WOLKITE UNIVERSITY

COLLEGE OF NATURAL AND COMPUTATIONAL SCIENCES

DEPARTMENT OF BIOTECHNOLOGY

SYNERGISTIC ANTIBACTERIAL ACTIVITY OF GARLIC(*Allium sativum*) AND NEEM(*Azadrichta indica*) LEAF EXTRACTS AGAINST SOME SELECTED PATHOGENIC BACTERIA AT WOLKITE UNIVERSITY

PREPARED BY:

NAME OF MEMBERS	ID
1. Amanual Endalew …….…..…..NSR/0214/13	
2. Belay Fanos……NSR/0377/13	
3. Ermiyas Wendafrew …….NSR/0774/13	
4. G/Egziabhier Birhan……..……...……NSR/ 0940/13	
5. Milkesa Tariku…….…….NSR/1522/13	
6. Sofia Kemal ……..……..……...….NSR/1925 /13	
7. Waktola Olijira…….…... NSR/ 2120/13	
8. Yohannes Endalamaw……..NSR/2239/13	
9.Yohannes Getie . ……..…… NSR/2252/13	

A Senior Research Project Submitted To Department Of Biotechnology For Partial Fulfillment Of The Requirement For The Degree Of Bachelor Science In Biotechnology

ADVISOR:MR. GETAW ABERA(MSC.) AND MR. GETU HAILU (MSC.)

SUBMITTED TO:DEPARTMENT OF BIOTECHNOLOGY

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DECLARATION

We declare that the research here by submitted to Wolkite University is our own original work, and all the sources that we have been used duly are acknowledged.

This research project entitled "Synergistic antibacterial activity of garlic and leafs of neem crud extracts against *some selected pathogenic bacteria*"is submitted by group four in the fulfillment of requirement the degree of bachler of science in biotechnology.

Submitted by :

Name of Students	**Signature**	**Date**
1.Amanual Endalew	**…….…**	
2.Belay Fanos…	**……..**	
3.Ermiyas Wendafrew	**…….**	
4.G/Egziabhier Birhan	**…...……**	
5.Milkesa Tariku	**…….…….**	
6. Sofia Kemal	**……..……**	
7. Waktola Olijira	**…….……**	
8. Yohannes Endalamaw	**…….**	
9.Yohannes Getie	**……..……**	

Approved by:

Name of Advisor	**Signature**	**Date**
1.……	**…….**	**……..**
2.……	**…….**	**…….**
Name of Examiner	**Signature**	**Date**
1. ……	**……**	**…….**
2….……	**……**	**……..**

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LIST of ABBREVIATIONS and ACRONYMS

B. cereus ……*Bacillus cereus*

CAM……complementary Alternative Medicine

MHA…… ……Mueller Hinton Agar

M.Pyogenes…..……mycobacterium pyogenes

M.tuberculosis ……...……. …mycobacterim tuberculosis

MIC……Minimum Inhibitory Concentration

Rpm…….……revolution per Minuit

S.Faecalis……...…….……*streptpcoccus Faecalis*

S.Aurasis……..……*staphylococcus aurosis*

STEC……..……*Shiga Toxin Escherichia coli*

WHO …….. ……World Health Organization

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ABSTRACT

Medicinal plants have been identified and used throughout human history. Chemical compounds in plants mediate their effect on the human body through process identical to those already will understood for the chemical compounds in conventional drugs thus herbal medicines don't differ guilty from conventional drugs in terms of how they work. This study was aimed to investigate the antibacterial properties of the mixture of Allium sativum(garlic) and Azadirachta indica (neem) against staphylococcus aureus ,bacilus cereus and Shiga Toxin E.coli . Medicinal plants were extracted with 99.9% (v/v) ethanol against tested pathogenic bacteria. MH agar disk and well diffusion method used for antibacterial susceptible tested.Antibiotic disk were used as positive control .Among the tested pathogenic bacterial both staphylococcus aureus and shiga toxin E.coli were susceptible to crude extracts. Bacillus cereus was resistance to crude extracts of both plant and individual extracts .The inhibition zone of ethanol crude extract of neem and garlic(18 mm) against Shiga toxin E.coli was significantly far greater than S.Aurasis . Natural species of neem and garlic possess effective anti-microbial activity could be used for prevention of pathogenic bacteria and further evaluation was necessary.

Keywords: *Allium sativum, Antibacterial activity, Azadirachta indica, Inhibition zone*

1. INTRODUCTION

1.1 Background of The Study

Traditional medicine serves as the most affordable and readily available form of healthcare in impoverished communities, where alternative therapies are the primary method of treatment(Yinger and Yewhalaw,2007). The effectiveness of traditional medicine among African traditional communities cannot be overstated. Over generations, indigenous societies have developed intricate social systems, and their traditional healers have gathered and preserved knowledge on the use of medicinal plants through oral tradition and empirical methods(Abel and Busia . This knowledge has been passed down from one generation to another, ensuring the continued existence of traditional and alternative therapies.

