of wound cells. In case of positive control one or two log reduction were recorded is because of cold effect on *E. coli* 01 cells.

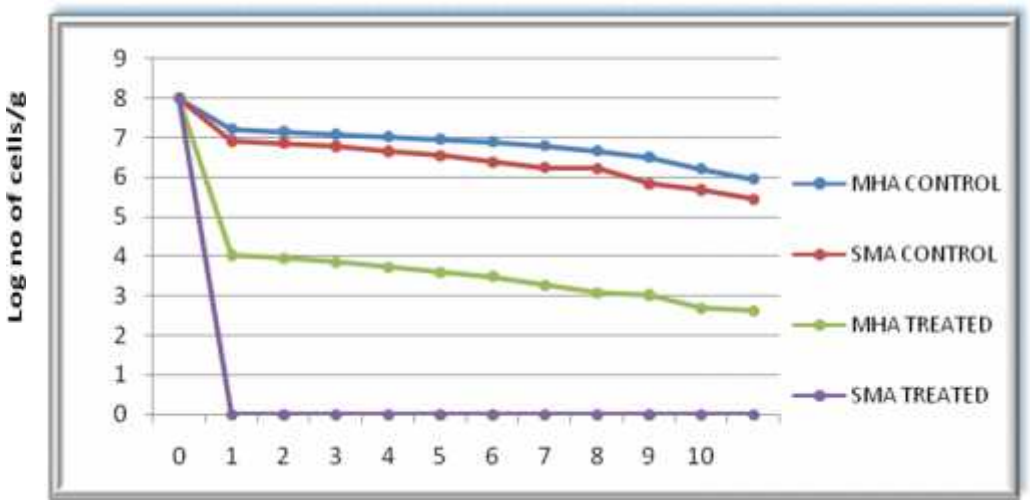
Inactivation of *E. coli* 01 in ground chicken meat exposed to 2.5% cinnamaldehyde and stored at -18° C for 10 days. The results are shown in Fig-13 a.



Incubation time (Day)

Fig 13a: Inactivation of *E. coli* 01 in ground chicken meat exposed to 2.5% cinnamldehyde

Inactivation of *E. coli* 01 in ground chicken meat exposed to 7.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig-13 b.



Incubation time (Day)

Fig 13b: Inactivation of *E. coli* 01 in ground chicken meat exposed to 7.5% cinnamaldehyde

Inactivation of E. coli 01 in ground chicken meat exposed to 12.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig-13 c.

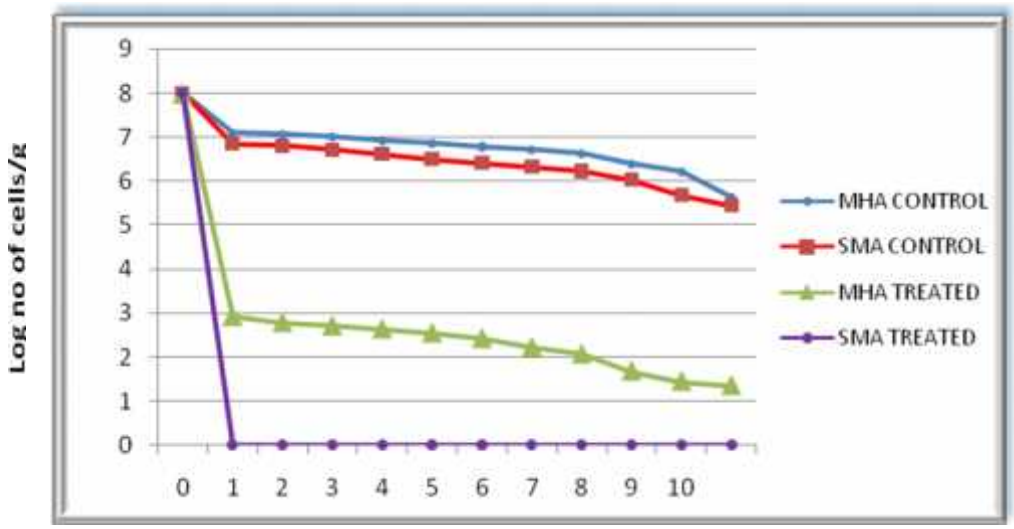


Fig 13c: Inactivation of *E. coli* 01 in ground chicken meat exposed to 12.5% cinnamaldehyde

From the figure 14 a, b and c it was apparent that only a few log reduction of *E. coli* 07 was found in selective medium in case of MIC level of cinnamaldehyde, whereas, in case of 3% and 5% cinnamaldehyde within 24 hours 7 log reduction of *E. coli* 07 was found but in non- selective medium (MHA) two or one log survival of *E. coli* 07 was found (Figure 14b and c) because of resuscitation of wound cells. In case of positive control one or two log reduction were recorded is because of cold effect on *E. coli* 07 cells.

Inactivation of E. coli 07 in ground chicken meat exposed to 2.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig-14 a.

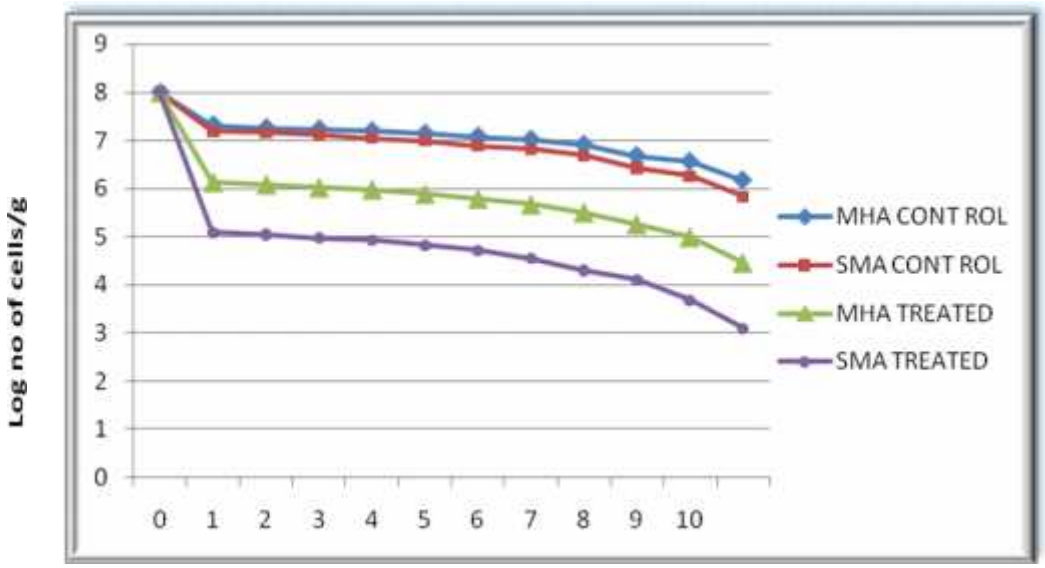
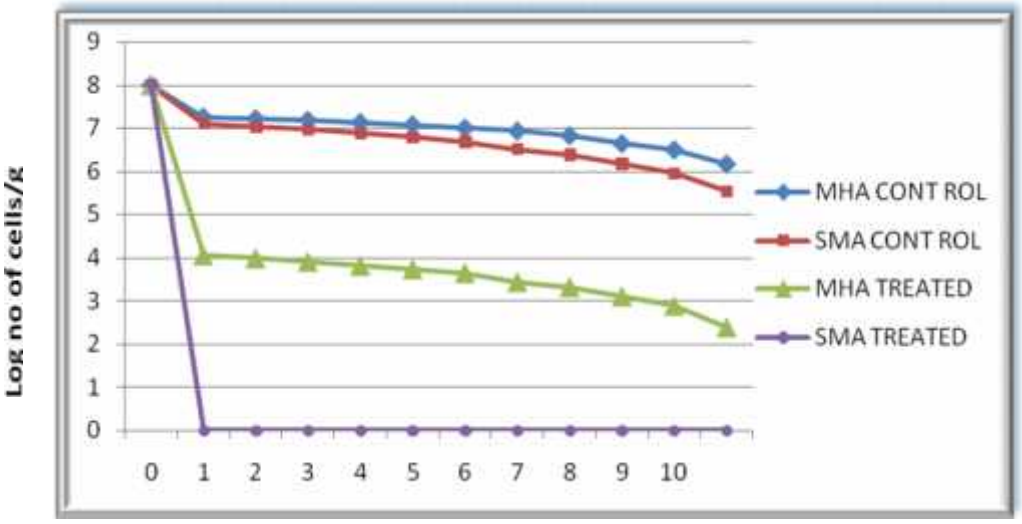


Fig 14a: Inactivation of *E. coli* 07 in ground chicken meat exposed to 2.5% cinnamaldehyde

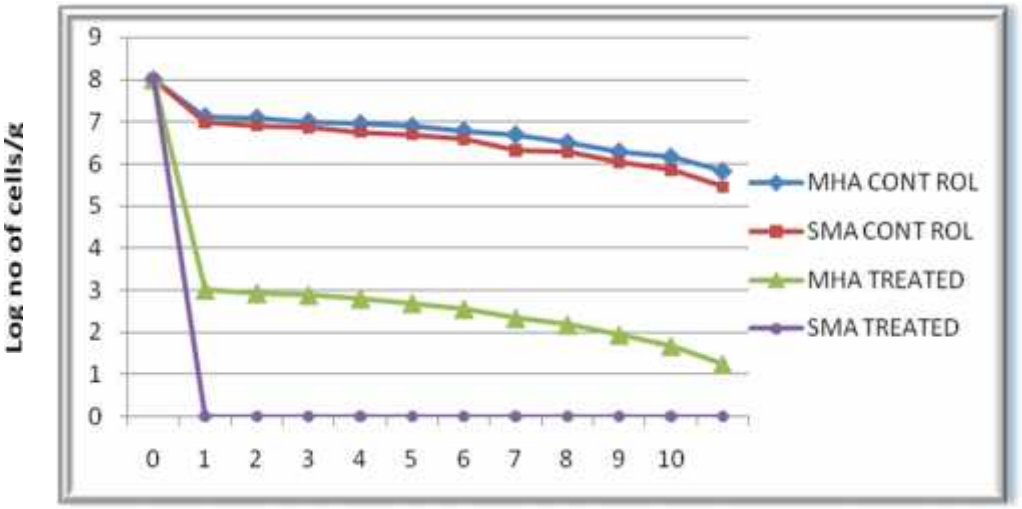
Inactivation of *E. coli* 07 in ground chicken meat exposed to 7.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig-14 b.



Incubation time (Day)

Fig 14b: Inactivation of *E. coli* 07 in ground chicken meat exposed to 7.5% cinnamaldehyde

Inactivation of *E. coli* 07 in ground chicken meat exposed to 12.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig-14 c.



Incubation time (Day)

Fig 14c: Inactivation of *E. coli* 07 in ground chicken meat exposed to 12.5% cinnamaldehyde

From the figure 15 a, b and c it was apparent that only a few log reduction of *E. coli* 17 was found in selective medium in case of MIC level of cinnamaldehyde, whereas, in case of 3% and 5% cinnamaldehyde within 24 hours 7 log reduction of *E. coli* 17 was found but in non- selective medium (MHA) two or one log survival of *E. coli* 17 was found(Fig- 15 b and c) because of resuscitation of wound cells. In case of positive control one or two log reduction were recorded is because of cold effect on *E. coli* 17 cells.

Inactivation of *E. coli* 17 in ground chicken meat exposed to 2.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig-15 a.

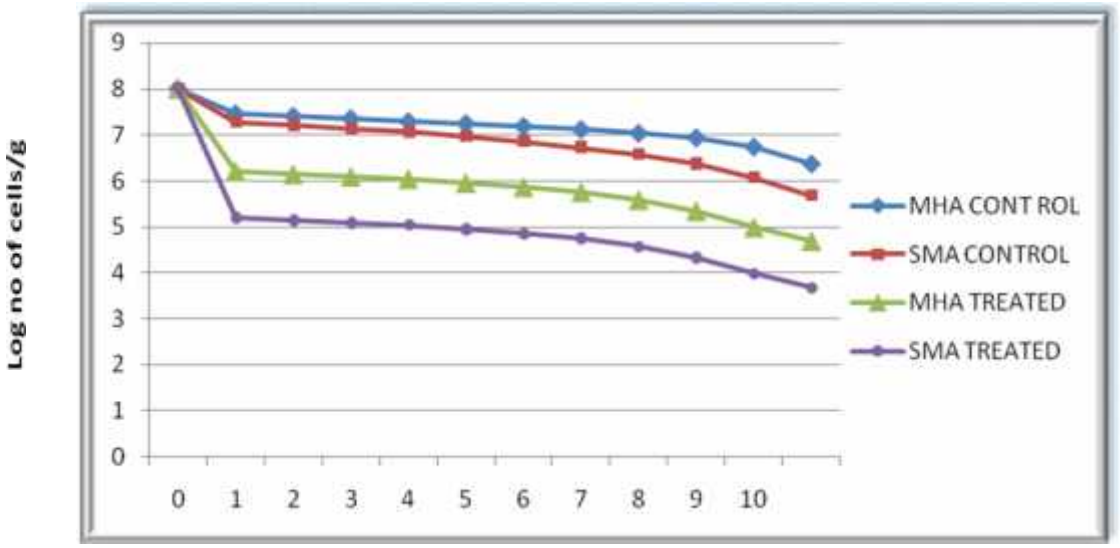


Fig 15a: Inactivation of *E. coli* 17 in ground chicken meat exposed to 2.5% cinnamaldehyde

Inactivation of *E. coli* 17 in ground chicken meat exposed to 7.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig-15 b.

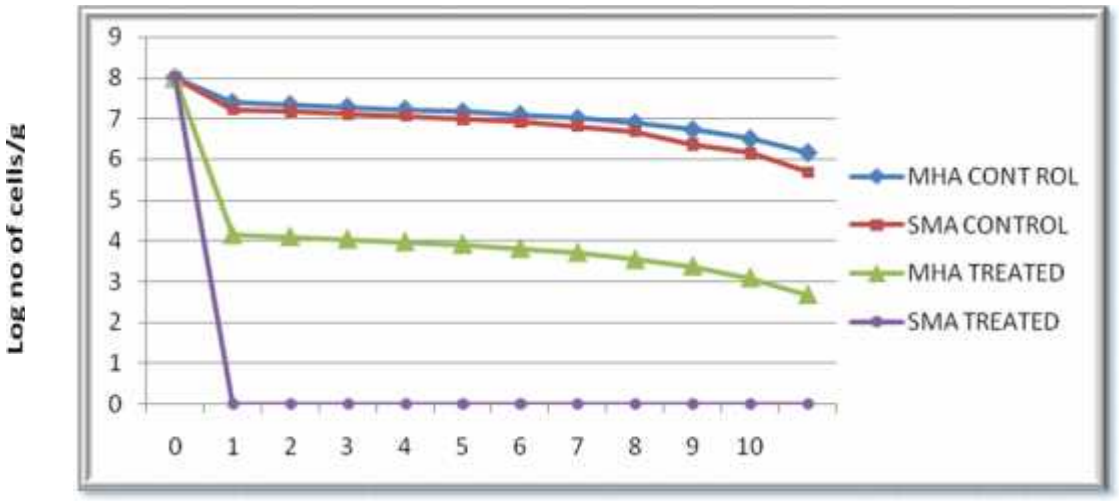


Fig 15b: Inactivation of *E. coli* 17 in ground chicken meat exposed to 7.5% Cinnamaldehyde

Inactivation of *E. coli* 17 in ground chicken meat exposed to 12.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig-15 c.

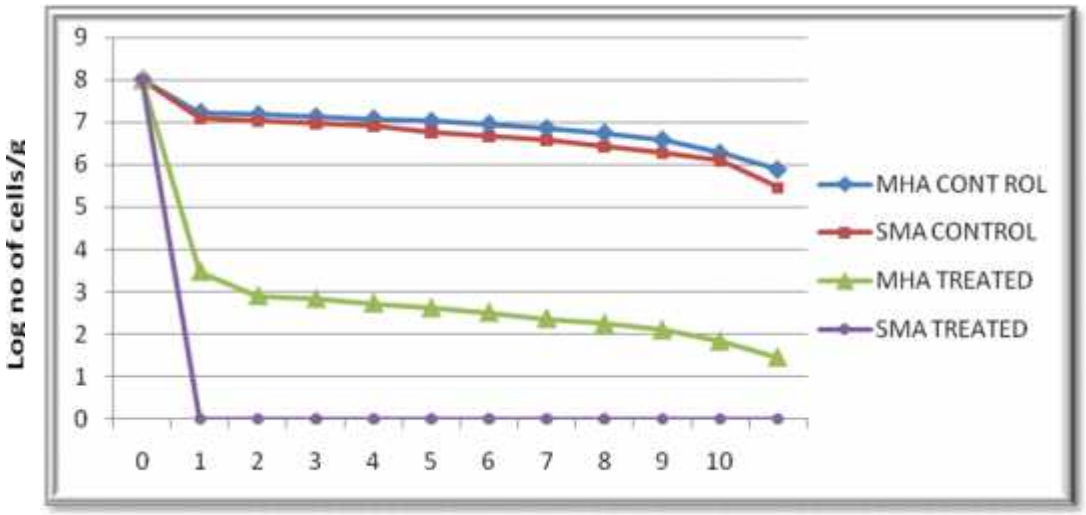
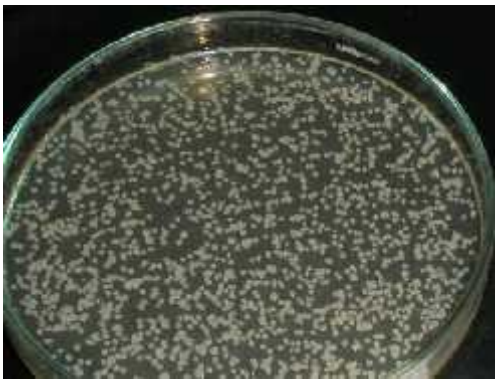
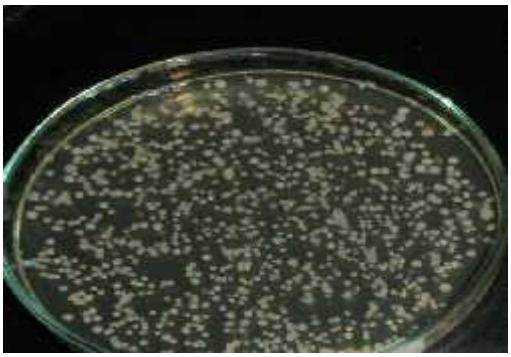


Fig 15c: Inactivation of *E. coli* 17 in ground chicken meat exposed to 12.5% cinnamaldehyde

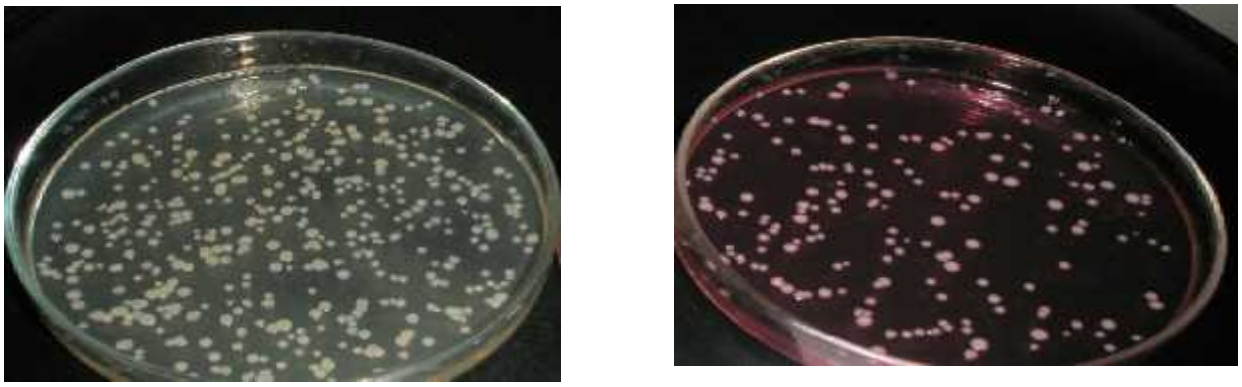


(A) Non selective media (MHA Control plate) and selective media (SMA Control plate)