The World Health Organization defines traditional medicine as a comprehensive approach that encompasses health practices, beliefs, and knowledge, incorporating plant, animal, and mineral-based medicines, spiritual therapies, manual techniques, and exercises. These methods are employed individually or in combination to treat, diagnose, prevent illness, and maintain overall well-being. In developed nations, the use of traditional medicine is often referred to as complementary and alternative medicine (CAM). CAM, also known as non-conventional or parallel medicine, includes a wide range of healthcare practices that are not part of a country's own traditions or integrated into its primary healthcare system(WHO,2011)

Conversely, African Traditional Medicine encompasses the entirety of knowledge and practices, whether explainable or not, utilized in the diagnosis, prevention, or treatment of physical, mental, or social diseases. It relies solely on past experiences and observations that have been handed down through generations, either verbally or in written form. In many developing countries across Asia, Latin America, and Africa, including Ethiopia, traditional medicine fulfills the primary healthcare needs of the population(Fokunang *et al* ,2011)

In Ethiopia specifically, traditional medicine plays a significant role in the healthcare system, with more than 80% of the population relying on it due to cultural acceptance, relatively low cost, and limited access to modern healthcare facilities. Given that the

majority of Ethiopia's population resides in rural areas with challenging access to healthcare services, traditional medicine serves as a crucial resource(Wubet Birhan *et al* 2011).Generally, in Ethiopian traditional medicine, medicinal plants and the indigenous knowledge are useful; yet, both of them are threatened due to different anthropogenic factors, which need appropriate conservation measures, and sustainable utilization. Natural compounds isolated from various parts of the plant parts such as leaves, fruits, stem, roots, and seeds have been shown to possess excellent medicinal values. Thousands of plant varieties used in folklore medicine have been studied for treatment of various diseases such as cancer, diabetes, arthritis, and infectious diseases.(Melaku Tafese 2021).

However, it currently remains as an area of research interest for presenting the medicinal values of several plant species that are not studied thoroughly. Therefore,our conducting studies focuses on identifying and documenting the important medicinal plants and their importance, means of indigenous knowledge transfer, and conservation practices used for managing medicinal plants in the country.In various study medicinal use garlic was obviously known through out in Ethiopian people.where as medicinal use of neem plants is not widely recognized and their synergistic activity of antibacterial of these plants not give attention and un exploited widely in our country and including the society of at guragie zones.So the aim of this study to determine their antibacterial activity of the two plants and recognize and advert for our campass students and society where lived in our university.

1.2 Objectives

1.2.1 General Objectives

To determine synergistic antimicrobial activity of garlic and leaf extracts of neem against *staphylococcus aureus,bacillus cereus and shiga toxin escherechia coli*

1.2.2 Specific Objectives

Specific objectives of this study is;

- ✧ To evaluate the effects of crude extract of garlic and neem leaves against selected pathogenic bacteria
- ✧ To determine the synergistic effects of crude extracts against selected pathogenic bacteria

1.3 STATEMENT of PROBLEM

The use of traditional medicine which is extract from plants such as garlic and neem is still worldwide in under developed and developing countries for their health care needs. Most people in Ethiopia is affected in case of Economical problems especially, those people found in rural areas. Because of highly observable shortage of money and financial problems to buy the medicine and also lack of health center and far from their linked home to arrive in a short period of time in order to save their life. So this study is conducted to solve such kind of problems by using Minimum Inhibitory Concentration ,Precise determination of antimicrobial susceptibility. MIC assays provide quantitative data on the lowest concentration of an antimicrobial agent that inhibits the growth of a specific pathogen, enabling precise determination of antimicrobial susceptibility.Guidance for antibiotic selection, MIC values help clinicians select the most effective antibiotics for treating infections, optimizing patient outcomes and reducing the risk of antimicrobial resistance.

1.4 Significance of Study

Conducting this research provide significance to solve the problems facing to the society in a day to day life and can solve the following societal problems.

- ❖ To change the traditional knowledge about the treatment of pathogen disease using natural products to scientific supported knowledge.

- To determine the concentration of the mixture of garlic and neem leaves used as medicine and help the society to use correct concentration which is effective to the microbial infections and less distress our body.
- To avoid the over dose of traditional medicine of mixture of garlic and leaves neem used by local healers without scientific measurement.
- Finally, to solve the societal problems by getting more viability of medicine in their near clinics with cost effective manner.

1.5 Scope of the Study

The study was conducted at wolkite University particularly in Biotechnology department and mainly focused on antibacterial activity of garlic(*Alliums ativum*)and neem (*Azadirachta indica*) in combination for the treatment of different pathogenic bacterial species that may cause a severe illness for human beings. Currently, there are so many bacteria that have the ability to resist the antibiotic drugs but by using the natural medicinal plants they may be inhibited.