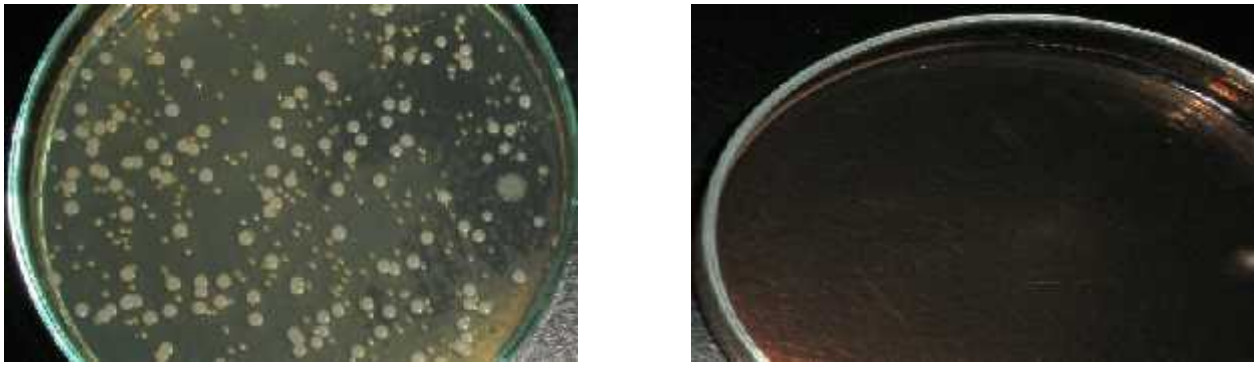


(B) Non selective media (MHA treatment plate) and selective media (SMA treatment plate)

Fig. 16: Inactivation of *E. coli* in ground chicken meat exposed to 2.5% cinnamaldehyde in (A) Non selective media (MHA Control plate) and selective media (SMA Control plate) (B) Non selective media (MHA treatment plate) and selective media (SMA treatment plate)

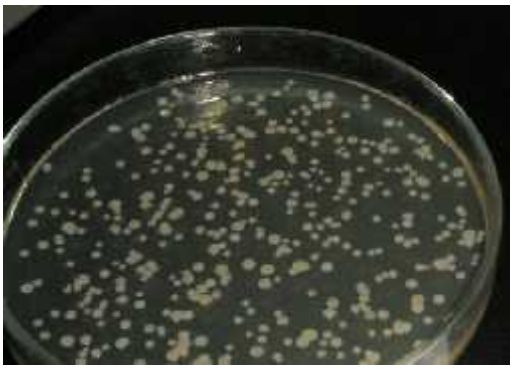


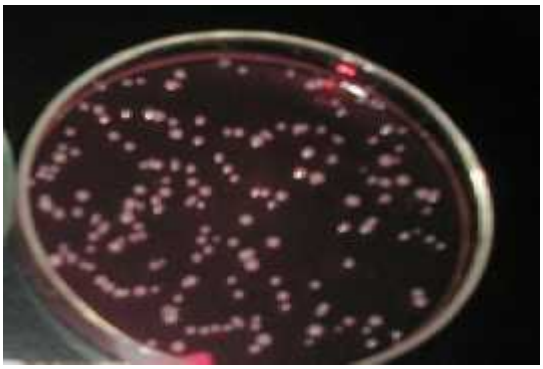
(A) Non selective media (MHA Control plate) and selective media (SMA Control plate)



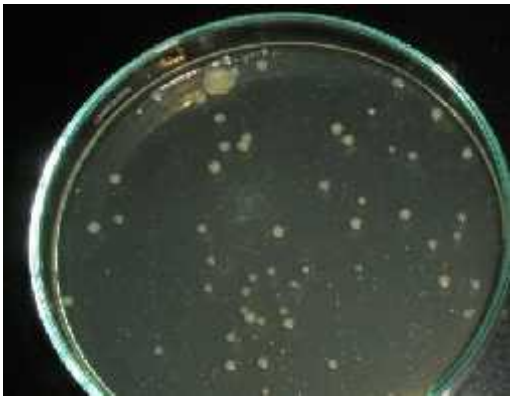
(B) Non selective media (MHA treatment plate) and selective media (SMA treatment plate)

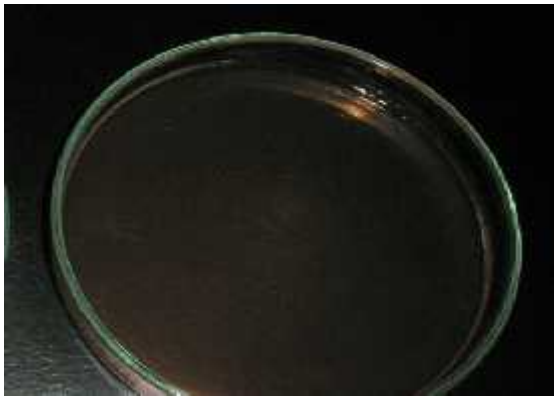
Fig. 16.1: Inactivation of *E. coli* in ground chicken meat exposed to 7.5% cinnamaldehyde in (A) Non selective media (MHA Control plate) and selective media (SMA Control plate) (B) Non selective media (MHA treatment plate) and selective media (SMA treatment plate).





(A) Non selective media (MHA Control plate) and selective media (SMA Control plate)





(B) Non selective media (MHA treatment plate) and selective media (SMA treatment plate)

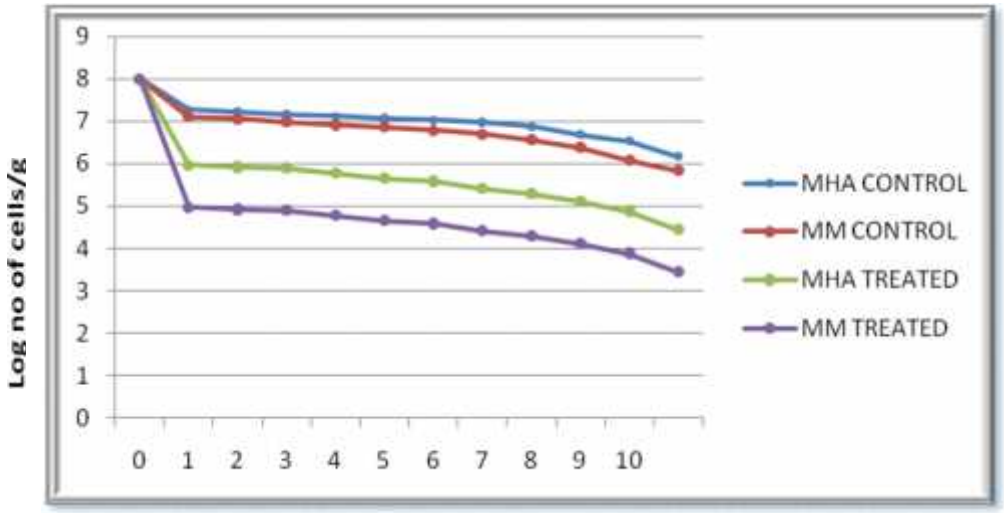
Fig. 16.2: Inactivation of *E. coli* in ground chicken meat exposed to 12.5% cinnamaldehyde (A) Non selective media (MHA Control plate) and selective media (SMA Control plate) (B) Non selective media (MHA treatment plate) and selective media (SMA treatment plate).

3.14. Inactivation of *Bacillus cereus* populations in inoculated ground chicken meat with essential oil:

Ground chicken meat was inoculated with each of 10^7 CFU/ml of *B. cereus* and the inoculated meat samples were challenged by MIC level, 3% MIC level and 5% MIC level of cinnamaldehyde. The results for *Bacillus cereus* are shown in Figure 17 a, b, c ; 18 a, b, c ; 19 a, b, c ; 20 a, b, c and 21 a, b, c respectively.

Firstly, treatment with the 2.5% cinnamaldehyde showed the 2.8 (5.92± 0.028) log reduction of *Bacillus cereus* 01 cells from 7 (7.275± 0.0353) log cells within 1 day of exposure when grown in non selective culture medium (MHA) while using MM medium (selective for *Bacillus cereus*) showed the 3.8 (4.92±0.028) log reduction of cells per gram (Fig 17a).. The experiment was carried out for 10 days.

Inactivation of *Bacillus cereus* 01 in ground chicken meat exposed to 2.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig- 17 a.



Incubation time (Day)

Fig 17a: Inactivation of *Bacillus cereus* (01) in ground chicken meat exposed to 2.5% cinnamaldehyde

Secondly, treatment with 7.5% cinnamaldehyde showed the 3.2 (3.99± 0.070) log reduction of *Bacillus cereus* 01 cells from 7 (7.185± 0.063) log cells within 1 day of exposure when grown in non selective culture medium (MHA) while using MM medium (selective for *Bacillus cereus*) no viable growth was observed within the same exposure (Fig.17b). The experiment was carried out for 10 days. After 10 days exposure 4.5 (2.45 ±0.212) log reduction was recorded in non selective medium (MHA) but no growth was recorded in selective medium. The *Bacillus cereus* 01 cells obtained in non selective medium might be the injured ones that could not grow in selective medium.

Inactivation of *Bacillus cereus* 01 in ground chicken meat exposed to 7.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig- 17 b.

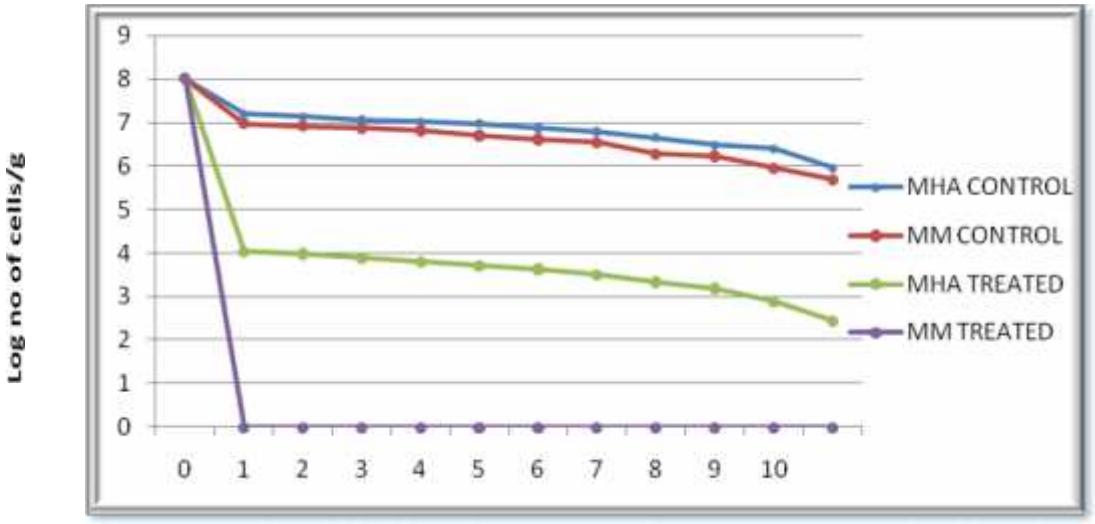


Fig 17b: Inactivation of *Bacillus cereus* 01 in ground chicken meat exposed to 7.5% cinnamaldehyde

Thirdly, treatment with the 12.5% cinnamaldehyde showed 4 (2.88± 0.042) log reduction of *Bacillus cereus* 01 cells from 7 (7.035± 0.049) log cells within 1 day of exposure when grown in non selective culture medium (MHA) while using MM medium (selective for *Bacillus cereus*) no viable growth was observed within the same exposure (Fig.17c). The experiment was carried out for 10 days. After 10 days exposure 5.8 (1.45 ±0.212) log reduction was recorded in non selective medium (MHA) but no growth was recorded in selective medium. The *Bacillus cereus* 01 cells obtained in non selective medium might be the injured ones that could not grow in selective medium.

Inactivation of *Bacillus cereus* 01 in ground chicken meat exposed to 12.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig- 17 c.

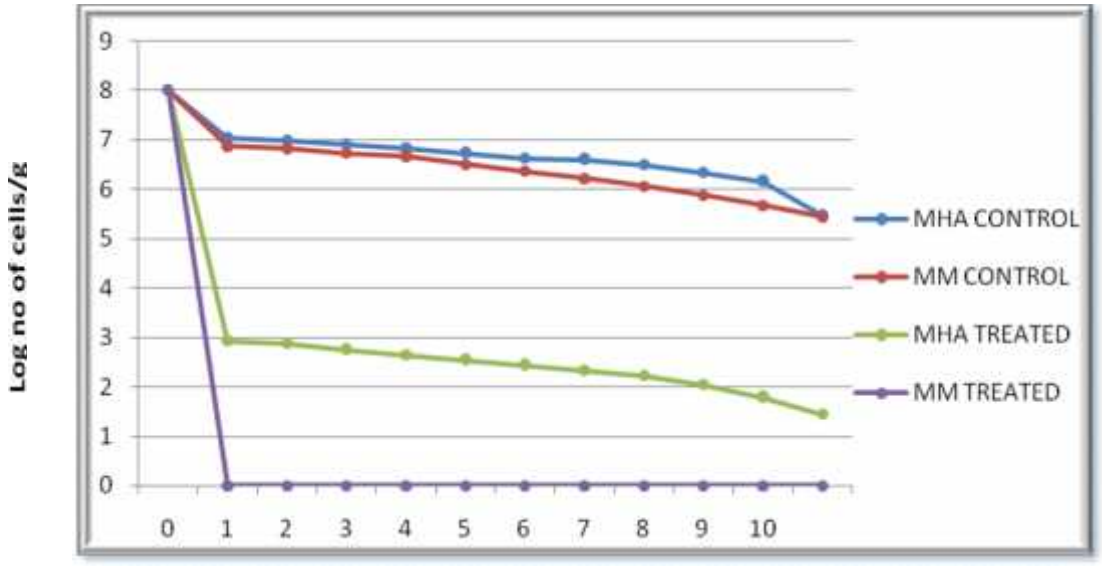


Fig 17c: Inactivation of *Bacillus cereus* 01 in ground chicken meat exposed to 12.5% cinnamaldehyde

3.14.1. Inactivation of *Bacillus cereus* 02 populations in inoculated ground chicken meat:

Firstly, treatment with the cinnamaldehyde 2.5% showed the 1.3 (5.945± 0.063) log reduction of *Bacillus cereus* 02 cells from 7.2 (7.275± 0.035) log cells within 1 day of exposure when grown in non selective culture medium (MHA) while using MM medium (selective for *Bacillus cereus*) showed the 2.2 (4.94±0.063) log reduction of cells per gram (Fig 18 a). The experiment was carried out for 10 days.

Inactivation of *Bacillus cereus* 02 in ground chicken meat exposed to 2.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig- 18 a.

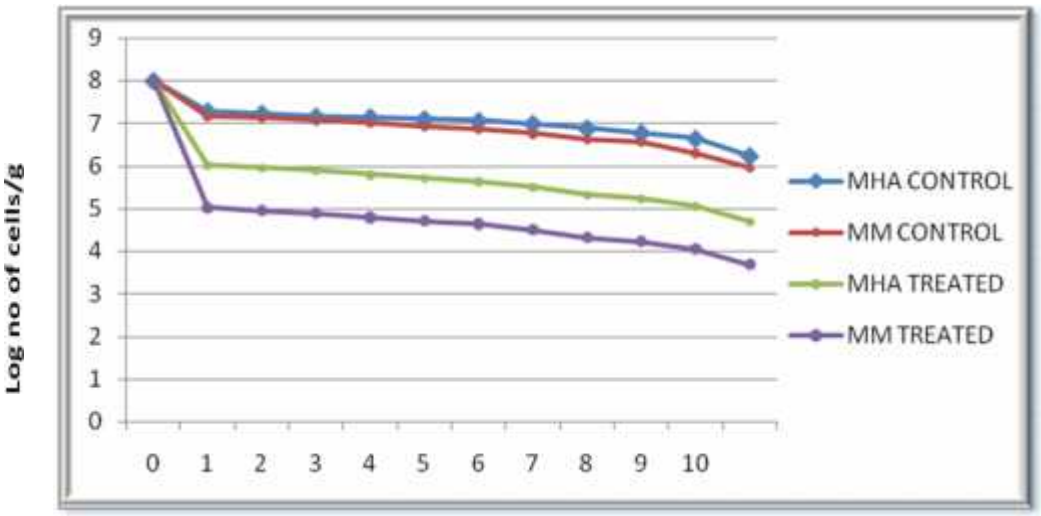


Fig 18a: Inactivation of *Bacillus cereus* 02 in ground chicken meat exposed to 2.5% cinnamaldehyde

Secondly, treatment with the 7.5% cinnamaldehyde showed the 3.5 (3.925± 0.035) log reduction of *Bacillus cereus* 02 cells from 7.2 (7.2± 0.042) log cells within 1 day of exposure when grown in non selective culture medium (MHA) while using MM medium (selective for *Bacillus cereus*) no viable growth was observed within the same exposure (Fig.18 b). The experiment was carried out for 10 days. After 10 days exposure near about 5 (2.35 ±0.070) log reductions was recorded in non selective medium (MHA) but no growth was recorded in selective medium. The *Bacillus cereus* 02 cells obtained in non selective medium might be the injured ones that could not grow in selective medium.

Inactivation of *Bacillus cereus* 02 in ground chicken meat exposed to 7.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig- 18 b.

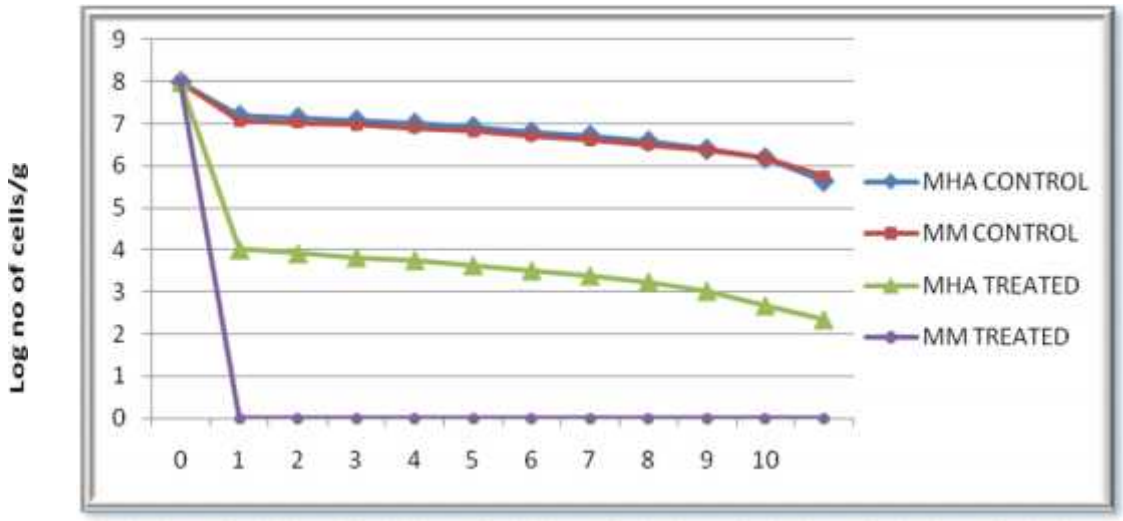


Fig 18b: Inactivation of *Bacillus cereus* 02 in ground chicken meat exposed to 7.5% cinnamaldehyde

Thirdly, treatment with the 12.5% cinnamaldehyde showed the 4.3 (2.865± 0.049) log reduction of *Bacillus cereus* 02 cells of from 7.2 (7.07± 0.049497) log cells within 1 day of exposure when grown in non selective culture medium (MHA) while using MM medium (selective for *Bacillus cereus*) no viable growth was observed within the same exposure (Fig.18 c). The experiment was carried out for 10 days. After 10 days exposure 5.8 (1.45±0.212) log reduction was recorded in non selective medium (MHA) but no growth was recorded in selective medium. The *Bacillus cereus* 02 cells obtained in non selective medium might be the injured ones that could not grow in selective medium.

Inactivation of *Bacillus cereus* 02 in ground chicken meat exposed to 12.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig- 18 c.