1.6 Limitation of the Study

In this study the researchers have been faced for several problems.Antibiotic disks used as a positive control were expired. Availability of the necessary media, paper disk and time were the major problems in the finding of this project which make the researchers not conduct further lab sessions to identify different types of pathogenic bacteria which may be present in the sample.

2 LITERATURE REVIEW

2.2. Traditional Medicinal Plants as Potential Sources of Antibiotics

The prevalence of plants in current healthcare practices should not come as a surprise, especially when one considers their predominance in ancient texts and in paleobotanical findings at archaeological sites. Many of these ancient medical practices persist in various forms of traditional medicine currently practised across the globe. Plants are capable of producing a vast array of structurally diverse compounds, each of which serves a specific role for the plant itself.Sometimes, these compounds are also active against human pathogens. There are four major groups of antimicrobial compounds made by plants: phenolics and polyphenols, terpenoids and essential oils, lectins and polypeptides, and alkaloids . In most cases, bioactive plant extracts contain complex mixtures of these groups, and their combined action can yield an enhanced effect(Abdallah,*et al*,2023). These compounds act on bacteria via a number of mechanisms, including inactivation of proteins, adhesins and enzymes, among other targets. More recent work has revealed that certain plant compounds can also block cell-to-cell signaling pathways and quench production of virulence factors and disrupt or inhibit the formation of biofilms which confer a protective advantage to pathogens during an infection . It is clear that we have only uncovered the tip of the iceberg in our understanding of the chemical diversity and bio activity of plant natural products.

2.3. Extraction Methods of Medicinal Plants

Alcohol is **t**he better activity of the ethanolic extracts compared to the aqueous extract can be attributed to the presence of higher amounts of polyphenols in comparison to aqueous extracts. It manner that they're greater efficient in cell walls and seeds degradation which have nonpolar character and cause polyphenols to be released from cells. More beneficial reason behind the decrease in activity of aqueous extract may be ascribed to the enzyme polyphenol oxidase, which degrade polyphenols in water extracts, while in methanol and ethanol they may be inactive(Milan Hait ,2019). Moreover, water is a better medium for the occurrence of the micro-organisms compared to ethanol . The higher concentrations of greater bioactive flavonoid compounds have been detected with ethanol 70% due to its higher

polarity than pure ethanol. By including water to the pure ethanol up to 30% for making ethanol 70% the polarity of solvent was elevated (Bimakr, 2010). Additionally, ethanol was found easier to penetrate the cell membrane to extract the intracellular ingredients from the plant material(Wang, 2010)

2.4. Antibiotic Resistance

The beginning of the era of antibiotics was one of the most important events and a turning point in the history of medicine.Antibiotics are powerful drugs that are used to combat once fatal diseases. As with any powerful medication, antibiotics carry a wide range of adverse effects. antibiotics disrupt the composition of the infectious agent, leading to bacterial adaptation or mutations, and in turn, to new strains that are resistant to the current antibiotic regimen.Antibiotic resistance, loss of susceptibility of bacteria to the killing or growth-inhibiting properties of an antibiotice agent. When a resistant strain of bacteria is the dominant strain in an infection, the infection may be untreatable and life-threatening. Antimicrobial resistance is natural phenomenon that occurs when microorganisms are exposed to antibiotic drugs. Under the selective pressure of antibiotics, susceptible bacteria are killed or inhibited, while bacteria that are naturally (or intrinsically) resistant or that have acquired antibiotic-resistant traits have a greater chance to survive and multiply (Prestinaci *et al* 2015).

Resistance can be defined as the temporary or permanent ability of an organism and its progeny to remain viable or multiply under environmental conditions that would destroy or inhibit them (Denyer *et al* 2005). Antimicrobial resistance makes it harder to eliminate infections from the body as existing drugs become less effective.The importance and value of antibiotics cannot be overestimated; we are totally dependent on them for treatment of infectious diseases and they should never be considered mere commodities..

2.5. Medicinal Plants and Its Uses

Medicinal plants are very vital in their uses for medication,The world primary means of treating diseases and fighting infections have been based on the use of medicinal

plants. From ancient times, plants have been rich sources of effective and safe medicines. Globally, about 64% of the total world population is reliant on traditional medicine for their healthcare needs.The plant parts used for medication preparation by the traditional healers are variables. Healers mostly used fresh specimens from commonly available plants to prepare remedies for their patients; this might be mostly due to the effectiveness of fresh medicinal plant parts in treatment since the contents are not lost before use compared to the dried ones(Singh, 2017).The traditional healers have harvested leaves, roots,seeds, fruits, stems . In Ethiopia medicinal plants have been used as traditional medicine to treat different human ailments by the local people from time immemorial.The main reason that many traditional medicine practitioners used the leaf parts compared to others for remedial preparation is due to their accessibility and for preventing them from extinction(Admasu Moges,and Yohannes Moges ,2020).

2.5.1.Garlic (Allium sativum)

The scientific name of garlic is *Allium sativum* and its common name in (English) is “garlic “and in Amharic is “Nech Shinkurt ” . It is a species in the onion genus, *Allium*. Garlic is a member of the onion family and is one of nature's most multi purpose medicinal plants. Garlic is native to Central Asia, and Mediterranean region as well as Africa and Europe. It was known to ancient Egyptian (Salunkhe and kadam,1998). *Allium sativum(Garlic)*is a bulbous plant. It grows up to 1.2 cm in height and it produces hermaphrodite flowers. Pollinated is through bees and other insects. Garlic is easy to grow and can be grown year round in mild climate. Biologicl classification of garlic:kingdom: Plantae, phylum: Tracheophyta,class: Liliopsida,order: Asparagales, family: Amaryllidaceae species: Allium sativum(Sethi Neeraj *et a*l 2014)

2.5.2.Medicinal Values of Garlic

. Garlic is more effective with least side effects as compared to commercial antibiotics; as a result, they are used as an alternative remedy for treatment of various infections . Garlic extract inhibits the growth of Gram positive and Gram negative bacteria, such as *Staphylococcus, Streptococcus, Micrococcus, Enterobacter, Escherichia, Klebsiella, Lactobacillus, Pseudomonas, Shigella, Salmonella, Proteus,* and

Helicobacter pylori. Its antibacterial activity is mainly due to the presence of allicin produced by the enzymatic activity of allinase on alliin. Allicin is considered to be the most potent antibacterial agent in crushed garlic extracts, but it can be unstable, breaking down within 16 h at 23°C. However, the use of a water-based extract of allicin stabilizes the allicin molecule due to the hydrogen bonding of water to the reactive oxygen atom in allicin or there may be water soluble components in crushed garlic that destabilize the molecule. The disadvantage of this approach is that allicin can react with water to form diallyl disulphide, which does not exhibit the same level of antibacterial activity of allicin(Gebreselema and Mebrahtu**,**2013)

2.5.3.Neem(Azadirachta indica)

Neem is a tree and the scientific name of neem tree is (*Azadirachta indica*) and Indian Lilac .It is a tree in mahogany family maliaiceae .It is one of the two genes of *Azadirachta indica* and is native to Indian subcontinent.Neem Trees will reach up to 30 m tall with limbs reaching half as wide. The shiny dark green pinnately compound leaves are up to 30 cm long. Each leaf has 10–12 serrated leaflets that are 7 cm long by 2.5 cm wide. It will grow where rainfall is as little, and thrives in areas that experience extreme heat of up to 48°C. Even some of the most cautious researchers are saying that neem deserves to be called a "wonder plant.The taxonomic classification of neem is as follows: Kingdom : Plantae, Order: Rutales, Suborder: Rutinae, Family: Meliaceae, Subfamily : Melioideae, Tribe : Melieae, Genus : Azadirachta, Species: indica(Imam Hashmat, *et al,*2012)

2.5.4.Medicinal Values of Neem

The crucial role of A. indica in antibacterial activity, further studies have been carried out and found that methanolic and acetone extracts were more effective against the bacteria compared to that of aqueous extract (Rajasekaran 2008, Singh et al. 2016).Studies at molecular level demonstrate that A.indica contains chemical constituents of alkaloids, terpenoids tannins and flavonoids(Makkar et al. 2007)responsible to overcome microbial infection specially having antioxidant andantimicrobial biological activities. These chemicas might show the antibacterial activity having the ability to make a complex with the bacterial cell walls. Inhibitory activity towards DNA topoisomerase enzyme II by azadiractin, a bioactive metabolite of neem might also involvein the antibacterial potential. Moreover, the Gram-positive bacterial strains were foundmore sensitivethan the Gram-negative ones(Sinaga et al. 2016)

2.6. Bacterial Test Organism

2.6.1 Staphylococcus Aureus

Staphylococcal infections, commonly called staph infections, are caused by a genus of bacteria called Staphylococcus.Staphylococcus aureus is a gram-positive bacteria that cause a wide variety of clinical diseases. Infections caused by this pathogen are common both in community-acquired and hospital-acquired settings.There are more than 30 strains (types) of Staphylococcus bacteria. The most common human pathogen is Staphylococcus aureus. A pathogen is an organism that causes disease.Healthcare providers prescribe antibiotics to treat staphylococcal infections(Giovanni Gherardi, 2023)

2.6.2 Bacillus Cereus

Bacillus cereus is a Gram-positive aerobic or facultatively anaerobic, motile, spore-forming, rod-shaped bacterium that is widely distributed environmentally. While B. cereus is associated mainly with food poisoning, it is being increasingly reported to be a cause of serious and potentially fatal non-gastrointestinal-tract infections. The pathogenicity of B. cereus, whether intestinal or non intestinal, is intimately associated with the production of tissue-destructive exoenzymes. Among these secreted toxins are four hemolysins, three distinct phospholipases, an emesis-inducing toxin, and proteases(Gharib,et al 2020).