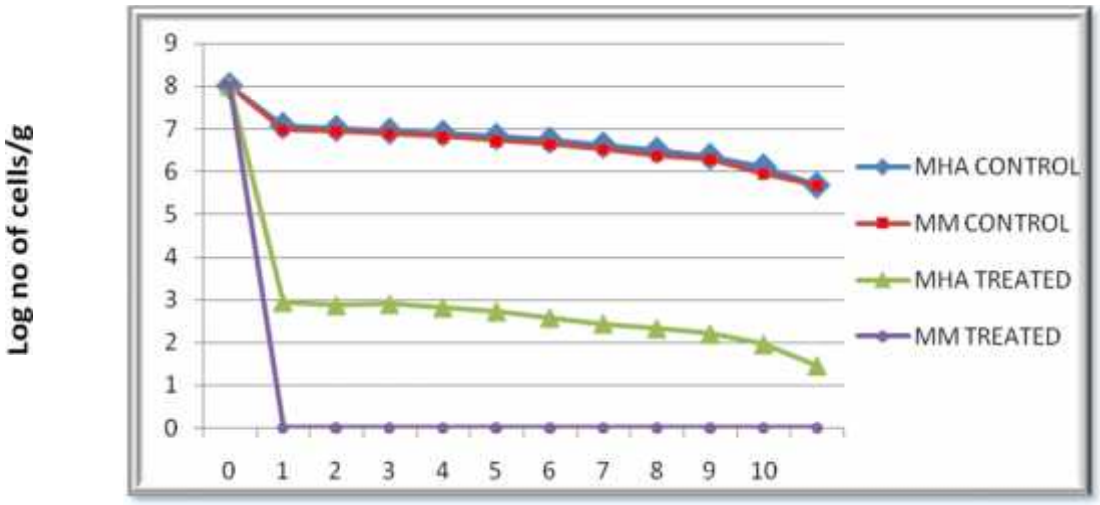


Fig 18c: Inactivation of *Bacillus cereus* 02 in ground chicken meat exposed to 12.5% cinnamaldehyde

3.14.2. Inactivation of *Bacillus cereus* 10 populations in inoculated ground chicken meat:

Firstly, treatment with the 2.5% cinnamaldehyde showed the 1 (6.135± 0.007) log reduction of *Bacillus cereus* 10 cells of from 7.3 (7.385± 0.007) log cells within 1 day of exposure when grown in non selective culture medium (MHA) while using MM medium (selective for *Bacillus cereus*) showed the 2 (5.135±0.007) log reduction of cells per gram (Fig 19 a). The experiment was carried out for 10 days.

Inactivation of *Bacillus cereus* 10 in ground chicken meat exposed to 2.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig- 19 a.

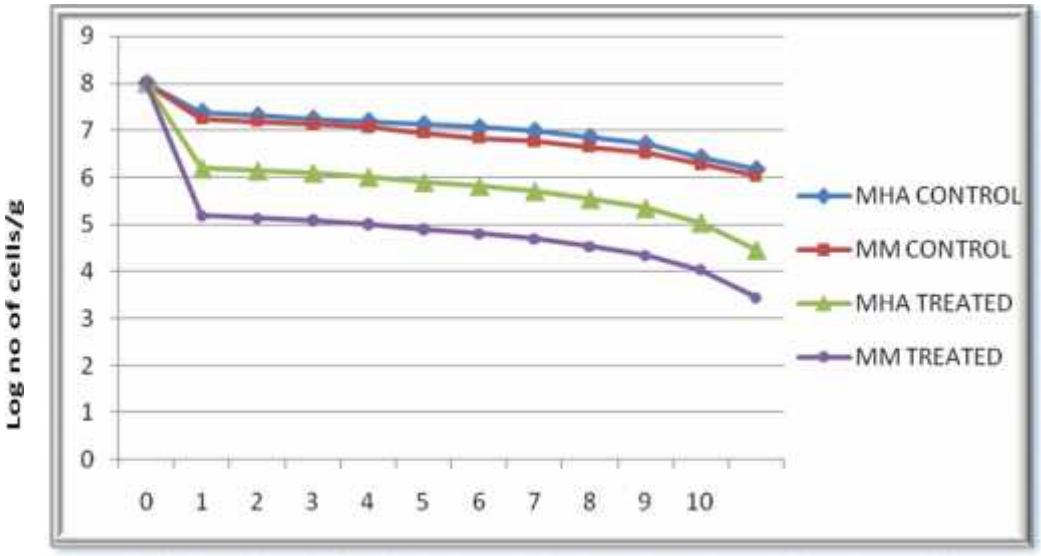


Fig 19a: Inactivation of *Bacillus cereus* 10 in ground chicken meat exposed to 2.5% cinnamaldehyde

Secondly, treatment with the 7.5% cinnamaldehyde showed the 3.3 (4.045± 0.035) log reduction of *Bacillus cereus* 10 cells from 7.3 (7.295± 0.035) log cells within 1 day of exposure when grown in non selective culture medium (MHA) while using MM medium (selective for *Bacillus cereus*) no viable growth was observed within the same exposure (Fig.19 b). The experiment was carried out for 10 days. After 10 days exposure 4.5 (2.685 ± 0.120) log reduction was recorded in non selective medium (MHA) but no growth was recorded in selective medium. The *Bacillus cereus* 10 cells obtained in non selective medium might be the injured ones that could not grow in selective medium.

Inactivation of *Bacillus cereus* 10 in ground chicken meat exposed to 7.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig- 19 b.

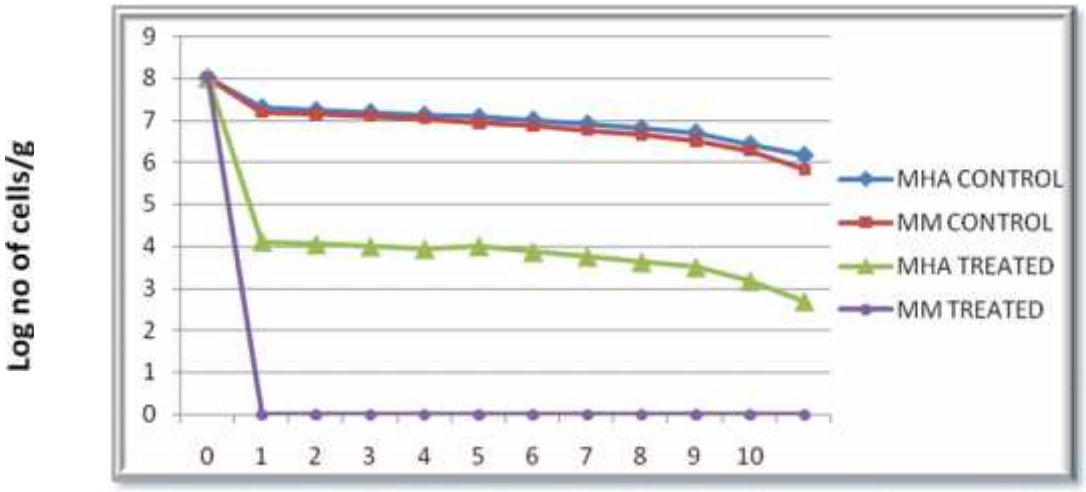


Fig 19b: Inactivation of *Bacillus cereus* 10 in ground chicken meat exposed to 7.5% cinnamaldehyde

Thirdly, treatment with the 12.5% cinnamaldehyde showed the 4 (2.91± 0.014) log reduction of *Bacillus cereus* 10 cells from 7.3 (7.22± 0.028) log cells within 1 day of exposure when grown in non selective culture medium (MHA) while using MM medium (selective for *Bacillus cereus*) no viable growth was observed within the same exposure (Fig.19 c). The experiment was carried out for 10 days. After 10 days exposure 5.5 (1.45±0.212) log reduction was recorded in non selective medium (MHA) but no growth was recorded in selective medium. The *Bacillus cereus* 10 cells obtained in non selective medium might be the injured ones that could not grow in selective medium.

Inactivation of *Bacillus cereus* 10 in ground chicken meat exposed to 12.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig- 19 c.

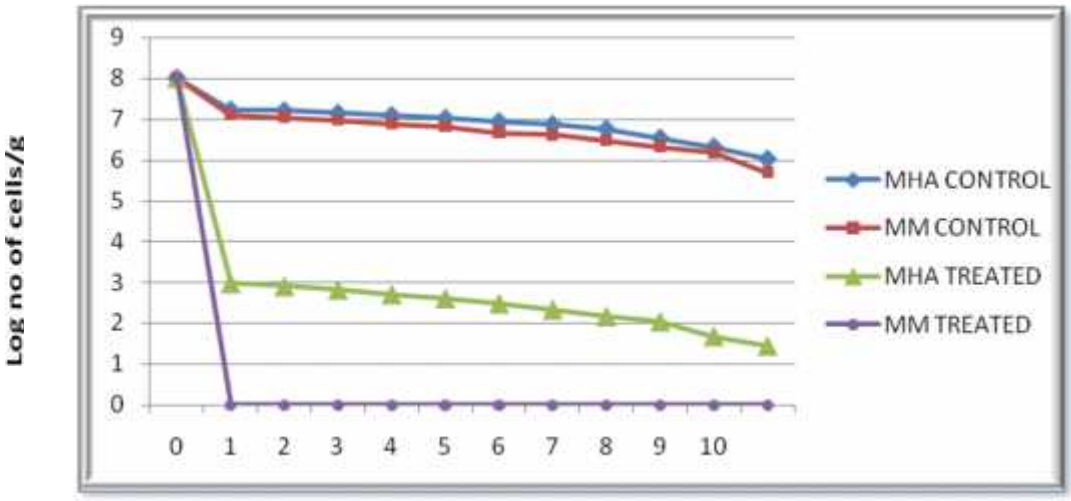


Fig 19c: Inactivation of *Bacillus cereus* 10 in ground chicken meat exposed to 12.5% cinnamaldehyde

3.14.3. Inactivation of *Bacillus cereus* 12 populations in inoculated ground chicken meat:

Firstly, treatment with the 2.5% cinnamaldehyde showed the 1.2 (6.055± 0.049) log reduction of *Bacillus cereus* 12 cells from 7.3 (7.32± 0.028) log cells within 1 day of exposure when grown in non selective culture medium (MHA) while using MM medium (selective for *Bacillus cereus*) showed the 2 (5.055±0.049) log reduction of cells per gram (Fig 20 a). The experiment was carried out for 10 days.

Inactivation of *Bacillus cereus* 12 in ground chicken meat exposed to 2.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig- 20 a.

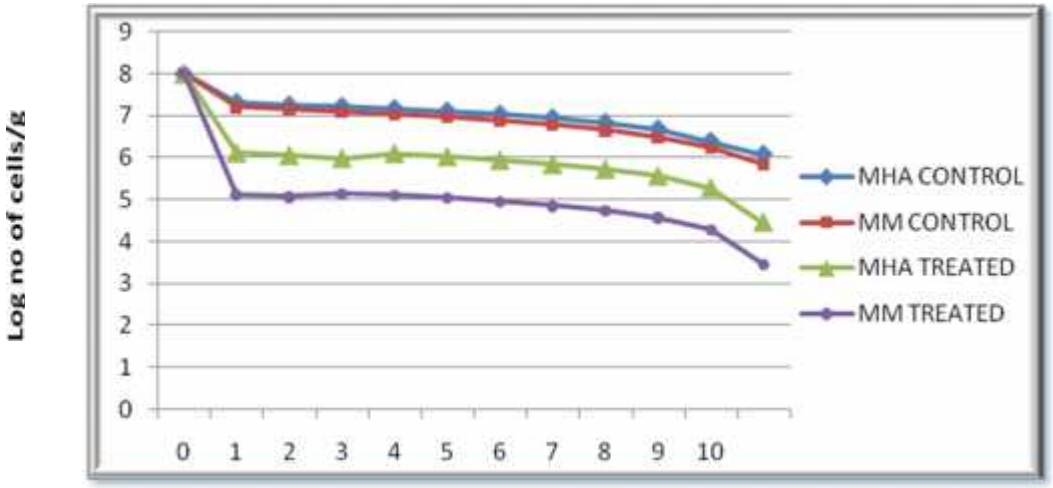


Fig 20a: Inactivation of *Bacillus cereus* 12 in ground chicken meat exposed to 2.5% cinnamaldehyde

Secondly, treatment with the 7.5% cinnamaldehyde showed the 3.5 (3.9± 0.028) log reduction of *Bacillus cereus* 12 cells from 7.3 (7.22± 0.028284) log cells within 1 day of exposure when grown in non selective culture medium (MHA) while using MM medium (selective for *Bacillus cereus*) no viable growth was observed within the same exposure (Fig.20 b). The experiment was carried out for 10 days. After 10 days exposure 4.8 (2.45 ± 0.212) log reduction was recorded in non selective medium (MHA) but no growth was recorded in selective medium. The *Bacillus cereus* 12 cells obtained in non selective medium might be the injured ones that could not grow in selective medium.

Inactivation of *Bacillus cereus* 12 in ground chicken meat exposed to 7.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig- 20 b.

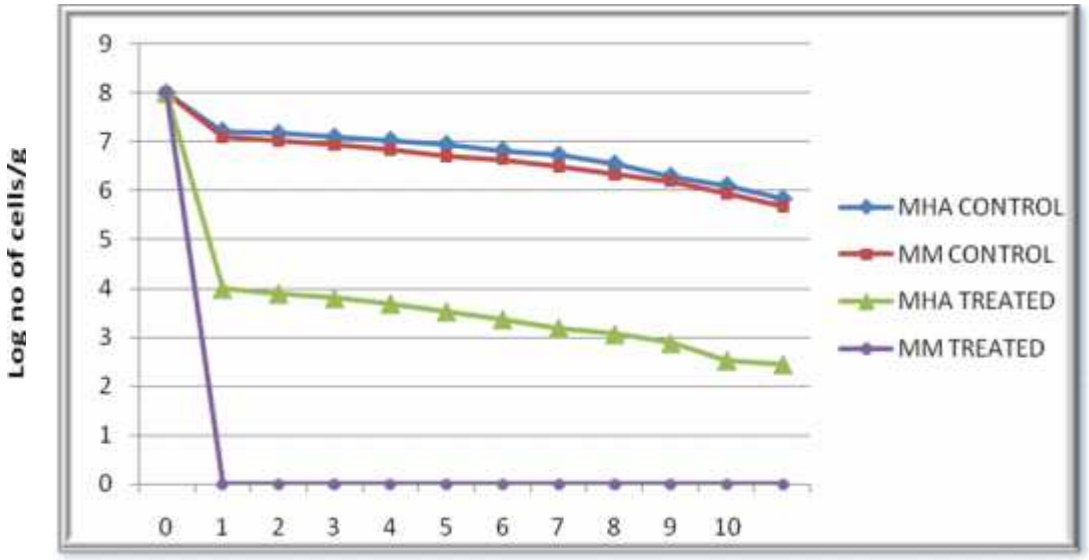


Fig 20b: Inactivation of *Bacillus cereus* 12 in ground chicken meat exposed to 7.5% cinnamaldehyde

Thirdly, treatment with the 12.5% cinnamaldehyde showed the 4.3 (2.865± 0.091) log reduction of *Bacillus cereus* 12 cells from 7.3 (7.125± 0.021) log cells within 1 day of exposure when grown in non selective culture medium (MHA) while using MM medium (selective for *Bacillus cereus*) no viable growth was observed within the same exposure (Fig.20 c). The experiment was carried out for 10 days. After 10 days exposure 5.9 (1.45±0.212) log reductions was recorded in non selective medium (MHA) but no growth was recorded in selective medium. The *Bacillus cereus* 12 cells obtained in non selective medium might be the injured ones that could not grow in selective medium.

Inactivation of *Bacillus cereus* 12 in ground chicken meat exposed to 12.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig- 20 c.

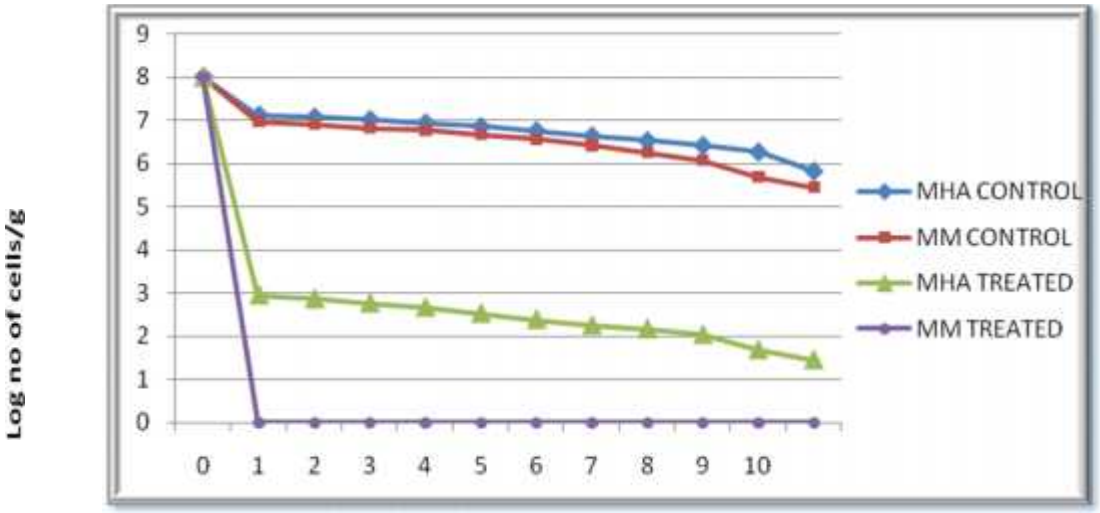


Fig 20c: Inactivation of *Bacillus cereus* 12 in ground chicken meat exposed to 12.5% cinnamaldehyde

3.14.4. Inactivation of *Bacillus cereus* 06 populations in inoculated ground chicken meat:

Firstly, treatment with the 2.5% cinnamaldehyde showed the 1.3 (5.07± 0.042) log reduction of *Bacillus cereus* 06 cells from 7.2 (7.34± 0.056) log cells within 1 day of exposure when grown in non selective culture medium (MHA) while using MM medium (selective for *Bacillus cereus*) showed the 2.3 (4.0±0.042) log reduction of cells per gram (Fig. 21 a). The experiment was carried out for 10 days.

Inactivation of Bacillus cereus 06 in ground chicken meat exposed to 2.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig- 21 a.

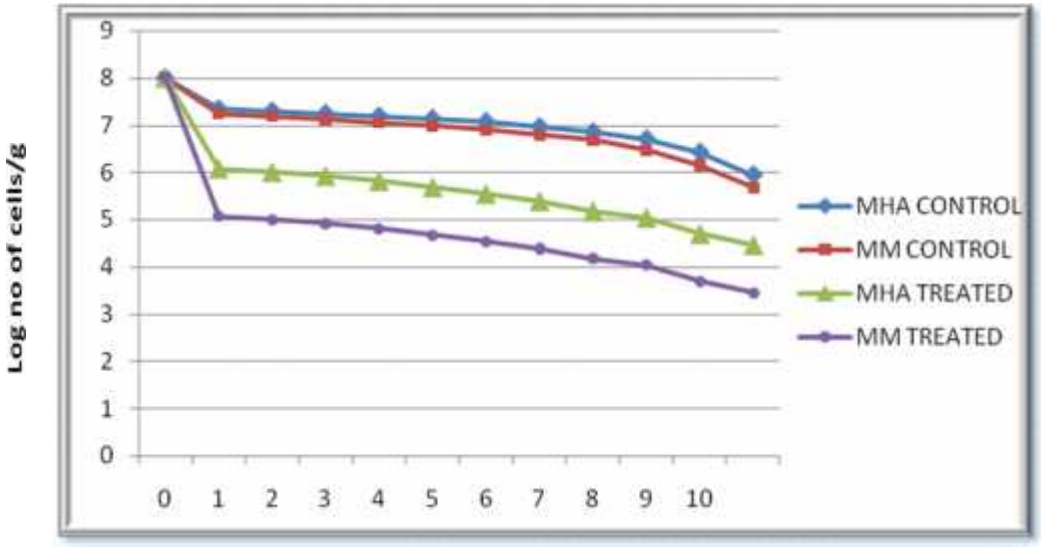


Fig 21a: Inactivation of *Bacillus cereus* 06 in ground chicken meat exposed to 2.5% cinnamaldehyde

Secondly, treatment with the 7.5% cinnamaldehyde showed the 3 (4.08± 0.042) log reduction of *Bacillus cereus* 06 cells from 7.2 (7.28± 0.021) log cells within 1 day of exposure when grown in non selective culture medium (MHA) while using MM medium (selective for *Bacillus cereus*) no viable growth was observed within the same exposure (Fig.21 b). The experiment was carried out for 10 days. After 10 days exposure 4.9 (2.45±0.212) log reductions was recorded in non selective medium (MHA) but no growth was recorded in selective medium. The *Bacillus cereus* (06) cells obtained in non selective medium might be the injured ones that could not grow in selective medium.