2.6.3 *Shiga Toxin Escherichia Coli*

Escherichia coli (E. coli) Shiga toxin-producing *Escherichia coli* (STEC) are zoonotic Gram-negative bacteria. While raw milk cheese consumption is healthful, contamination with pathogens such as STEC can occur due to poor hygiene practices at the farm level. STEC infections cause mild to serious symptoms in humans.Most strains of E. coli are harmless. Some strains however, such as Shiga toxin-producing E. coli (STEC), can cause severe food borne disease. It is transmitted to humans primarily through consumption of contaminated foods, such as raw or under cooked ground meat products, raw milk, and contaminated raw vegetables and sprouts.STEC produces toxins, known as Shiga-toxins because of their similarity to the toxins produced by Shigella dysenteriae(Cheleste M. Thorpe, 2004).

3.MATERIALS and METHODS

3.1. Description of the Study Area

The study was conducted at Wolkite, Ethiopia, The administrative center of the Central Ethiopia regional state ,at biotechnology laboratory in Wolkite University College of natural and computational science which is found in Gurage zone. The town has a latitude and longitude of 8°17′N 37°47′E and an elevation between 1910 and 1935 meters above sea level.The distance between Addis Ababa to Wolkite University is 166 Km by road.

3.2 Study Design

The study design was experimental factorial design: This design allows to simultaneously evaluate the effects of multiple factors neem leaf extract concentration, garlic extract concentration on bacterial growth inhibition. It also enables the assessment of potential interactions between these two factors. In his design provides comprehensive information on the individual and combined effects of the extracts.

3.3. Collection of Samples

Bulb of garlic (Allium Sativum) was obtained from local farmers of herbal products around gubrie town and fresh form of neem leaf used for this study was collected from welkite university compound around male dormitory near to social library building .The test organisms , S. aureus , B. cereus and *shiga toxin* E.coli was obtained from our microbial biotechnology laboratory . These test organisms was grown on Nutrient agar and maintained at $4^{o}C$ in the refrigerator for 48 hours before use our study.

3.4. Preparation of Plant Extracts

The plant materials was collected thoroughly washed in running tap water to remove debris and dust particles and then was rinsed in distilled water. The plants(neem leaf) was dried in the shade area of the laboratory in an open air at room temperature for about seven days and it was protected from sun light. After completely dry, these was grounded to a fine powder by using electric grinder and fresh garlic bulbs was crushed by using mortar and pestle. The plant samples 50g(25g of garlic and 25gof neem leaf powder) were weighed by using an electronic balance and then synergetic

powder of plant samples were added into a conical flask contained 300 ml of 99.9% ethanol for synergistic crude extract , 25g of galic and 25g of neem leaf powder were poured into each separate conical flask contained 150ml of 99.9% of ethanol(. Abubakar and Haque,2020). then after shacked for about 3 days manually (by hand) and instrumental laboratory shaker. The crude extracts of garlic and neem were filtered through Whatman filter paper and then it were evaporated the alcohols by rotary evaporator. Each extracts were transferred to glass jar and labeled accordingly and then stored at 4^0C refrigerator until use.(Jeyaseelan, *et,al*,2012)

3.5. Preparation of Bacterial Suspension

3.5.1. Preparation of Suspension for *Staphylococcus Aureus,Bacillus Cereus and Shiga Toxin Escherichia Coli*

The test microorganisms were cultured on nutrient agar at 37°C for 24 hrs.The Colonys were Selected by using a sterile inoculating loop used to picked up a single bacterial colony from the original culture plate. Ensure the loop was sterilized by flaming it until red hot and it was allowed to cool.The selected colonys were Streaked onto a fresh agar plate.The quadrant streak method was performed to obtained isolated colonies. The newly inoculated plate was Incubated again at 37^0C.After incubation, the plate was observed to ensure that single, isolated colonies were formed.By streaking the inculcating loop containing bacteria at the top end of the agar plate moving in a zigzag horizontal pattern until 1/3 of the plate was covered.Then, three to five well isolate overnight and culture colonies of the same morphological type was selected from an agar plate culture. The top of each colony were touched with sterile bent wire-loop and the growth were transferred into the test tube contained 5ml of saline solution .Then after saline suspension was incubated for 30 minutes to initiate the bacterial suspension(Nadia Belkebla,2022).