Inactivation of *Bacillus cereus* 06 in ground chicken meat exposed to 7.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig- 21 b.

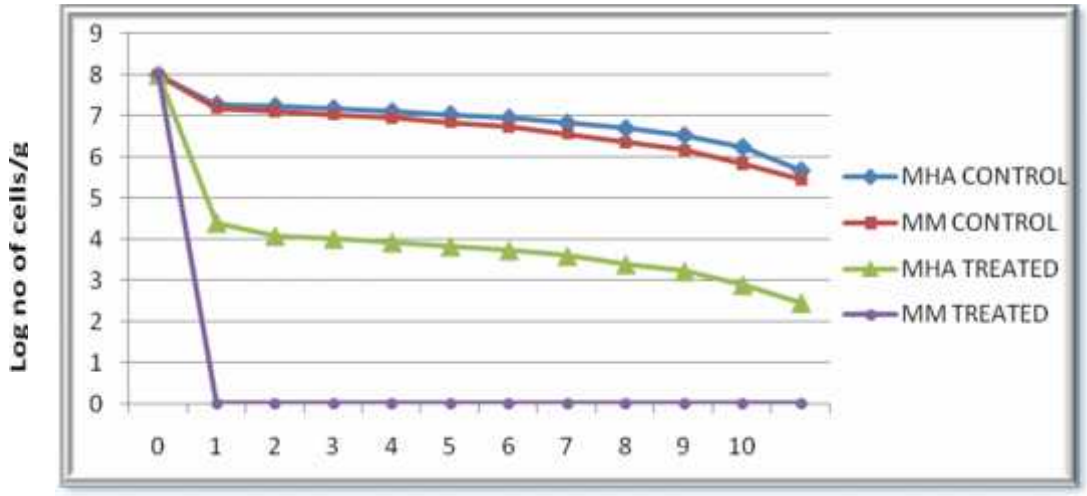


Fig 21b: Inactivation of *Bacillus cereus* 06 in ground chicken meat exposed to 7.5% cinnamaldehyde

Thirdly, treatment with the 12.5% cinnamaldehyde showed the 4.2 (2.93± 0.042) log reduction of *Bacillus cereus* 06 cells from 7.2 (7.18 ± 0.021) log cells within 1 day of exposure when grown in non selective culture medium (MHA) while using MM medium (selective for *Bacillus cereus*) no viable growth was observed within the same exposure (Fig.21 c). The experiment was carried out for 10 days. After 10 days exposure 6 (1.25±0.070) log reductions was recorded in non selective medium (MHA) but no growth was recorded in selective medium. The *Bacillus cereus* 06 cells obtained in non selective medium might be the injured ones that could not grow in selective medium.

Inactivation of *Bacillus cereus* 06 in ground chicken meat exposed to 12.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig- 21 c.

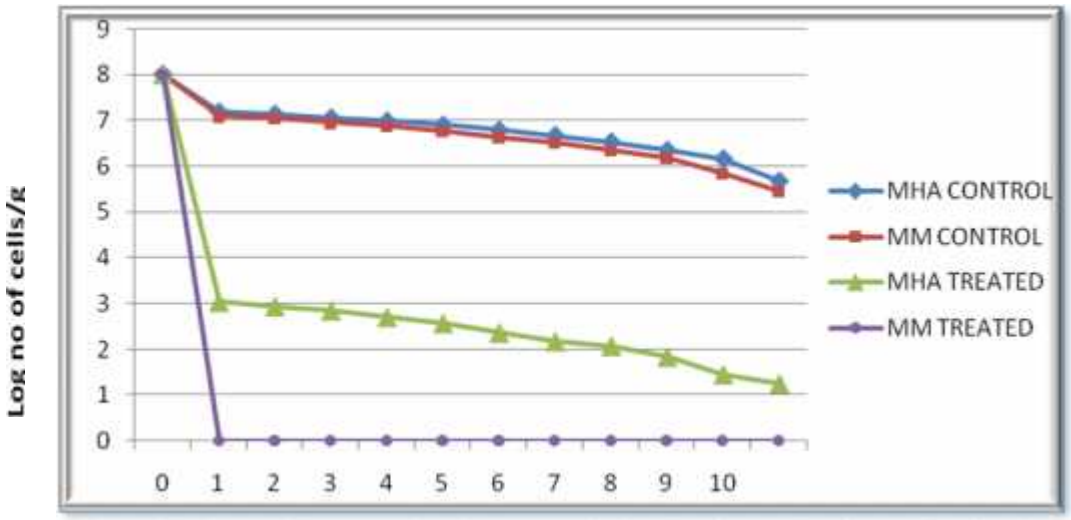
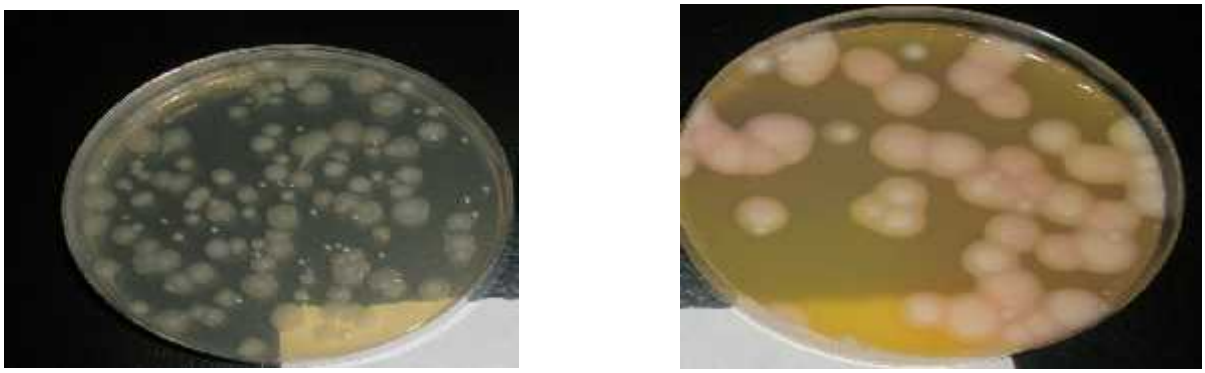


Fig 21c: Inactivation of *Bacillus cereus* 06 in ground chicken meat exposed to 12.5% cinnamaldehyde

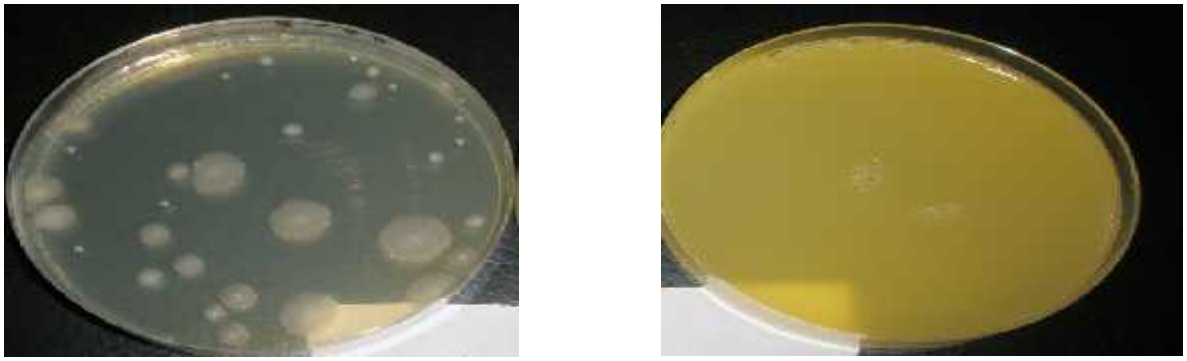
(A) Non selective media (MHA Control plate) and selective media (MM Control plate)

(B) Non selective media (MHA Treatment plate) and selective media (MM Treatment plate)

Fig 22: Inactivation of *B. cereus* in ground chicken meat exposed to 2.5% cinnamaldehyde in (A) Non selective media (MHA Control plate) and selective media (MM Control plate) (B) Non selective media (MHA Treatment plate) and selective media (MM Treatment plate)

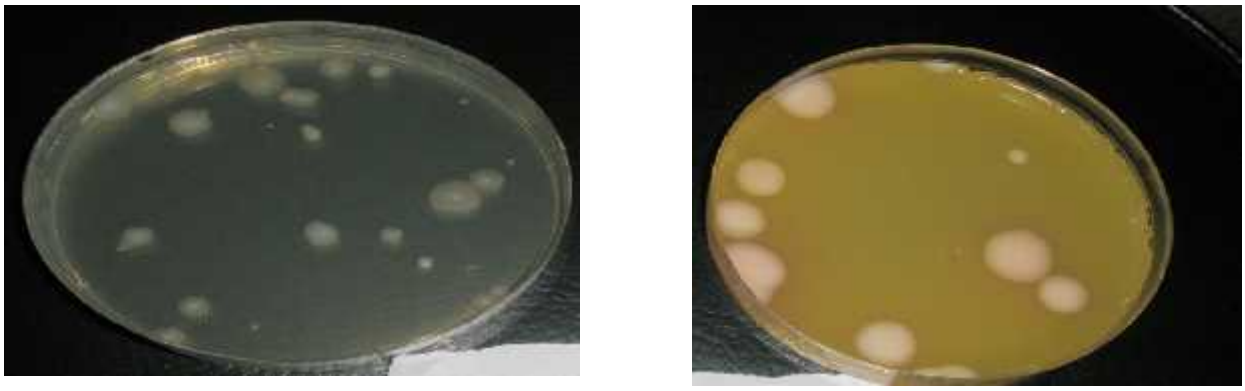


(A) Non selective media (MHA Control plate) and selective media (MM Control plate)

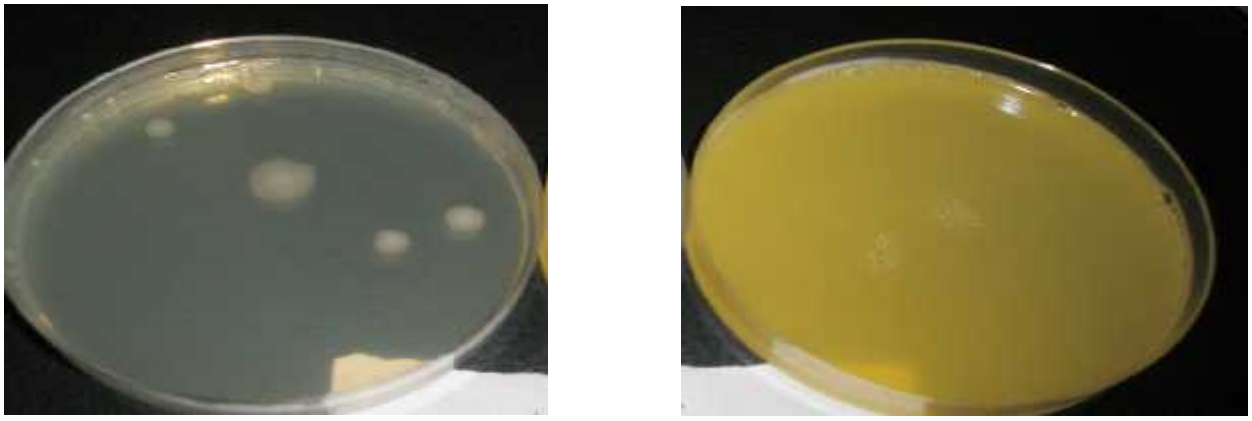


(B) Non selective media (MHA Treatment plate) and selective media (MM Treatment plate)

Fig 22.1: Inactivation of *B. cereus* in ground chicken meat exposed to 7.5% cinnamaldehyde in (A) Non selective media (MHA Control plate) and selective media (MM Control plate) (B) Non selective media (MHA Treatment plate) and selective media (MM Treatment plate).



(A)Non selective media (MHA Control plate) and selective media (MM Control plate)



(B)Non selective media (MHA Treatment plate) and selective media (MM Treatment plate)

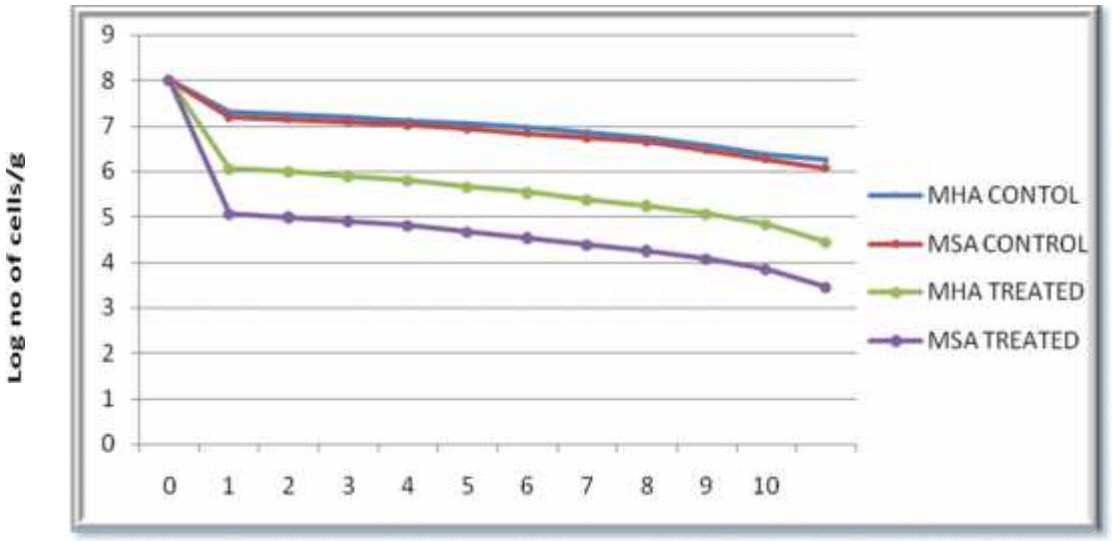
Fig 22.2: Inactivation of *B. cereus* in ground chicken meat exposed to 12.5% cinnamaldehyde in (A) Non selective media (MHA Control plate) and selective media (MM Control plate) (B) Non selective media (MHA Treatment plate) and selective media (MM Treatment plate)

3.15. Inactivation of *S. aureus* populations in inoculated ground chicken meat

Ground chicken meat was inoculated with each of 10^7 CFU/ml of *S. aureus* and the inoculated meat samples were challenged by MIC level, 3% MIC level and 5% MIC level of cinnamaldehyde. The results for *S. aureus* are shown in Figure 23 a, b, c; 24 a, b, c; 25 a, b, c; 26 a, b, c and 27 a, b, c, respectively.

Firstly, treatment with the 2.5% cinnamaldehyde showed the 1.2 (5.99± 0.028) log reduction of *S. aureus* 05 cells from 7.2 (7.285±0.021) log cells within 1 day of exposure when grown in non selective culture medium (MHA). After 10 days exposure 2.8 (4.45±0.212) and 3.9 (3.45±0.212) log reductions was recorded in non selective medium (MHA) and MSA medium (Selective medium) respectively (Fig 23 a).

Inactivation of *S. aureus* 05 *in* ground chicken meat exposed to 2.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig-23 a.



Incubation time (Day)

Fig 23a: Inactivation of *S. aureus* 05 in ground chicken meat exposed to 2.5% cinnamaldehyde

Secondly, treatment with the 7.5% cinnamaldehyde showed the 3 (3.89± 0.014) log reduction of *S. aureus* 05 cells from 7.2 (7.22± 0.028) log cells within 1 day of exposure when grown in non selective culture medium (MHA) while using MSA medium (selective for *S. aureus*) no viable growth was observed within the same exposure (Fig.23 b). The experiment was carried out for 10 days. After 10 days exposure 4.5 (2.45±0.212) log reductions was recorded in non selective medium (MHA) but no growth was recorded in selective medium. The *S. aureus* 05 cells obtained in non selective medium might be the injured ones that could not grow in selective medium.

Inactivation of *S. aureus* 05 in ground chicken meat exposed to cinnamaldehyde 7.5% and stored at -18°C for 10 days. The results are shown in Fig-23 b.

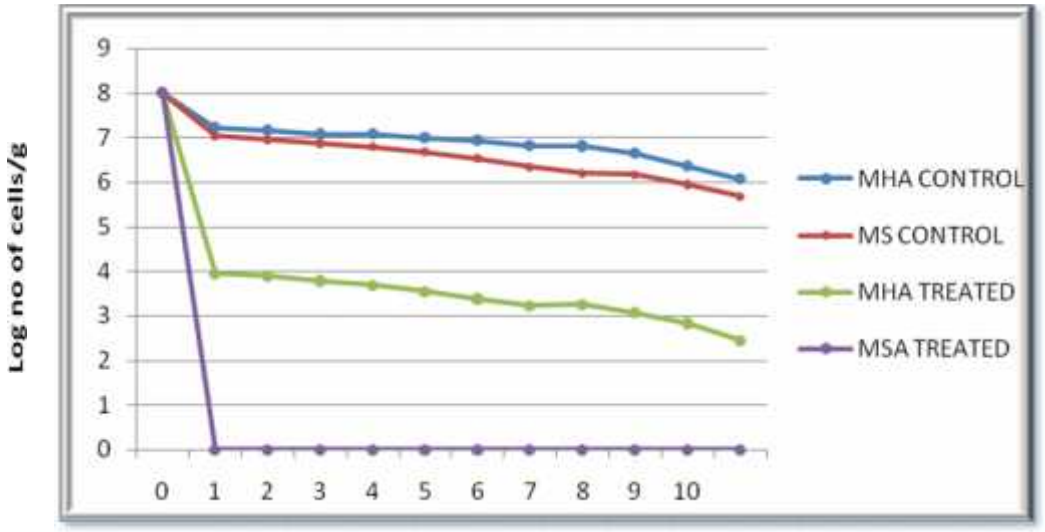


Fig 23b: Inactivation of *S. aureus* 05 in ground chicken meat exposed to 7.5% cinnamaldehyde

Thirdly, treatment with the 12.5% cinnamaldehyde showed the 4 (2.825± 0.077) log reduction of *S. aureus* 050 cells from 7.2 (7.115±0.636) log cells within 1 day of exposure when grown in non selective culture medium (MHA) while using MSA medium (selective for *S. aureus*) no viable growth was observed within the same exposure (Fig.23 c). The experiment was carried out for 10 days. After 10 days exposure 5.8 (1.45±0.212) log reductions was recorded in non selective medium (MHA) but no growth was recorded in selective medium. However, the MSA medium colony count was zero from the initial count and remains constant in the following days.

Inactivation of *S. aureus* 05 in ground chicken meat exposed to 12.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig-23 c.