3.6 Preparation of Media

Meuler Hinton agar was prepared from a commercially available dehydrated base according to the manufacturer's instructions. About 23.4g of nutrient agar powder was weighed using a clean electronic weighing balance and then about 600 mL of sterile distilled water poured into a conical flask . Then,covered by aluminium foil shacked by hand and heated by hot oven for 60 second to mix properly and finally autoclaved for 15 min at 121^0C. Soon after autoclaving, the Meuler Hinton agar medium was allowed to cool to room temperature prior to poured it into the petri-plate.

Plates were dried faster in low humidity. The freshly prepared and cooled medium was poured into flat-bottomed petri dishes on a level, horizontal surface to give a uniform depth of approximately 4 mm. This was achieved by pouring 25 mL of the medium for plates with diameters of 100 mm.

3.7 Determination of Antibacterial Activity of Garlic and Neem Leaves Extracts by MHA Agar Well Diffusion Method

Suspensions of bacterial isolates were prepared in sterile normal saline, and sterile swabs were used to inoculate the entire surface of the MHA plates by streaking in three directions to evenly distribute the inoculum. This was repeated two more times, rotating the plate to ensure even distribution, and finally swabbing the rim of the agar. MH agar wells were prepared by using blue colour sterilize micropipette instead of cork borer with 6 mm diameter and about 2.5 cm apart to minimize overlapping of zones . 50 µL of garlic and neem individually and mixture of them were carefully added to the respective wells in the plate in triplicate by using micropipette,and the antibiotic disc was dispensed by sterile pair of forceps onto the surface of the inoculate MH agar plate and presse down to ensure complete contact with the agar surface. Crude extracts and antibiotic discs will be allowed to diffuse for about 40 minutes before incubation and then the plates were incubate in an upright position at 37^0C for 24 hours(Edward ,*et al*,2017**)**.

3.8 Determination of Antibacterial Activity of Garlic and Neem Leaves Extracts by MHA Agar Disk Diffusion Method

Aseptically apply 25 microliters (µL) of each extract (garlic and neem) were added onto separate sterile disks Instead of Sterilize filter paper disks using antibiotic disk then Using sterile forceps.The solvent was Allowed to evaporate completely in a sterile cabinet. the extract-impregnated disks were carefully placed onto the inoculated MHA plates ,by using sterile forceps. the plates were allowed to stand for 30 minutes at room temperature to allow for diffusion of the extract and antibiotics.the plates were up right position and incubated them at 37°C for 24 hours(Zihadi ,et al,2019).

4. RESULT

After done the above procedure properly ,the data was analyzed through Quantitative Analysis: Measuring inhibition zones and interpreting the diameters quantitatively can help compare the effectiveness of the extracts against selected pathogenic bacteria as illustrated in the following figures and tables below

4.1 Evaluation of Crude Extract of Garlic Against Selected Pathogenic Bacteria

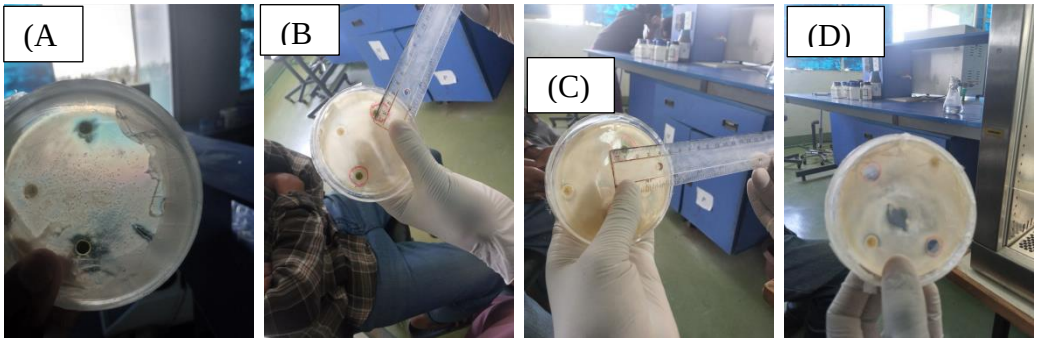

Figure 1 The diameter of inhibition zone of garlic ethanolic crude extracts against selected pathogenic bacteria:(A) Diameter inhibition of garlic extract on *Shiga Toxin E.coli* bacteria by well Diffusion Method ,(B) Diameter inhibition of garlic extract on *Shiga Toxin E.coli* bacteria by disk Diffusion Method,(C) Diameter inhibition of garlic extract on *S. aureus* bacteria by disk Diffusion method,(D)Diameter inhibition of garlic extract on *S. aureus* bacteria by well Diffusion method

Table 1: Antibacterial activity testing garlic extract using MH agar well and disk diffusion assay