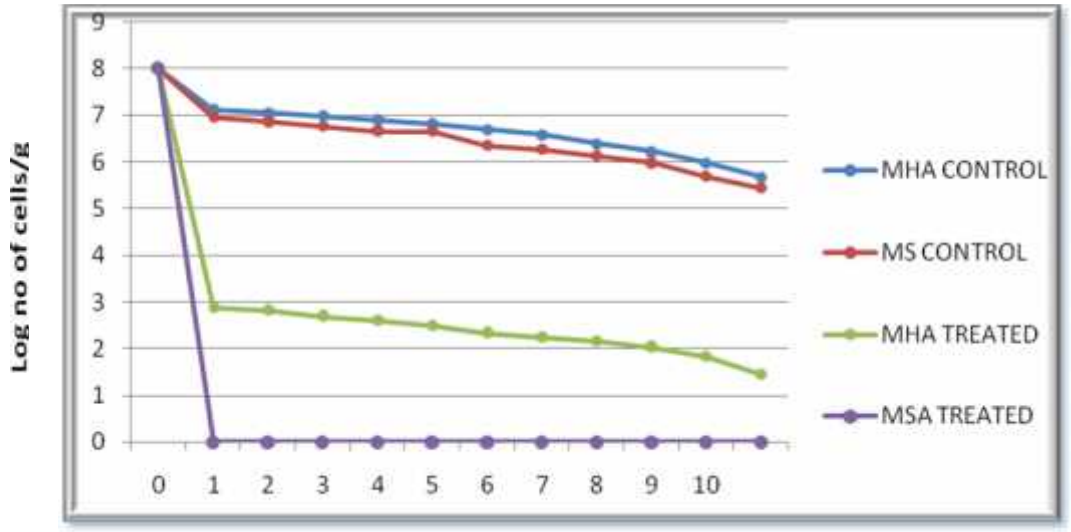


Fig 23c: Inactivation of *S. aureus* 05 in ground chicken meat exposed to 12.5% cinnamaldehyde

3.15.1. Inactivation of *S. aureus* 09 populations in inoculated ground chicken meat

Firstly, treatment with the 2.5% cinnamaldehyde showed the 1 (6.1± 0.035) log reduction of *S. aureus* 09 cells from 7.3 (7.34±0.028) log cells within 1 day of exposure when grown in non selective culture medium (MHA). After 10 days exposure 2.2 (4.68±0.12) and 3.1 (3.68±0.12) log reductions was recorded in non selective medium (MHA) and MSA medium (selective medium) respectively (Fig- 24 a).

Inactivation of *S. aureus* 09 *in* ground chicken meat exposed to 2.5% cinnamaldehyde and stored at -18°C C for 10 days. The results are shown in Fig-24 a.

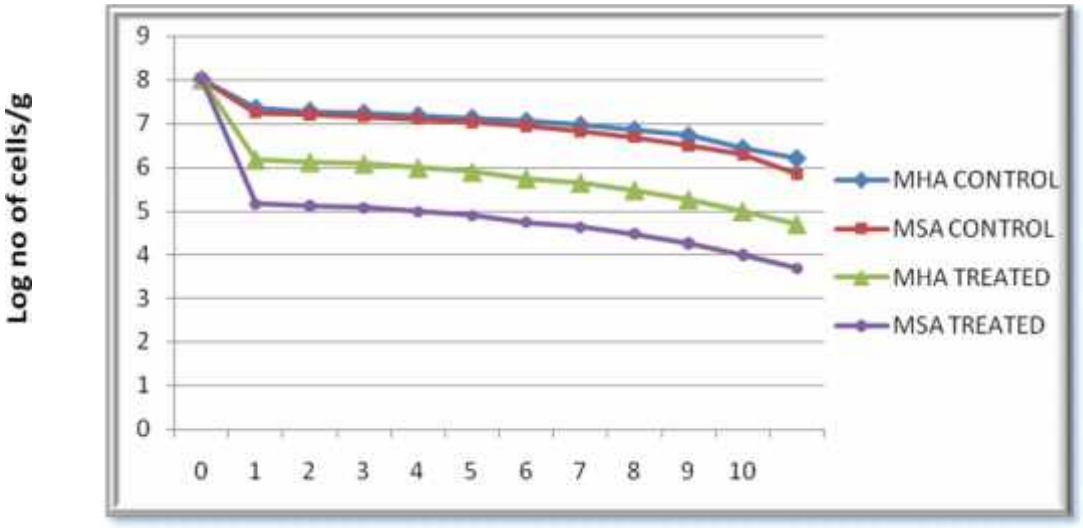


Fig 24a: Inactivation of *S. aureus* 09 in ground chicken meat exposed to 2.5% cinnamaldehyde

Secondly, treatment with the 12.5% cinnamaldehyde showed the 3.2 (3.89± 0.014) log reduction of *S. aureus* 09 cells from 7.2 (7.26±0.14 log) cells within 1 day of exposure when grown in non selective culture medium (MHA) while using MSA medium (selective for *S. aureus*) no viable growth was observed within the same exposure (Fig.24b). The experiment was carried out for 10 days. After 10 days exposure 4.5 (2.68±0.120) log reductions was recorded in non selective medium (MHA) but no growth was recorded in selective medium.

Inactivation of *S. aureus* 09 in ground chicken meat exposed to 7.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig-24 b.

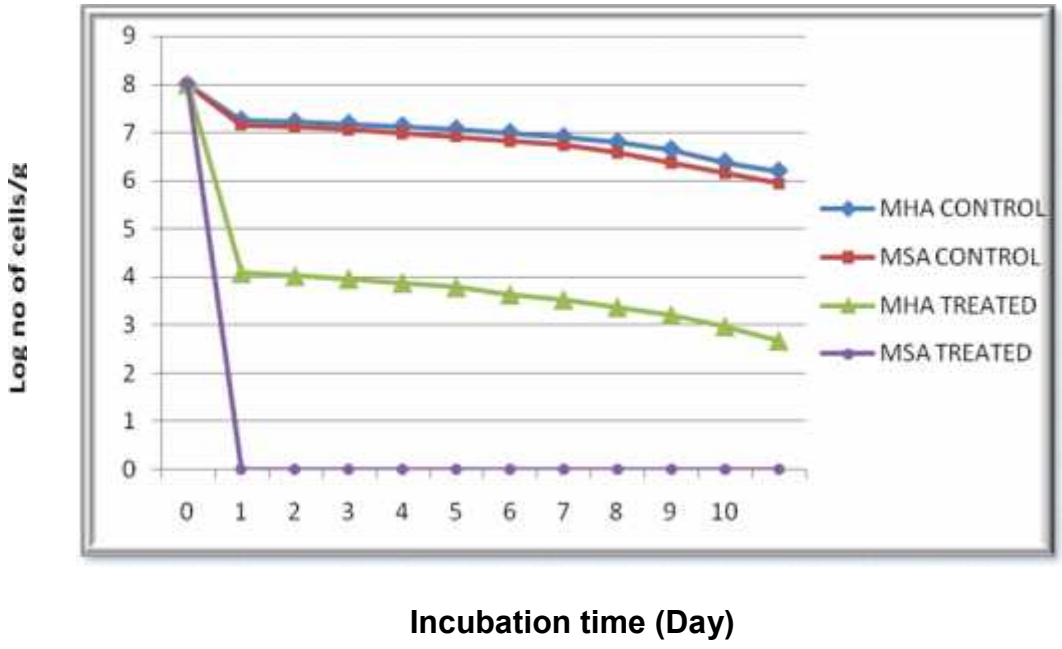


Fig 24b: Inactivation of *S. aureus* 09 in ground chicken meat exposed to 7.5% cinnamaldehyde

Thirdly, treatment with the 12.5% cinnamaldehyde showed the 4.3 (2.96± 0.056) log reduction of *S. aureus* 09 cells from 7.2 (7.19±0.035log) cells within 1 day of exposure

when grown in non selective culture medium (MHA) while using MSA medium (selective for *S. aureus*) no viable growth was observed within the same exposure (Fig.24 c). The experiment was carried out for 10 days. After 10 days exposure 6 (1.45±0.212) log reductions was recorded in non selective medium (MHA) but no growth was recorded in selective medium. However, the MSA medium colony count was zero from the initial count and remains constant in the following days.

Inactivation of *S. aureus* 09 in ground chicken meat exposed to 12.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig-24 c.

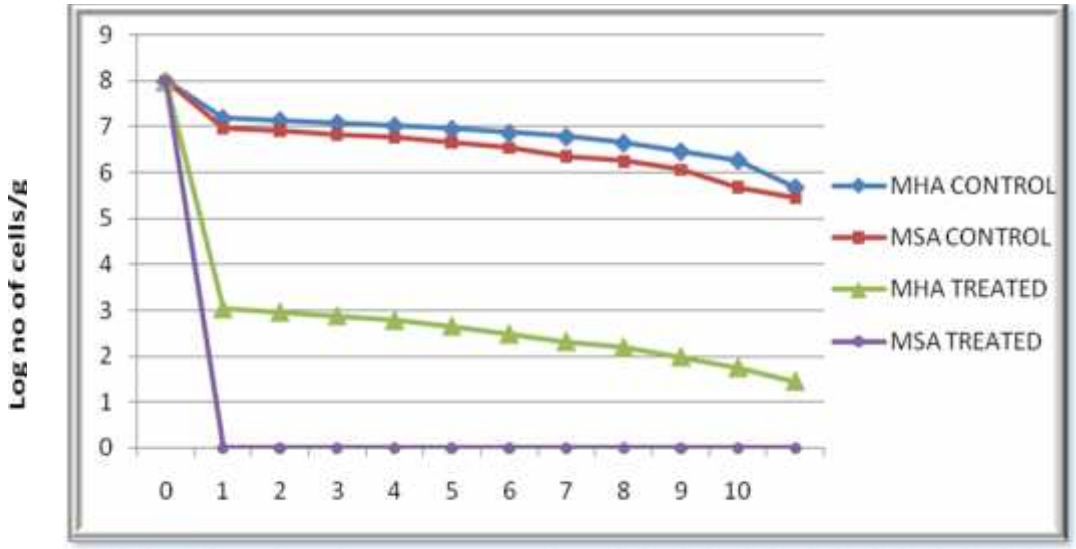


Fig 24c: Inactivation of *S. aureus* 09 in ground chicken meat exposed to 12.5% cinnamaldehyde

3.15.2. Inactivation of *S. aureus* 08 populations in inoculated ground chicken meat

Firstly, treatment with the 2.5% cinnamaldehyde showed the 1 (6.03± 0.043) log reduction of *S. aureus* 08 cells from 7.1 (7.14±0.016) log cells within 1 day of exposure when grown in non selective culture medium (MHA). After 10 days exposure 2.2 (4.8±0.282) and 3.1 (3.8±0.282) log reductions was recorded in non selective medium (MHA) and MSA medium (selective medium) respectively (Fig- 25 a).

Inactivation of *S. aureus* 08 in ground chicken meat exposed to 2.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig-25 a.

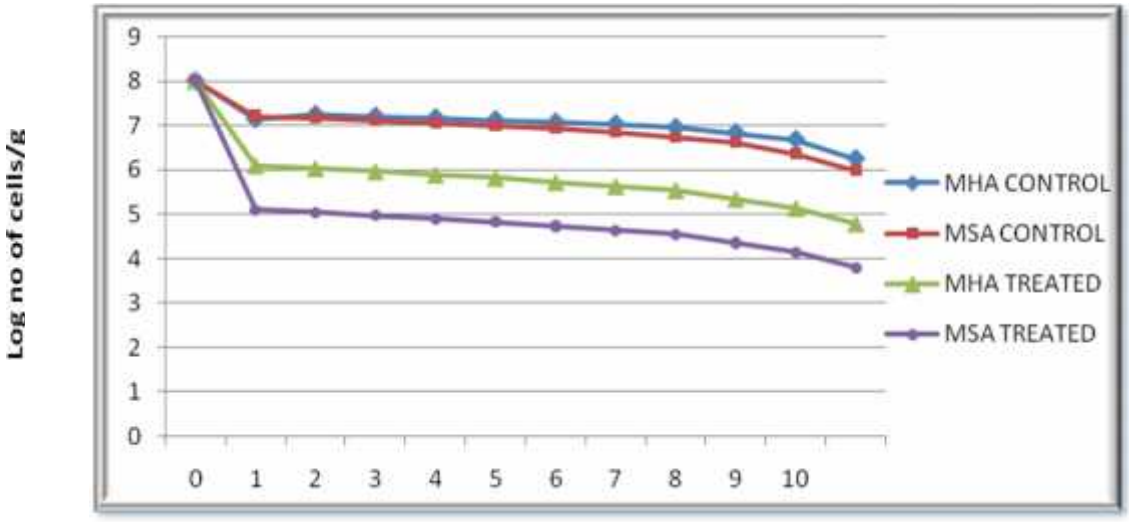


Fig 25a: Inactivation Survival of *S. aureus* 08 in ground chicken meat exposed to 2.5% cinnamaldehyde

Secondly, treatment with the 7.5% cinnamaldehyde showed the 3.5 (3.95± 0.04) log reduction of *S. aureus* 08 cells from 7.2 (7.23±0.035 log) cells within 1 day of exposure when grown in non selective culture medium (MHA) while using MSA medium (selective

for *S. aureus*) no viable growth was observed within the same exposure (Fig. 25 b). The experiment was carried out for 10 days. After 10 days exposure 4.5 (2.75±0.212) log reductions was recorded in non selective medium (MHA) but no growth was recorded in selective medium.

Inactivation of *S. aureus* 08 in ground chicken meat exposed to 7.5% cinnamaldehyde and stored at -18°C C for 10 days. The results are shown in Fig-25 b.

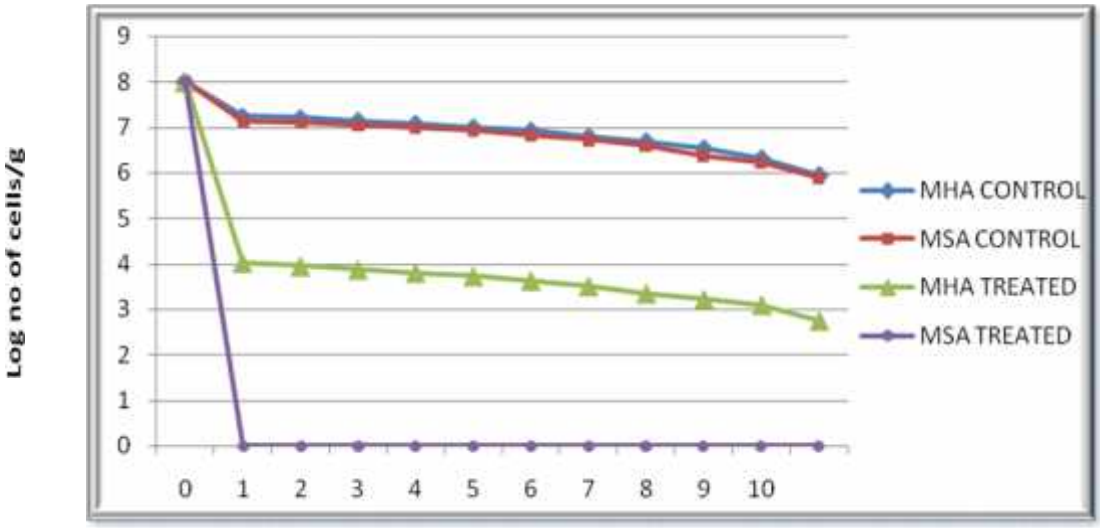


Fig 25b: Inactivation of *S. aureus* 08 in ground chicken meat exposed to 7.5% cinnamaldehyde

Thirdly, treatment with the 12.5% cinnamaldehyde showed the 4 (2.85± 0.063) log reduction of *S. aureus* 08 cells from 7.2 (7.17±0.042) log cells within 1 day of exposure when grown in non selective culture medium (MHA) while using MSA medium (selective for *S. aureus*) no viable growth was observed within the same exposure (Fig. 25 c). The

experiment was carried out for 10 days. After 10 days exposure 5.9 (1.45±0.212) log reductions was recorded in non selective medium (MHA) but no growth was recorded in selective medium. However, the MSA medium colony count was zero from the initial count and remains constant in the following days.

Inactivation of *S. aureus* 08 in ground chicken meat exposed to 12.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig- 25 c.

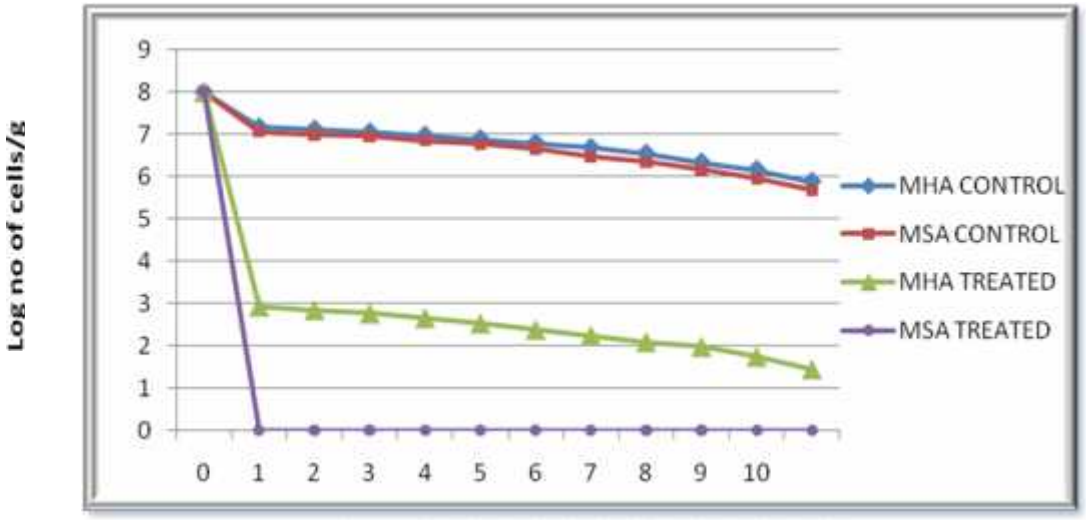


Fig 25c: Inactivation of *S. aureus* 08 in ground chicken meat exposed to 12.5% cinnamaldehyde

3.15.3. Inactivation of *S. aureus* 12 populations in inoculated ground chicken meat

.Firstly, treatment with the 2.5% cinnamaldehyde showed the 2.3 (6.09± 0.028) log reduction of *S. aureus* 12 cells from 7.3 (7.31±0.014) log cells within 1 day of exposure

when grown in non selective culture medium (MHA). After 10 days exposure 2.8 (4.68±0.12) and 3.3 (3.68±0.12) log reductions was recorded in non selective medium (MHA) and MSA medium (selective medium) respectively (Fig- 26 a).

Inactivation of *S. aureus* 12 in ground chicken meat exposed to 2.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig-26 a.

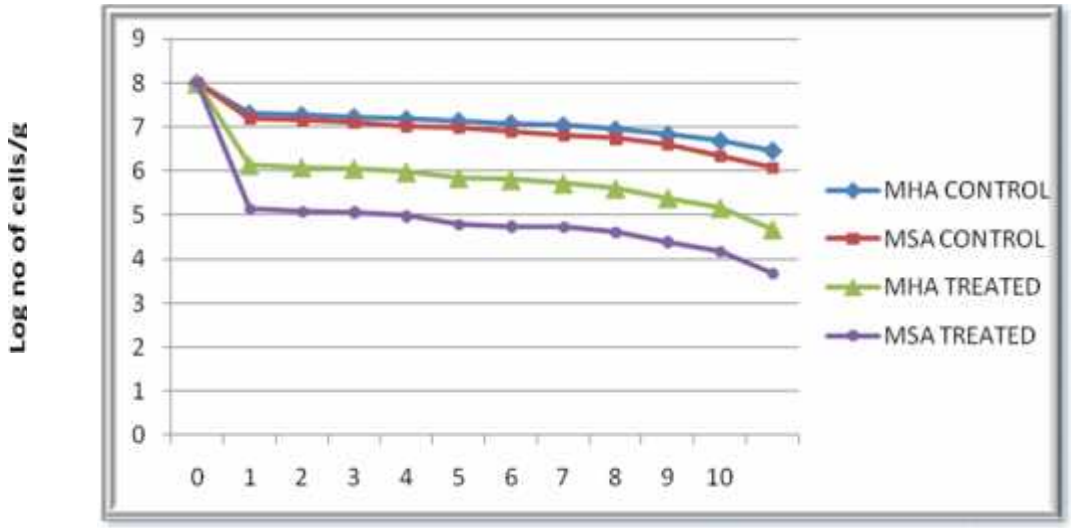


Fig 26a: Inactivation of *S. aureus* 12 in ground chicken meat exposed to 2.5% cinnamaldehyde

Secondly, treatment with the 7.5% cinnamaldehyde showed the 3.1 (3.97± 0.98) log reduction of *S. aureus* 12 cells from 7.3 (7.24±0.014) log cells within 1 day of exposure when grown in non selective culture medium (MHA) while using MSA medium (selective for *S. aureus*) no viable growth was observed within the same exposure (Fig. 26 b). The experiment was carried out for 10 days. After 10 days exposure 4.2 (2.68±0.12) log reductions was recorded in non selective medium (MHA) but no growth was recorded in selective medium.