Test organisms	Determination of Antibacterial Activity	
	Inhibition zone of garlic extract By well Diffusion Method (mm)	Inhibition zone of garlic extract by Disk Diffusion Method (mm)
S. aureus	15	14.5
B. cereus	No inhibition	No inhibition
Shiga Toxin E.coli	17	13

4.2 Evaluation of Crude Extract of Neem Against Selected Pathogenic Bacteria

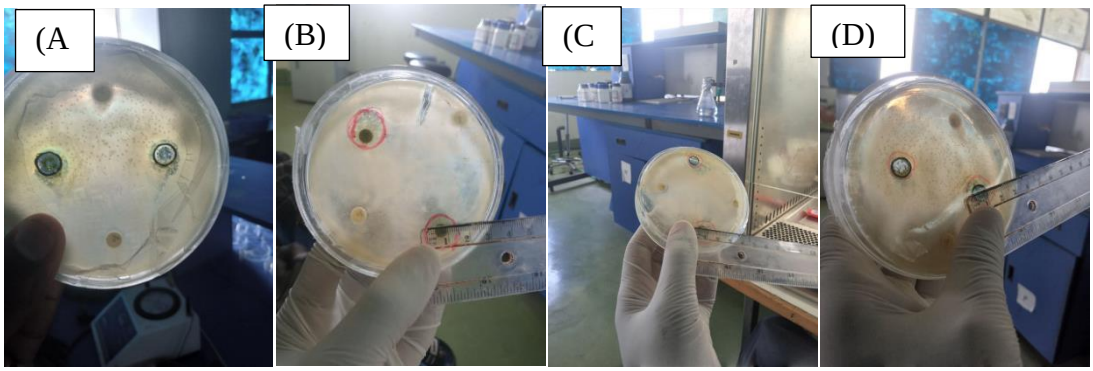

Figure 2 The diameter of inhibition zone of neem leaf crude extracts against pathogenic bacteria:(A) Diameter inhibition of neem leaves extract on *Shiga Toxin E.coli* bacteria by well Diffusion Method ,(B) Diameter inhibition of neem leaves extract on *Shiga Toxin E.coli* bacteria by disk Diffusion Method,(C) Diameter inhibition of neem leaves extract on *S. aureus* bacteria by disk Diffusion method,(D)Diameter inhibition of neem leaves extract on *S. aureus* bacteria by well Diffusion method

Table 2 : Antibacterial activity testing neem extract by using MH agar well and disk diffusion assay

Test organisms	Determination of Antibacterial Activity	
	Inhibition zone of neem leaves extract By well Diffusion Method (mm)	Inhibition zone of neem leaves extract by Disk Diffusion Method (mm)
S. aureus	14	13
B. cereus	No inhibition	No inhibition
Shiga Toxin E.coli	15	12

4.3 Determination of the Synergistic Effects of Crude Extracts Against Selected Pathogenic Bacteria

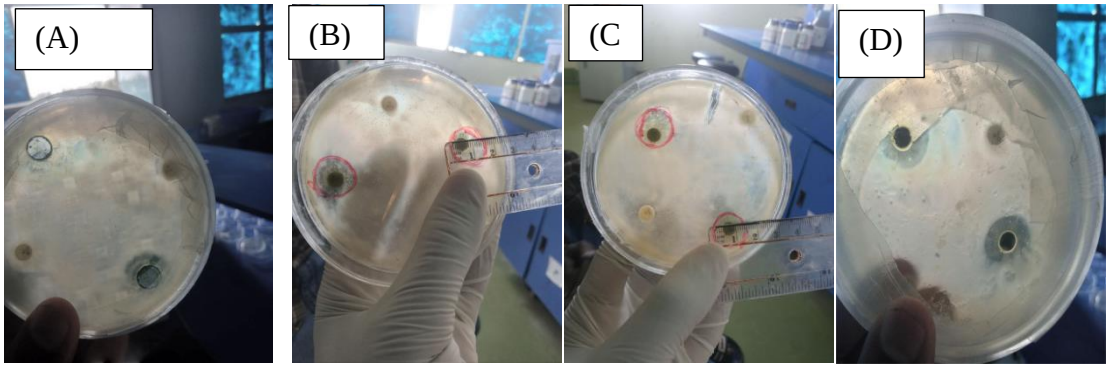

Figure 3 The diameter of inhibition zone of synergetic effect of crude extracts against tested pathogenic bacteria:(A) Diameter inhibition of synergetic crude extracts on *Shiga Toxin E.coli* bacteria by well Diffusion Method ,(B) Diameter inhibition of synergetic crude extracts extract on *Shiga Toxin E.coli* bacteria by disk Diffusion Method,(C) Diameter inhibition of synergetic crude extracts extract on *S. aureus* bacteria by disk Diffusion method,(D)Diameter inhibition of synergetic crude extracts extract on *S. aureus* bacteria by well Diffusion method.