Inactivation of *S. aureus* 12 in ground chicken meat exposed to 7.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig- 26 b.

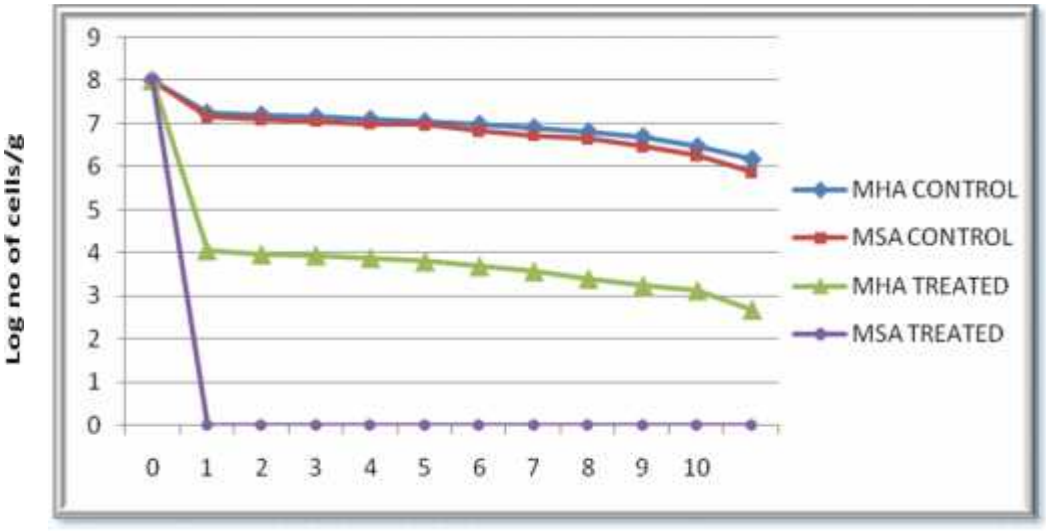


Fig 26b: Inactivation of *S. aureus* 12 in ground chicken meat exposed to 7.5% cinnamaldehyde

Thirdly, treatment with the 12.5% cinnamaldehyde showed the 4 (2.95± 0.028) log reduction of *S. aureus* 12 cells of from 7.2 (7.18±0.028) log cells within 1 day of exposure when grown in non selective culture medium (MHA) while using MSA medium (selective for *S. aureus*) no viable growth was observed within the same exposure (Fig- 26 c). The experiment was carried out for 10 days. After 10 days exposure 5.8 (1.45±0.212) log reductions was recorded in non selective medium (MHA) but no growth was recorded in selective medium. However, the MSA medium colony count was zero from the initial count and remains constant in the following days.

Inactivation of *S. aureus* 12 in ground chicken meat exposed to 12.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig- 26 c.

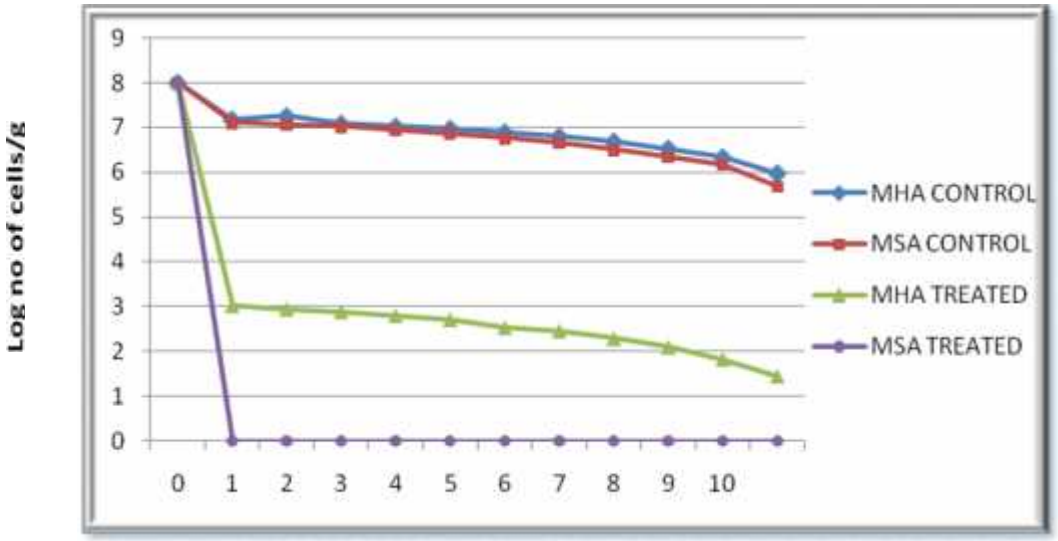


Fig 26c: Inactivation of *S. aureus* 12 in ground chicken meat exposed to 12.5% cinnamaldehyde

3.15.4. Inactivation of *S. aureus* 22 populations in inoculated ground chicken meat

Firstly, treatment with the 2.5% cinnamaldehyde showed the 1 (6.03± 0.043) log reduction of *S. aureus* 22 cells from 7.2 (7.27±0.042) log cells within 1 day of exposure when grown in non selective culture medium (MHA). After 10 days exposure 2.4 (4.75±0.212) and 3.2 (3.75±0.212) log reductions was recorded in non selective medium (MHA) and MSA medium (selective medium) respectively (Fig- 27 a).

Inactivation of *S. aureus* 22 *in* ground chicken meat exposed to 2.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig-27 a.

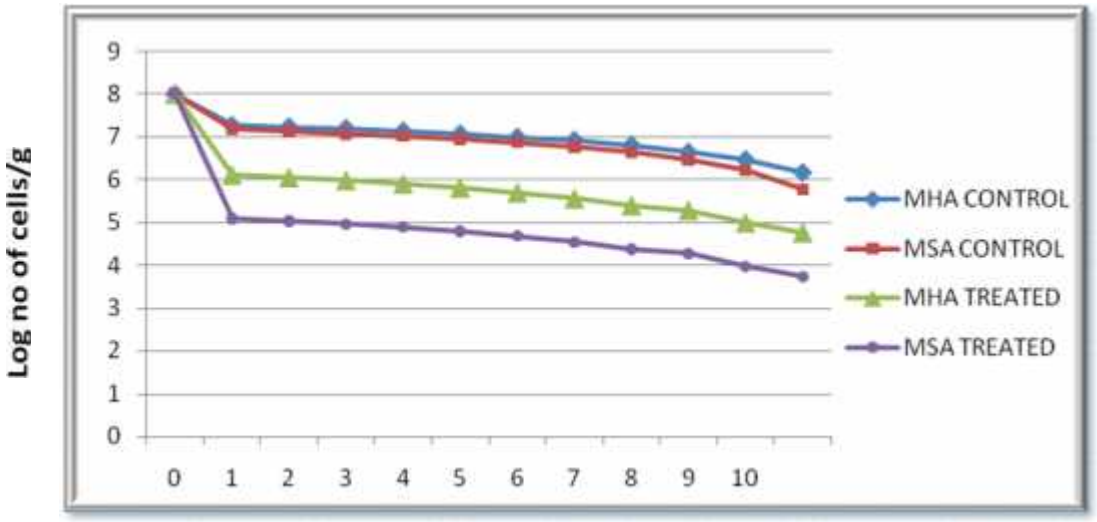


Fig 27a: Inactivation of *S. aureus* 22 in ground chicken meat exposed to 2.5% cinnamaldehyde

Secondly, treatment with the 7.5% cinnamaldehyde showed the 4 (3.92± 0.035) log reduction of *S. aureus* 22 cells of from 7.2 (7.22±0.035) log cells within 1 day of exposure when grown in non selective culture medium (MHA) while using MSA medium (selective for *S. aureus*) no viable growth was observed within the same exposure (Fig. 27 b). The experiment was carried out for 10 days. After 10 days exposure 4.8 (2.53 ±0.332) log reductions was recorded in non selective medium (MHA) but no growth was recorded in selective medium.

Inactivation of *S. aureus* 22 in ground chicken meat exposed to 7.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig- 27 b.

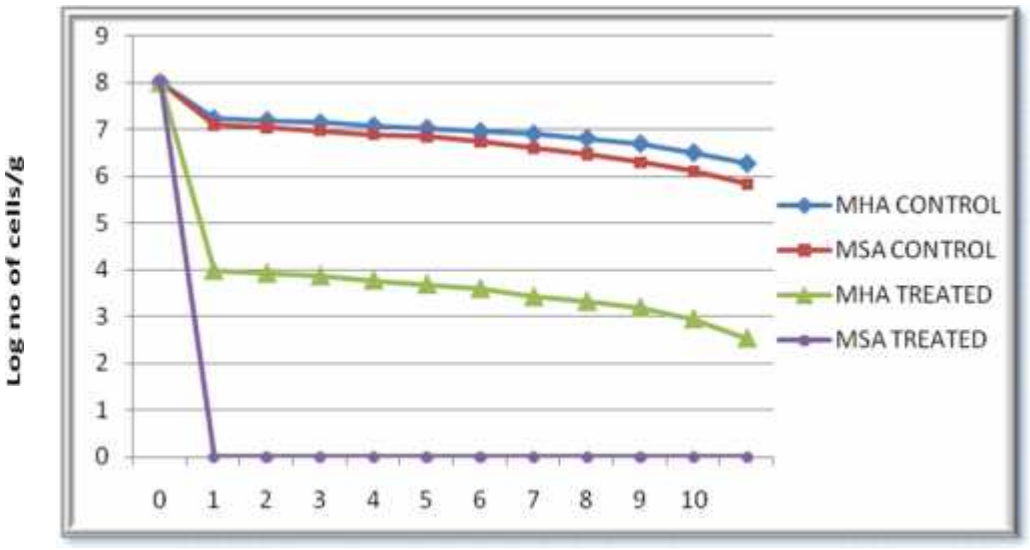


Fig 27b: Inactivation of *S. aureus* 22 in ground chicken meat exposed to 7.5% cinnamaldehyde

Thirdly, treatment with the 12.5% cinnamaldehyde showed the 3.9 (2.84± 0.07) log reduction of *S. aureus* 22 cells from 7.2 (7.14±0.056) log cells within 1 day of exposure when grown in non selective culture medium (MHA) while using MSA medium (selective for *S. aureus*) no viable growth was observed within the same exposure (Fig. 27 c). The experiment was carried out for 10 days. After 10 days exposure 5.9 (1.45±0.212) log reductions was recorded in non selective medium (MHA) but no growth was recorded in selective medium. However, the MSA medium colony count was zero from the initial count and remains constant in the following days.

Inactivation of *S. aureus* 22 in ground chicken meat exposed to 12.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig- 27 c.

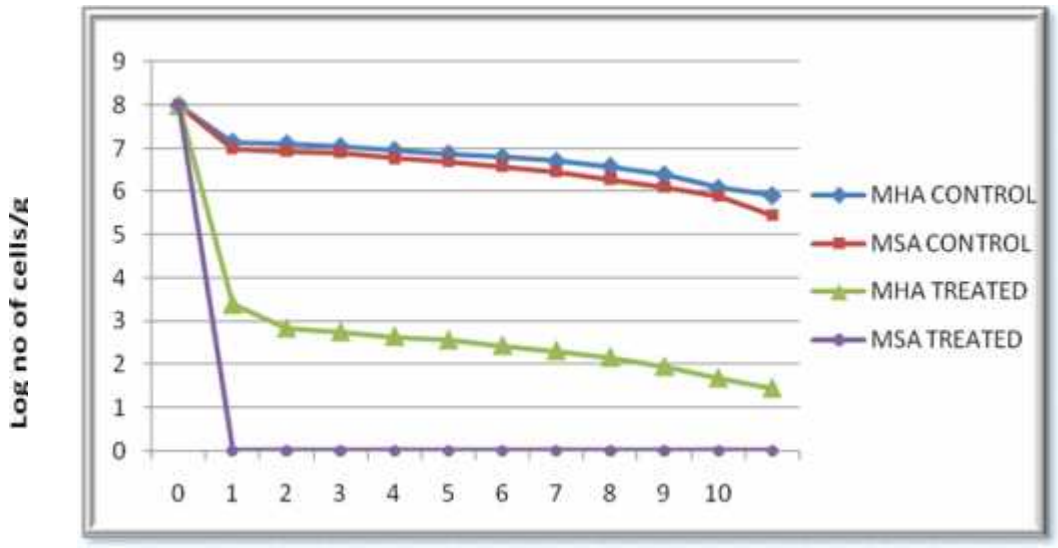


Fig 27c: Inactivation of *S. aureus* 22 in ground chicken meat exposed to 12.5% cinnamaldehyde

3.16. Inactivation of *E. coli* (O1+ 07 +17) populations in inoculated ground chicken meat

Firstly, treatment with the 2.5% cinnamaldehyde showed the 1.2 (5.90± 0.021) log reduction of *E. coli* (O1+ 07 +17) cells from 7.2 (7.18±0.021) log cells within 1 day of exposure when grown in non selective culture medium (MHA). After 10 days exposure 2 (5.10±0.049) and 3 (4.10±0.049) log reductions was recorded in non selective medium (MHA) and SMA medium (selective medium) respectively (Fig- 28 a).

Inactivation of *E. coli* (O1+ 07 +17) *in* ground chicken meat exposed to 2.5% cinnamaldehyde and stored at -18°C C for 10 days. The results are shown in Fig-28 a.

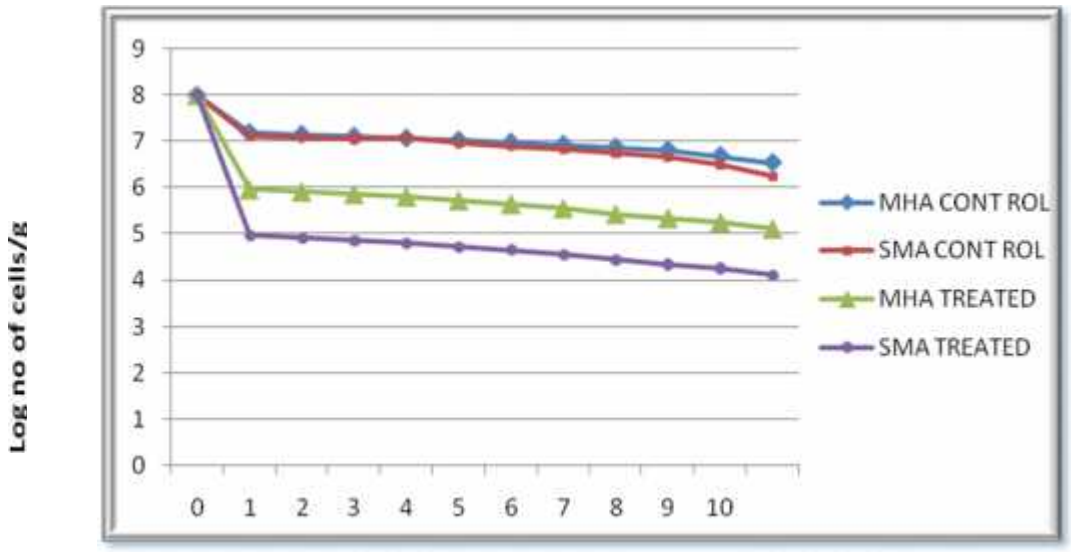


Fig 28a: Inactivation of *E. coli* (O1+ 07 +17) in ground chicken meat exposed to 2.5% cinnamaldehyde

Secondly, treatment with the 7.5% cinnamaldehyde showed the 3.2 (3.87± 0.056) log reduction of *E. coli* (O1+ 07 +17) cells from 7.2 (7.12±0.028) log cells within 1 day of exposure when grown in non selective culture medium (MHA) while using SMA medium (selective for *E. coli)* no viable growth was observed within the same exposure (Fig. 28 b). The experiment was carried out for 10 days. After 10 days of exposure 4 (3.02±0.169) log reductions was recorded in non selective medium (MHA) but no growth was recorded in selective medium.

Inactivation of *E. coli* (O1+ 07 +17)) in ground chicken meat exposed to 7.5% cinnamaldehyde and stored at -18°C C for 10 days. The results are shown in Fig-28 b.

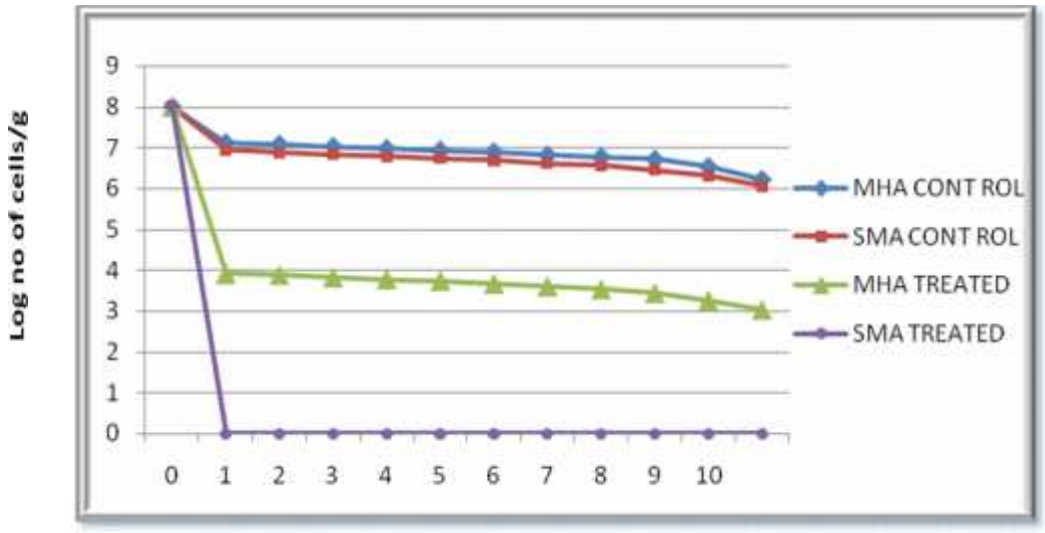


Fig 28b: Inactivation of *E. coli* (O1+ 07 +17) in ground chicken meat exposed to 7.5% cinnamaldehyde

Thirdly, treatment with the 12.5% cinnamaldehyde showed the 4.5 (2.59± 0.012) log reduction of *E. coli* (O1+ 07 +17) cells from 7.3 (7.44±0.572) log cells within 1 day of exposure when grown in non selective culture medium (MHA) while using SMA medium (selective for *E. coli*) no viable growth was observed within the same exposure (Fig. 28 c). The experiment was carried out for 10 days. After 10 days exposure 5.8 (1.5±0.282) log reductions was recorded in non selective medium (MHA) but no growth was recorded in selective medium. However, the SMA medium colony count was zero from the initial count and remains constant in the following days.

Inactivation of *E. coli* (O1+ 07 +17) in ground chicken meat exposed to 12.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig- 28 c.