Table 3 :Antibacterial activity of synergetic effect tested on selected pathogenic bacteria by using MH agar well and disk diffusion assay

Test organisms	Determination of Antibacterial Activity	
	Inhibition zone of synergetic extract By well Diffusion Method (mm)	Inhibition zone of synergetic extract By disk Diffusion Method (mm)
S. aureus	16	13.5
B. cereus	No inhibition	No inhibition
Shiga Toxin E.coli	18	15.5

5. DISCUSSION

The traditional knowledge about the treatment of infectious disease using natural products or substances should be investigated using scientific methods. Larger inhibition zones indicate greater antibacterial activity. In this study, mixture of garlic and neem crude extract against tested pathogenic bacteria had larger inhibition zone than the inhibition zones of garlic and neem crude extracts used separately According to (Makhulu Edmond Edward *et al* 2017)

Although mixture of garlic and neem extract had high antimicrobial activity, garlic extract and neem extract alone also had good potential of a broad spectrum of activity against both Gram-positive and Gram-negative bacteria .Both disk diffusion and well diffusion assays can be used to compare the antibacterial activity of garlic, neem and their potential synergistic extract.But according to this study disk diffusion assays their inhibition zone was less than well diffusion method there was due to in disk diffusion method extract diffuse radially outward from the disk(Farrah Nurkhaliza *et al*,2023)

But individual and synergetic garlic and neem leaf crude extracts respectively not against *Bacillus Cereus* pathogenic bacteria(there was not obtained inhibition zone) ,this due to may be several factor such as ,bacillus cereus can be naturally resistant to some compounds found in garlic and neem extracts and also intrinsic resistance could be due to factors like ,bacillus cereus can form spores that are highly resistant to various environmental stresses, including some antimicrobial,The cell wall of Bacillus Cereus might be less susceptible to the action mechanisms of the plant extracts and *Bacillus Cereus* was not specifically against by individually and synergetic effect of crude extracts(Yang,*et al* 2023) .Antibiotic disks were used as a positive control to compare inhibition zone of ethanol crude extracts but the antibiotic disks were expired their inhibition zone were not obtained and it was given unreliable result.

The garlic ethanolic crude extracts has potential antibacterial activity against some bacteria, but not all. S. aureus showed the most susceptibility, while B. cereus was resistant.The neem leaves ethanolic crude extract has potential antibacterial activity against some bacteria, but not all. Based on the above tables ,*Shiga Toxin* E. coli

appears to be the most susceptible, while B. cereus was resistant.The synergistic ethanolic crude extract demonstrates potential as a broad-spectrum antibacterial agent. It seems effective against S. aureus and shiga toxin E. coli, with Shiga Toxin E. coli showing slightly higher susceptibility. However, B. cereus appears resistant(Agnieszka Magryś1 *et al* 2021)

6. CONCLUSION and RECOMMENDATION

6.1 Conclusion

From the overall study it can be concluded that, the mean inhibition zones of the combined antibacterial activity of garlic and neem crude extracts of the plants have significant antibacterial activity against the tested pathogenic bacteria were greater than the mean inhibition zones of crude extracts of garlic and neem used individually. And also the antibacterial activity of garlic and neem crude extracts were greater than the activity of currently used antibiotics against the selected organisms.This experiment investigated the antibacterial activity of a garlic and neem leaves ethanolic crude extracts against three different bacteria,S. aureus, B. cereus, and Shiga Toxin E. coli.Larger diameter of inhibition Zone indicates stronger antibacterial activity.Well diffusion method and disk diffusion method results were provided for comparison.The garlic,neem and their synergetic ethanolic crude extracts has potential antibacterial activity against some bacteria, but not all. S. aureus and shiga toxin e. coli showed the susceptibility, while B. cereus was resistant.

6.2 Recommendation

Based on the findings of this study, the following recommendations have been forwarded:

- Further *in vivo* studies should be conducted to evaluate the effect of antimicrobial agent to treat bacterial infections.
- There is a need for detailed scientific study of traditional medical practices to ensure that valuable therapeutic knowledge of the society on some plants and thereby to preserve such important natural antimicrobial resources.
- It is necessary to study other locally produced and prepared drugs from different sources for their antimicrobial activities.
- Further research should be conducted to investigate the synergistic antimicrobial effect of the mixture of garlic and neem crude extracts against many other bacterial pathogens and fungal species because this study was carried out only on three selected pathogenic bacteria.

- Investigate the specific bioactive compounds responsible for the observed effects and their mode of action against the target bacteria.
- Explore optimal concentrations and ratios of garlic and neem leaf extracts to maximize their synergistic effect while minimizing any potential cytotoxicity or adverse effects
- Further research and validation are essential to harness the full therapeutic potential of these plant extracts in combating bacterial infections and addressing the global challenge of antibiotic resistance.

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