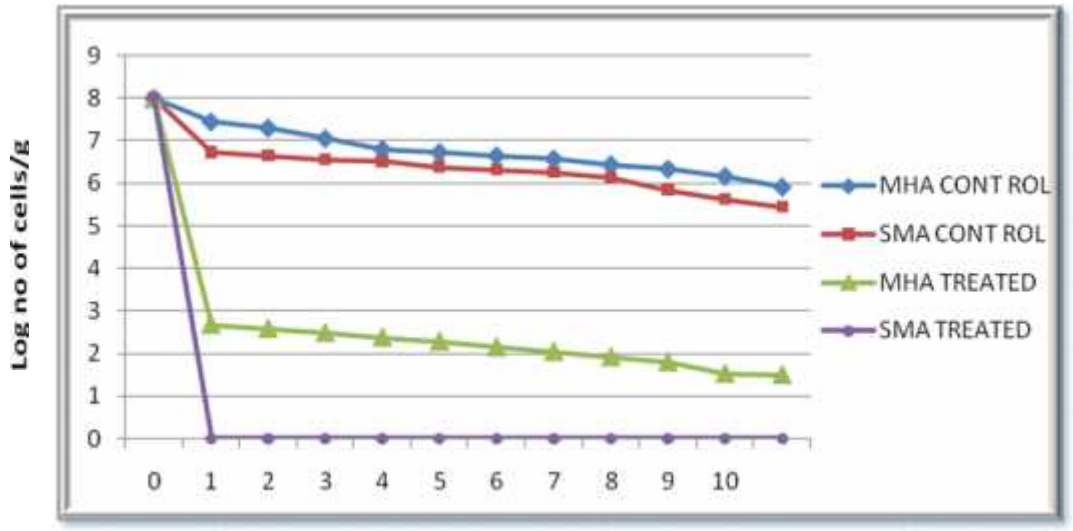


Fig 28c: Inactivation of *E. coli* (O1+ 07 +17) in ground chicken meat exposed to 12.5% cinnamaldehyde

3.17. Inactivation of *Bacillus cereus* (01+02+ 06+ 10+ 12) populations in inoculated ground chicken meat:

Firstly, treatment with the 2.5% cinnamaldehyde showed the 1 (5.97± 0.035) log reduction of *Bacillus cereus* (01+02+ 06+ 10+ 12) cells from 7.2 (7.2± 0.042) log cells within 1 day of exposure when grown in non selective culture medium (MHA) while using MM medium (selective for *Bacillus cereus)* showed the 2.2 (4.97±0.035) log reduction of cells per gram (Fig 29 a). The experiment was carried out for 10 days.

Inactivation of *Bacillus cereus* (01+02+ 06+ 10+ 12) in ground chicken meat exposed to 2.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig- 29 a.

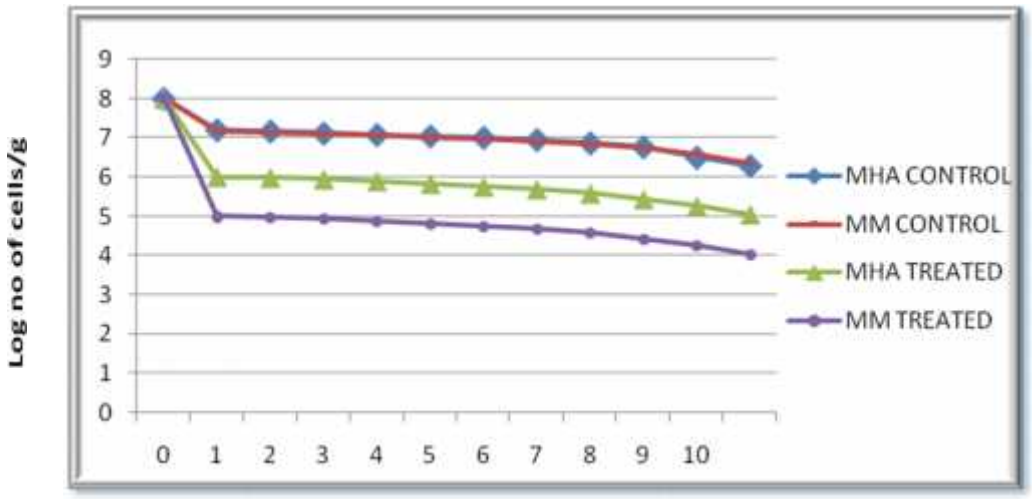


Fig 29a: Inactivation of *Bacillus cereus* (01+02+ 06+ 10+ 12) in ground chicken meat exposed to 2.5% cinnamaldehyde

Secondly, treatment with the 7.5% cinnamaldehyde showed the 3.3 (3.91± 0.014) log reduction of *Bacillus cereus* (01+02+ 06+ 10+ 12) cells from 7.2 (7.10± 0.049) log cells within 1 day of exposure when grown in non selective culture medium (MHA) while using MM medium (selective for *Bacillus cereus*) no viable growth was observed within the same exposure (Fig. 29b). The experiment was carried out for 10 days. After 10 days exposure 4.2 (2.92±0.212) log reductions was recorded in non selective medium (MHA) but no growth was recorded in selective medium. The *Bacillus cereus* (01+02+ 06+ 10+ 12) cells obtained in non selective medium might be the injured ones that could not grow in selective medium.

Inactivation of *Bacillus cereus* (01+02+ 06+ 10+ 12) in ground chicken meat exposed to 7.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig- 29 b.

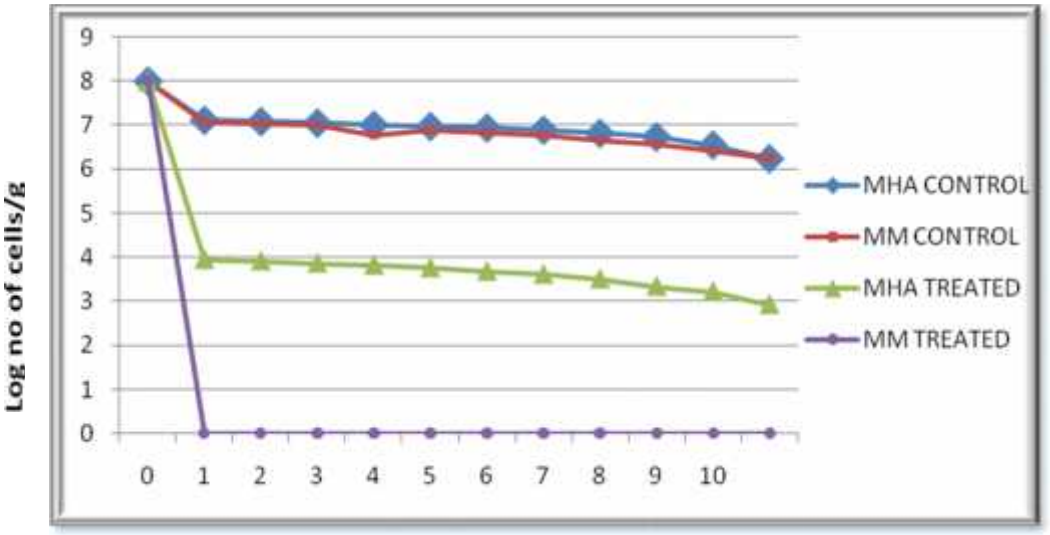


Fig 29b: Inactivation of *Bacillus cereus* (01+02+ 06+ 10+ 12) in ground chicken meat exposed to 7.5% cinnamaldehyde

Thirdly, treatment with the 12.5% cinnamaldehyde showed the 4.2 (2.78± 0.021) log reduction of *Bacillus cereus* (01+02+ 06+ 10+ 12) cells from 7.2 (7.01 ± 0.014) log cells within 1 day of exposure when grown in non selective culture medium (MHA) while using MM medium (selective for *Bacillus cereus*) no viable growth was observed within the same exposure (Fig. 29c). The experiment was carried out for 10 days. After 10 days exposure 5.1 (1.98±0.12) log reductions was recorded in non selective medium (MHA) but no growth was recorded in selective medium. The *Bacillus cereus* (01+02+ 06+ 10+ 12) cells obtained in non selective medium might be the injured ones that could not grow in selective medium.

Inactivation of *Bacillus cereus* (01+02+ 06+ 10+ 12) in ground chicken meat exposed to 12.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig-29 c.

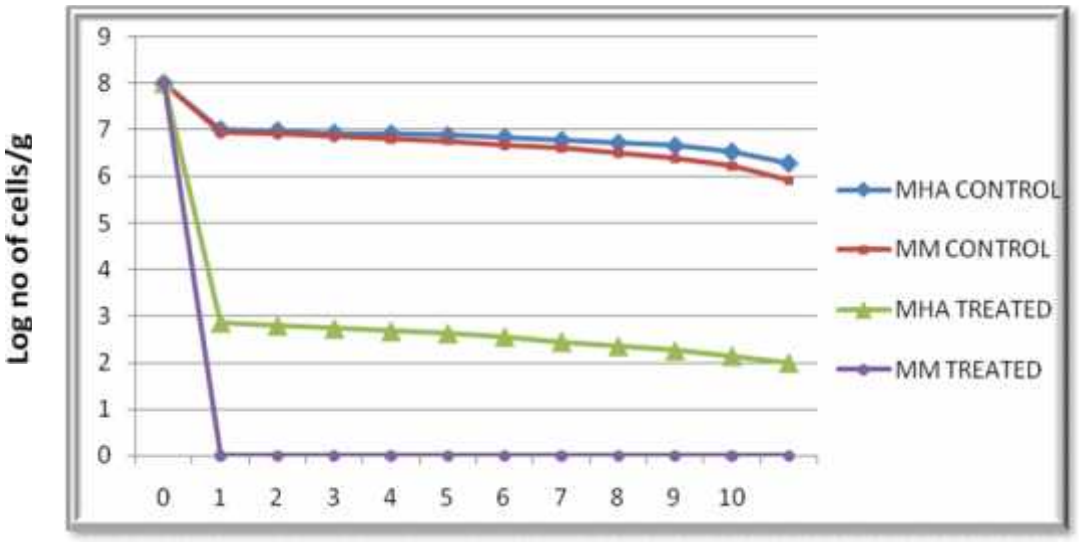


Fig 29c: Inactivation of *Bacillus cereus* (01+02+ 06+ 10+ 12) in ground chicken meat exposed to 12.5% cinnamaldehyde

3.18. Inactivation of *S. aureus* (09+05+22+08+12) populations in inoculated ground chicken meat

Firstly, treatment with the 2.5% cinnamaldehyde showed the 1 (5.99± 0.014) log reduction of *S. aureus* (09+05+22+08+12) cells from 7.2 (7.21±0.021) log cells within 1 day of exposure when grown in non selective culture medium (MHA). After 10 days exposure 2 (5.07±0.098) and 3 (4.07±0.098) log reductions was recorded in non selective medium (MHA) and MSA medium (selective medium) respectively (Fig. 30 a).

Inactivation of *S. aureus* (09+05+22+08+12) in ground chicken meat exposed to 2.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig-30 a.

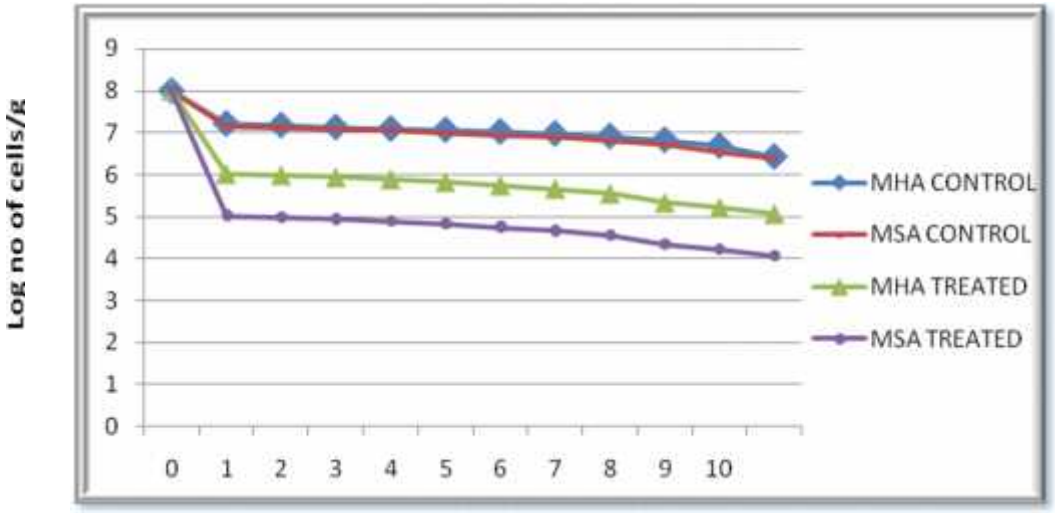


Fig 30a: Inactivation of *S. aureus* (09+05+22+08+12) in ground chicken meat exposed to 2.5% cinnamaldehyde

Secondly, treatment with the 7.5% cinnamaldehyde showed the 3.4 (3.92± 0.035) log reduction of *S. aureus* (09+05+22+08+12) cells from 7.2 (7.15±0.028) log cells within 1 day of exposure when grown in non selective culture medium (MHA) while using MSA medium (selective for *S. aureus*) no viable growth was observed within the same exposure (Fig. 30 b). The experiment was carried out for 10 days. After 10 days exposure 4 (3.02±0.169) log reductions was recorded in non selective medium (MHA) but no growth was recorded in selective medium.

Inactivation of *S. aureus* (09+05+22+08+12) in ground chicken meat exposed to 7.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig-30 b.

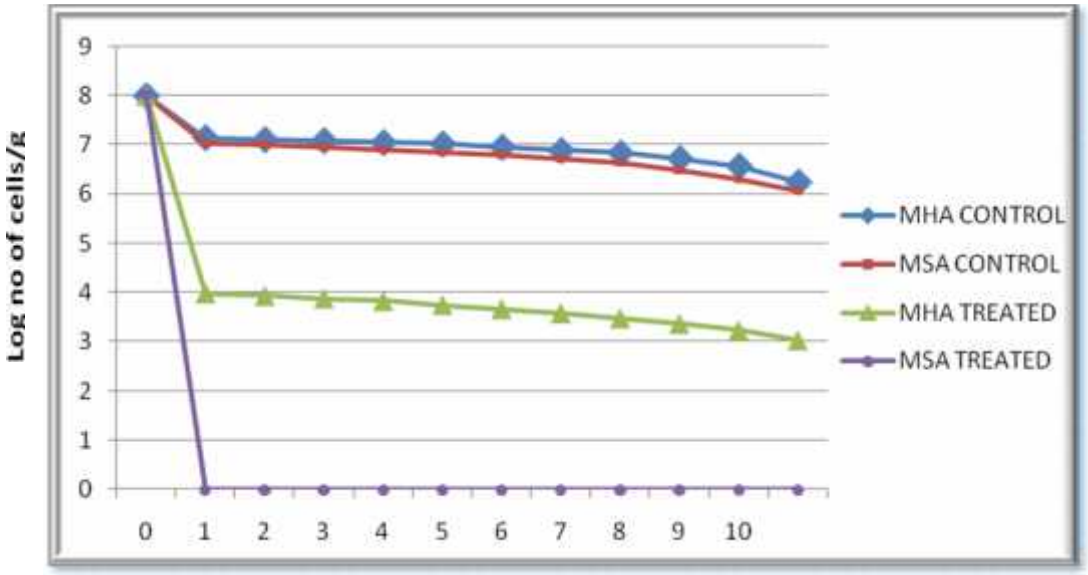


Fig 30b: Inactivation of *S. aureus* (09+05+22+08+12) in ground chicken meat exposed to 7.5% cinnamaldehyde

Thirdly, treatment with the 12.5% cinnamaldehyde showed the 4.1 (2.82± 0.042) log reduction of *S. aureus* (09+05+22+08+12) cells from 7.2 (7.06±0.035) log cells within 1 day of exposure when grown in non selective culture medium (MHA) while using MSA medium (selective for *S. aureus*) no viable growth was observed within the same exposure (Fig.30 c). The experiment was carried out for 10 days. After 10 days exposure 5 (1.98±0.12) log reductions was recorded in non selective medium (MHA) but no growth was recorded in selective medium. However, the MSA medium colony count was zero from the initial count and remains constant in the following days.

Inactivation of *S. aureus* (09+05+22+08+12)) in ground chicken meat exposed to 12.5% cinnamaldehyde and stored at -18°C for 10 days. The results are shown in Fig-30 c.

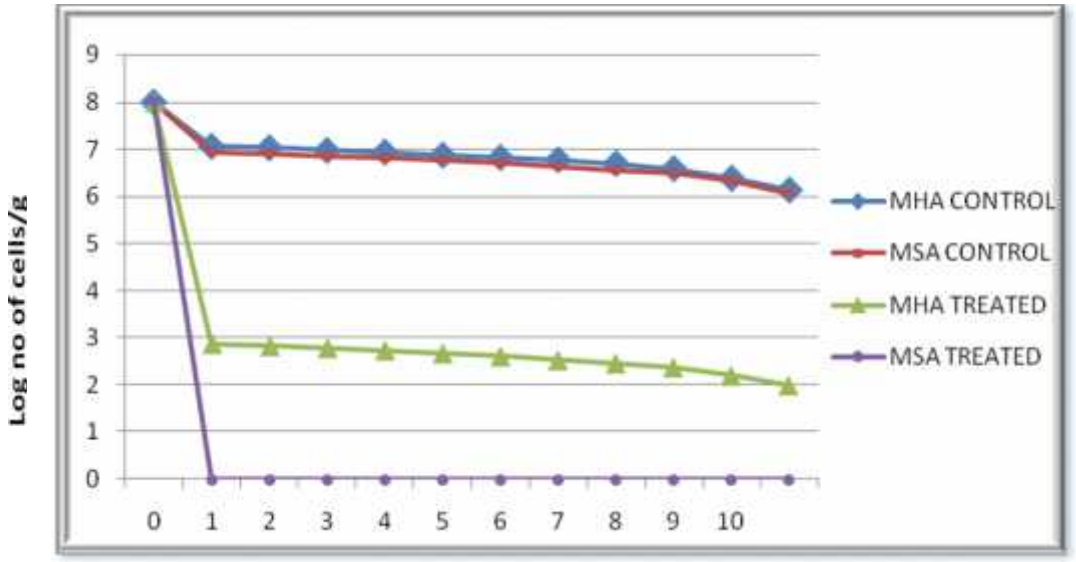


Fig 30c: Inactivation of *S. aureus* (09+05+22+08+12) in ground chicken meat exposed to 12.5% cinnamaldehyde

In this study both non selective and selective media was used for the enumeration of bacteria on treated and untreated meat. Regardless of meat condition or treatments higher population of bacteria were recovered on non selective medium than selective one. So the cells grown on the MHA might be the injured that does not possess health hazard.

Chapter Four

Discussion

4.1. **Discussion**

There is currently a great interest in antibacterial compounds naturally present in foods and food ingredients (Beuchat and Golden, 1989). It has been known since ancient times that spices and their essential oils have varying degrees of antimicrobial activity (Shelef, 1983; Zaika, 1988; Beuchat and Golden, 1989; Ting and Deibel, 1992; Juven, *et al*., 1994; Tassou, *et al*., 1995; Chang, 1995; Sivropoulou, *et al*., 1996; Wan*, et al*., 1998; Lachowicz, *et al*., 1998).Plant essential oils and extracts have been used for thousands of years (Jones, 1996) in food preservation, pharmaceuticals, alternative medicine and natural therapies (Reynolds, 1996 and Balchin, 1997). Essential oils are potential source of novel antimicrobial compounds (Mitscher, 1987) especially against bacterial pathogens. Essential oils have been previously reviewed and classified as strong, medium or weak antimicrobials (Zaika, 1988). Thymol, cinnamon oil and carvacrol have previously been demonstrated as broad spectrum antimicrobials (Nirdiry 1998; Stammati, *et al*., 1999). Most recently, Friedman, *et al*., (2002) examined 96 essential oils and 23 oil compounds for their antimicrobial activity. They found that cinnamaldehyde, thymol, carvacrol and eugenol were most active against *Escherichia coli, Salmonella enterica* and *Liseteria. Monocytogens* (Friedman, *et al*., 2002).

The present investigation can be classified into four major parts. The first part is the isolation and identification of *E*. *coli*, *S*. *aureus* and *B*. *cereus* from fast food samples collected from various part of Dhaka city of Bangladesh. This was done following the the criteria put forth in Bergey's manual of Determinative Bacteriology, 9 th edition (John, 1994). The second part is the detection of antibacterial activity of cinnamaldehyde against three food borne pathogens by the disc diffusion method (Bauer, *et al*., 1966).The third part the detection of antibacterial activity of cinnamaldehyde against three food borne pathogens in different temperature and pH. The fourth and final part is inactivation of isolated organism in ground chicken meat with cinnamaldehyde.

Food samples were collected from different areas of Dhaka city of Bangladesh. Samples were collected from different food shop and street food of Newmarket, Gulshan, Mirpur and Dhanmondi. About 100g of food sample was collected for each test and the samples were kept in the sterile bags and tagged with labels, indicating the place, date and time of sampling of fast foods. For isolation of *Escherichia coli, Bacillus cereus* and *Staphylococcus aureus* from food samples three types of selective media was used such as Sorbitol MacConkey agar (SMA), *Bacillus cereus* selective agar base medium supplemented with egg yolk, Polymyxin B and Mannitol salt agar (MSA), From the selective medium, the selected colonies were isolated and then sub cultured to maintain pure culture. From the collected food samples, 14 *Escherichia coli,* 09 *Bacillus cereus* and 17 *Staphylococcus aureus* were subjected to cultural, morphological, and microscopic and biochemical study for presumptive identification process.

For primary isolation, Gram staining was done and the result was Gram positive for *Bacillus cereus* and *Staphylococcus aureus* and Gram negative for *Escherichia coli* which is the most important characteristic. *Escherichia coli, Staphylococcus aureus* and *Bacillus cereus* colonies were characterized morphologically and physiologically following the directions given by the Bergey's manual of Systemic Bacteriology, 9^{th} edition (John, 1994).

Biochemical tests are also important in categorizing *Escherichia coli, Staphylococcus aureus* and *Bacillus cereus*. *Escherichia coli* are MR, Indole and Catalase positive and VP, Citrate, Urease, H_2S, Gelatin, and Oxidase negative (Table -3.7.1). All the isolates produce acid and gas from glucose and they are sorbitol positive (Table-3.7.2).
Staphylococcus aureus are positive for MR, VP and Catalase test and negative for Oxidase, Indole, Starch and Urease test (Table -3.7.3). The ability of *Staphylococcus aureus* to utilize various carbohydrates source of carbon energy can be a diagnostic value. The isolates were tested for their capability to ferment different types of carbohydrates such as lactose and sucrose and H_2S production. All isolates suspected as *Staphylococcus aureus* were found to produce acid but no gas from Lactose, Sucrose and negative response to H_2S production (Table-3.7.4). *Bacillus cereus* are

positive for VP, Nitrate, Citrate, Catalase and Gelatin hydrolysis test ; negative for MR, and Indole production (Table -3.7.5). All isolates studied showed negative response to arabinose, rhamnose, galactose and xylose and positive response to glucose and fructose but variable response to mannose (Table -3.7.6). All isolates studied showed positive response to maltose and starch, and negative response to raffinose and mannitol but variable response to lactose and sucrose (Table -3.7.7).

Antibiotic sensitivity pattern is an important criterion in identifying the isolates. Isolated *Escherichia coli* were studied for their sensitivity to 16 antibiotics at concentration selected to be of diagnostic value. All the isolates of *Escherichia coli* were sensitive to Amikacin (30µg), Gentamycin (10µg), Imipenem (10µg), Meropenem (10µg) and Netilmycin(30µg). All the isolates were resistant to Nalidixic acid (30µg). Except for Ec-12 all isolates displayed sensitivity to Nitrofurantoin (30µg). As for the rest of the antibiotics, the observed sensitivity or resistance pattern was mixed (Table -3.8.1).
Isolated *Staphylococcus aureus* were studied for their sensitivity to 14 antibiotics at concentration selected to be of diagnostic value. All the isolates of *Staphylococcus aureus* were sensitive to Amikacin (30µg) and Ciprofloxacin (5µg). All the isolates were resistant to Amoxyclave (30µg), Ceftriaxzone(30 µg), Aztreonam(30µg), Cefixme (5µg), and Penicillin (10µg). Except for Sa-22 all isolates displayed resistant to Cloxacillin (5µg). Most of the isolates were totally resistant to Cotrimoxazole (25µg) except Sa-04, Sa-01, which showed zones of inhibition. As for the rest of the antibiotics, the observed sensitivity or resistance pattern was mixed (Table -3.8.2.). Isolated *Bacillus cereus* was studied for their sensitivity to 15 antibiotics at concentration selected to be of diagnostic value. All the isolates of *Bacillus cereus* were sensitive to Amikacin (30µg) and Gentamycin (10µg), Netilmycin (30µg), Imipenem (10µg) and Erythromycin (15µg). All the isolates were resistant to Penicillin (10µg), Cefixime (5µg), and Cefotaxime (30 µg). Most of the isolates were totally resistant to Cotrimoxazole (25µg) except Bc-07, Bc-01, which showed zones of inhibition. As for the rest of the antibiotics, the observed sensitivity or resistance pattern was mixed (Table -3.8.3.).

In this study the antibacterial activity of cinnamaldehyde, carvacrol and euginol was determined by *E. coli*, *S. aureus* and *B. cereus* by the disc diffusion method (Bauer, *et al*., 1966). The results presented in Table 3.9.1, 3.9.2 and 3.9.3 showed that the oils under investigation exhibited marked antibacterial activity against all the test organisms. The findings of cinnamaldehyde correlated with the findings of Hoque, *et al*., (2008) who applied essential oils of cloves and cinnamon against food borne pathogens and spoilage bacteria. The essential oils under this study consist of phenolic components, which render them effective against the tested microorganisms. This was confirmed by Farag, *et al*., (1989).

The diameters of the zones of inhibition of cinnamaldehyde obtained against by disc diffusion method were compared to those obtained against commonly used antibiotic Imipenem (10 µg/disc) which was used as standard (Table 3.9.1, 3.9.2 and 3.9.3). Cinnamaldehyde showed maximum inhibition for *S. aureus* (39 mm) and minimum inhibition for *E. coli* (28.5) with zones of inhibition larger than those observed against the Imipenem (10 µg/disc). Gram positive and Gram negative bacteria were found to be effective but in this study there was an only Gram negative bacterium out of the three bacteria. If this study was done with several Gram negative and Gram positive bacteria, there will be possibility of finding a variation between Gram positive and Gram negative bacteria which was found by Hoque, *et al*., (2008).

The antibacterial activity of cinnamaldehyde was found at all temperature employed (25°C,37°C,50°C,75 °C and 100°C) suggesting that cinnamaldehyde activity was not inactivated at high temperature even at 100°C for 30 min treatment (Table-3.11.1). An insignificant decrease in activity of cinnamaldehyde was observed at 50°C after that the activity was slightly increased. This might be due to partial exhaustion of solvent (ethanol) in oils at high temperature above 60°C.Similar type of findings was reported by Hoque, *et al*., (2008).

In normal temperature, cinnamaldehyde inhibit cell separation that is this essential oil interferes with the cell division of the microorganisms. Cinnamaldehyde is a natural product from spices that inhibits cell separation in *Bacillus cereus*. Cell division is

regulated by FtsZ, a prokaryotic homolog of tubulin. FtsZ assembles into Z ring at the site of cell division. Cinnamaldehyde decreases the *in vitro* assembly reaction and binding of FtsZ. It is found that cinnamaldehyde perturbs the Z ring morphology *in vivo* and reduces the frequency of the Z ring per unit cell length of *E.coli*. In addition, GTP dependent FtsZ polymerization is inhibited by cinnamaldehyde. On the other hand, the possibility that the mechanism of cinnamaldehyde activity involves inhibition of cell wall synthesis (*B. cereus*) (Kwon, *et al*., 2003) or inhibition of biosynthesis of enzymes histidine decarboxylase (Wendakoon, *et al*., 1995) is unlikely because of the rapidity of ATP inhibition or depletion.

In contrast, starting at 60°C, pure cinnamaldehyde undergoes a temperature dependent transformation to benzaldehyde and glyoxal under the influence of heat (Friedman*, et al*., 2000).Benzaldehyde has the antimicrobial activity which hampers the membrane integrity of the microorganisms .Effective antibacterial activity this compound at 100°C suggested that these compound may be used as potential preservatives for the foods processed at high temperature.

The antibacterial activity was not affected at pH 5.0, though a significant decrease of inhibitory effect was found. The pH 7.0 and 9.0 favored the antibacterial activity of cinnamaldehyde against most of the tested organisms, where as the highest activity of cinnamaldehyde against most of the tested organisms were found at pH 7.0 (Table-3.12.1.).This agreed the result of Hoque, *et al*., (2008), they found higher activity of cinnamon oil against cocktail of *S. aureus* at pH 7.0 compared to pH 5.0 and 9.0 and disagreed with the Thompson (1990). At high pH cinnamaldehyde will not dissociate rather its hydrophobicity increase. Due to this hydrophobic nature, they interact with the cell wall increasing cellular permeability and finally loss of cellular constitution.
MIC of cinnamaldehyde was determined by the broth dilution method at 37°C and pH 7. MIC values of cinnamaldehyde against the test bacteria ranged from 2.5 to 10 % (Table- 3.10.2).

Cinnamaldehyde is a plant extract and it has a very good antibacterial activity. It shows significant MIC level against *E. coli*, *S*. *aureus and B. cereus*. Normally this bacterium inhabits the food and cause serious food borne infection. This experiment is to challenge the bacteria to grow in food against cinnamaldehyde. If it is possible for the extract to inhibit the bacteria in food than it will give a good finding of the application of cinnamaldehyde against food borne pathogens.

Application of cinnamaldehyde was experimentally done to inhibit the bacteria *E. coli*, *B. cereus* and *S. aureus* in ground chicken meat individually and combined. Firstly, chicken meat was grinded and fat was removed because bacteria can grow heavily in presence of fat. Then meat was treated with 95% ethanol to inhibit the all other organism present in the meat. After alcohol treatment appropriate amount of test organism (10^7 CFU/g) were applied in the meat to grow and cinnamaldehyde were used to evaluate the inhibition of the test organism. Three types of concentration are MIC level (2.5%), 3 times of MIC (7.5%) and 5 times of MIC (12.5%). Four types of media were used to evaluate the growth. One is non selective (MHA) and another is selective (MSA, SMA and MM) for every concentration one control for each media was evaluated. Ground chicken meat was inoculated with each of 10^7 CFU/ml of *E*. *coli*, *S*. *aureus* and *B*. *cereus* and the inoculated meat samples were challenged by MIC level, 3% MIC level and 5% MIC level of cinnamaldehyde. After treatment with MIC, 3% and 5% levels of cinnamaldehyde against *E*. coli in ground chicken meat, the aliquot from the samples were inoculated in MHA and MSA, SMA and MM media together with control samples.

From the Figure 13 a to 27 c, it was apparent that only a few log reduction of *E*. *coli* (Figure 13 a to 15 a)*, B*. *cereus* (Figure 17 a to 21 a) and *S*. *aureus* (Figure 23 a to 27 a), was found in selective media used in case of MIC level of cinnamaldehyde (2.5%). 3 times (7.5%) and 5 times (12.5%) MIC level of cinnamaldehyde employed resulted 7 log reduction of *E*. *coli* (Figure 13 b and c to 15 b and c), *B*. *cereus* (Figure 17 b and c to 21 b and c) and *S*. *aureus* (Figure 23 b and c to 27 b and c), respectively, growing on selective media after 24 hours of exposure. In case of non- selective culture medium (MHA), 1 to 2.5 log survivals of *E*. *coli, B*. *cereus* and *S*. *aureus* were recorded, as this

was due resuscitation of wound cells. In case of positive control one or two log reductions were recorded, this was due to cold effect on the test organisms employed.

From the experimental findings, it was found that there is no significant difference between 3 times and 5 times MIC level of cinnamaldehyde when applied on ground chicken meat against *E. coli*, *B. cereus* and *S. aureus*. So, it is wise to consider 3 times MIC level rather than 5 times MIC level of cinnamaldehyde against those pathogens studied in ground chicken meat.

But for application as preservative in food five fold concentration of the MIC value of the respective antimicrobial agents are used as the antibacterial activity is decreased when they are added to food materials containing protein, carbohydrate and fat. Therefore, antimicrobial agent with lower MIC value is expected as preservative which will not affect the color, flavor, taste and other parameter of food. In this context cinnamaldehyde is a more potential agent. However, the component of the food may affect the activity of the antimicrobial compound either antagonistically or synergistically. As a result, antibacterial activities of the cinnamaldehyde against food spoilage and /or pathogenic bacteria are an important finding. These findings may be applied in controlling organisms in chicken and meat based products.

Chapter Five

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Appendices

Appendix A

Mueller-Hinton Agar

Ingredients	Grams per litre
Meat infusion	6.0
Casein hydrolysate	17.5
Starch	1.5
Agar	10.0
p^H	7.4±0.2

Mueller-Hinton Broth

Ingredient	Grams per litre
Beef infusion	300
Casein hydrolysate	17.5
Starch	1.5
p^H	7.4±0.2

Eosin Methylene Blue Agar

Ingredients	Grams per litre
Peptone	10.0
Lactose	10.0
Dipotassium hydrogen phosphate	2.0
Eosin- Y	0.4
Methylene blue	0.065
Agar	15
Distilled water	1000ml
p^H	6.8±0.2

Bacillus cereus Agar Base

Ingredients	Grams per litre
Peptone	1.0
Mannitol	10.0
Sodium chloride	2.0
Magnesium sulphate	0.1
Disodium hydrogen phosphate	2.5
Potassium dihydrogen phosphate	0.25
Bromothymol blue	0.12
Sodium Pyruvate	10.0
Agar	14.0
p^H	7.2±0.2

Supplements

Egg Yolk Emulsion 100 ml

Polymyxin B (50000 IU) 5ml

MacConkey Agar

Ingredients	Grams per litre
Peptone	20.0
Lactose	10.0
Bile salts	5.0
Sodium chloride	5.0
Neutral red	0.075
Agar	12.0
p^H	7.4±0.2

Mannitol Salt Agar

Ingredients	Grams per litre
'Lab-Lemco' Powder	1.0
Peptone	10.0
Mannitol	10.0
Sodium chloride	75.0
Phenol red	0.025
Agar	15.0
p^H	7.5±0.2

Sorbitol MacConkey Agar

Ingredients	Grams per litre
Peptone	20.0
Sorbitol	10.0
Bile salt no.3	1.5
Sodium chloride	5.0
Neutral red	0.03
Crystal violet	0.001
Agar	15.0
p^H	7.1±0.2

Tryptone Soya Broth

Ingredients	Grams per litre
Pancreatic digest of casein	17.0
Enzymatic digest of soya bean	3.0
Sodium chloride	5.0
Dipotassium hydrogen phosphate	2.5
Glucose	2.5
pH	7.3 ± 0.2

Tryptone Soya Agar

Ingredients	Grams per litre
Tryptone	15.0
Soya peptone	5.0
Sodium Chloride	5.0
Agar	15.0
p^H	7.3±0.2

Simmons Citrate Agar

Ingredients	Grams per litre
Magnesium sulphate	0.2
Ammonium dihydrogen phosphate	0.2
Sodium ammonium phosphate	0.8
Sodium citrate,tribasic	2.0
Sodium chloride	5.0
Bromothymol blue	0.08
Agar	15.0
p^H	7.0±0.2

Nutrient Agar

Ingredients	Grams per litre
'Lab- Lemco' Powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
Agar	15.0
p^H	7.4±0.2

Strach Agar

Ingredients	Grams per litre
Beef extract	3.0
Soluble starch	2.0
Peptone	5.0
Agar	18.0
Dis. water	1.0
p^H	7.5 ± 0.2

Urea Broth

Ingredients	Grams per litre
Peptone	1.0
Dextrose	1.0
Disodium phosphate	1.2
Potassium dihydrogen phosphate	0.8
Sodium chloride	5.0
Phenol red	0.004
p^H	6.8 ± 0.2

Nutrient Gelatin

Ingredients	Grams per litre
'Lab- Lemco' Powder	3.0
Peptone	5.0
Gelatin	120.0
p^H	6.8 ± 0.2

Blood Agar Base

Ingredients	Grams per litre
'Lab- Lemco' Powder	10.0
Peptone	10.0
Sodium chloride	5.0
Agar	15.0
p^H	7.3 ± 0.2

For Blood agar, the base was cooled at 50°C and 7% defibrinated Sheep blood was added.

Peptone water

Ingredients	Grams per litre
Peptone	10.0
Sodium chloride	5.0
Dis. water	1000ml
p^H	7.0±0.2

Nitrate Broth

Ingredients	Grams per litre
Peptone	3.0
Potassium nitrate	0.2
Dis. water	1000ml
p^H	7.0±0.2

Nutrient Gelatin

Ingredients	Grams per litre
Beef extract	3.0
Peptone	5.0
Gelatin	04.0
Dis. water	1000ml
p^H	7.0±0.2

MR-VP medium

Ingredients	Grams per litre
Glucose	5.0
Peptone	5.0
Dipotassium hydrogen phosphate	5.0
Dis. water	1000ml
p^H	7.0±0.2

SUGAR BROTH BASE

Ingredients	Amount in g /100mL
Peptone	1.0
Meat extract	0.3
Carbohydrate	0.5
Sodium chloride	0.5
Phenol red	0.008
Dis.water	100.0
p^H	7.0±0.2

Appendix B

Composition of chemical used:

McFarland standard

Ingredients	Amount in g /100mL
1% sulfuric acid	99.5mL
1% $BACl_2$	0.5mL

Normal saline

Ingredients	Amount in g /100mL
Sodium Chloride(Table salt)	8.5g

Alcohol

Ingredients	Amount in g /100mL
Alcohol (95%)	95mL
Alcohol (70%)	70mL

Reagents for testing biochemical characteristics of bacterial strains

Kovac's reagent (bio Merieux sa, France)

Ingredients	Amount in g/L
p- Dimethylaminobenzaldehyde	5.0
Isoamyl alcohol	75.0
HCL (37%)	25.0

Reagents for Nitrate reduction test

NITI ((bio Merieux sa, France)

Ingredients	Amount in g/100mL
Sulphonic Acid	0.80
Acetic acid(5N)	100

NIT2 (bio Merieux sa, France)

Ingredients	Amount in g/100mL
N-N Dimethyl-1- napthylamine	0.60
Acetic acid	100

Catalase test reagent

Ingredients	Amount in g/100mL
Hydrogen per oxide	3.0
Dis. water	100.0

Reagents for VP test

Barritt's reagent A

Ingredients	Amount in g/100mL
a-naphtholin	6.0
95% ethyl alcohol.	100.0

Barritt's reagent B

Ingredients	Amount in g/100mL
potassium hydroxide	16.0
Dis. water	100.0

Reagents for Oxidase test

Ingredients	Amount in g/10mL
N,N-dimethyl-p-phenylenediamine hydrochloride	0.15 g
Dis. water	10.0

Solution for Gram stain

Ammonium Oxalate- Crystal Violate Solution

2.0 gm crystal violet was dissolved in 20.0 ml of 95% ethanol and 0.8g ammonium oxalate was dissolved in 80.0 ml Dis. water in separate container. Both solutions were mixed together and stored in a dropping bottle. The solution was filtered through Whatman No. 1 filter paper before use as the primary stain in the gram staining method.

Gram's Iodine Solution

The fresh solution of gram's iodine was required as a mordant in the Gram staining method. 1.0g iodine crystal and 2.0 g potassium iodine were kept in a screw –capped bottle. 300.0 ml distilled water was added to the mixture to make the fresh solution, when required.

Safranin solution

2.5 g safranin was dissolved in 10.0 ml of 95% ethanol.100.0 ml distilled water was added to the solution and stirred in a dropping bottle. This solution was filtered through Whatman filter before use as a counter stain in the Gram staining method.

Tris-HCL Buffer

The 0.2 M Tris-HCL buffer was prepared in the following way

Solution A:

24.2 g Tris was dissolved in 1000 ml distilled water.

Solution B:

0.2m HCL

50 ml of solution A and 5 ml of solution B was mixed and finally diluted to 200 ml.

Citrate Buffer

0.1 M citrate buffer was prepared in the following way

Solution A:

19.21 gm citric acid was dissolved in 1000 ml distilled water.

Solution B:

53.65 gm disodium hydrogen phosphate was dissolved in 1000 ml distilled water.

24.3 ml of solution A and 25.7 ml of solution B was mixed and finally diluted to 100 ml.

Phosphate Buffer

0.2 M Phosphate buffer was prepared in the following way

Solution A:

27.8 gm monobasic sodium hydrogen phosphate was dissolved in 1000 ml distilled water.

Solution B:

53.65 gm of disodium hydrogen phosphate was dissolved in 1000 ml distilled water.

39 ml of solution A and 61 ml of solution B was mixed and finally diluted to 200 ml.