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Synergistic Antibacterial Activity of Black Seed (*Nigella sativa*) and Clove (*Syzigium Aromaticum*) Against Some Selected Pathogenic Bacteria

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Abstract: At present, the paucity of new antimicrobials coming into the market has led to the problem of antibiotic resistance fast escalating into a global health crisis. Diverse on metabolic, genetic and physiological fronts, rapid progression of resistant microbes and the lack of a strategic management plan have led researchers to consider plant-derived substances (PDS) as alternative or in complementing antibiotics against the diseases, thus the aim of this study was to assess the antibacterial effect of black seed (*Nigella. sativa)* and clove (*Siyzigium. aromaticum)* seed extracts and their synergistic action against some selected pathogenic bacteria; namely: *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Salmonell typhi* and *Escherichia coli* clinical isolates and their standard derivatives. Ethanol, hexane, acetone and chloroform crude extracts of *N. sativa* and *S. aromaticum* were evaluated against tested pathogenic bacteria using agar well diffusion method; the inhibitory zones were recorded in millimeters. Ciprofloxacin was used as positive controls, while dimethyl sulfooxide (DMSO) was served as negative control. The minimal inhibitory concentration (MIC) of the plant extracts against test bacteria were assessed using agar well dilution and broth dilution method; and then Minimum Bactericidal Concentration (MBC) was evaluated. The inhibition zone of all *N. sativa* crude extract against all clinical isolate and standard pathogenic bacteria ranged from (12-30mm). The inhibition zone of all *S. aromaticum* crude extract against all clinical and standard pathogenic bacteria ranged from (12-32mm). *N. sativa* hexane extract against *E. coli (clinical isolate)* exhibited the lowest inhibition zone while acetone extract against *S. aureus (ATCC25923)* exhibited the highest inhibition zone. *S. aromaticum* chloroform extract against *S. aurues (clinical isolate)* exhibited the lowest inhibition zone while ethanol extract against *S. aureus (ATCC25923)* exhibited the highest inhibition zone. The synergistic antibacterial effect of *N. sativa* and *S. aromaticum* crude extract against both clinical isolate and standard pathogenic bacteria ranged from (12-33mm). The inhibition zone of the synergistic antibacterial effect of *N. sativa* and *S. aromaticum* seed extracts against tested pathogenic bacteria was significantly (P value ranges from 0.01 to 0.03) greater than the extracts used separately. Thus, the present finding supports the traditional use of these plants in combination for treating pathogens. And also there is a need for detailed scientific study of traditional knowledge to ensure that valuable therapeutic knowledge of some plants is preserved as well as to provide scientific evidence for their efficacies. The result of phytochemical screening also showed that the plants contain trepenoids, tannin, flavonoids and saponins except acetone extract of both plants which could not show the presence of flavonoids.

Keywords: Nigella Sativa, Syzigium Aromaticum, Synergistic Effect, Antibacterial Activity, Inhibition Zone, Phytochemical Screening

1. Introduction

1.1. Background of the Study

At present, the paucity of new antimicrobials coming into the market has led to the problem of antibiotic resistance fast escalating into a global health crisis [39]. The selective pressure exerted by the use of antibiotics (particularly overuse or misuse) has been deemed the major factor in the emergence of bacterial resistance to these antimicrobials [18]. Resistant organisms in health-care and community settings pose a threat to survival rates from serious infections, including neonatal sepsis and health-care-associated infections, and limit the potential health benefits from surgeries, transplants, and cancer treatment [38].

Diverse on metabolic, genetic and physiological fronts, rapid progression of resistant microbes and the lack of a strategic management plan have led researchers to consider plant-derived substances (PDS) as alternative or in complementing antibiotics against the diseases [8]. Folk knowledge has become a form of complementary medicine and holds great promise as a source of successful therapy for multiple drug resistant strains of bacteria [45].

The interest in the use of medicinal herbs or plants in the treatment of different diseases is currently increasing worldwide because of their positive results and less side effects [1]. According to the World Health Organization (WHO), 60-80 percent of the world's population relies on herbal remedies or traditional medicine for their primary health care and treatment, particularly in developing countries [25].

In addition, the WHO urged developing countries to use their medicinal plants as a method to generate successful programs for their primary health care [60]. In terms of natural structure, many of the antimicrobials being used today are related. In certain instances, from nature and/or adapted from herbal sources, chemically synthesized medicines have obtained their modulated form [29].

Approximately one-half of all licensed drugs that were registered worldwide in the 25 year period prior to 2007 were natural products or their synthetic derivatives [37]. Plants contain a wide array of phytochemicals such as tannins, terepenoids, flavonoids and alkaloids which have traditionally been utilized for centuries in folk medicines or ethno medicines [51].

About 80% of the total population in Ethiopia depends on conventional remedies as a primary source of health care [16]. Despite Western medicine becoming more widespread, Ethiopians tend to rely more on complementary and alternative medicine [15].

The primary parts of the plants used for the preparation of traditional medicines are leaves and roots [34]. Local practitioners have offered different traditional medicines for the diseases of their patients, such as stomachaches, asthma, dysentery, malaria, evil eyes, cancer, skin diseases, and headaches [44]. Since time immemorial, the use of medicinal plants for human and animal treatments has been practiced [41].

Significant habitats for medicinal plants are stream/riverbanks, agricultural fields, disturbed sites, bushlands, forested areas and their margins, woodlands, grasslands, and home gardens [46]. In general, in the health care of the majority of people in Ethiopia, medicinal plants used for traditional medicine play a significant role.

Spices are the most common plant materials with potential antimicrobial properties that are used in foods; and they have been used traditionally for thousands of years by many cultures for preserving foods and as food additives to enhance aroma and flavor [42].

Spices may be indigenous or exotic, aromatic or with strong taste, but used in all cultures to enhance the taste of foods. Spices possess antimicrobial activity due to the presence of Essential oil, alkaloids, glycosides etc. that are present in abundance herbs and spices commonly used in Indian food preparation. The presence of these bioactive substances is responsible for antimicrobial properties [40].

Black seed is an annual herbaceous plant that is believed to be native to the Mediterranean region, but has been grown in other parts of the world, including the Arab desert, Northern Africa, and some parts of Asia. The black seed is derived from the common buttercup (Ranunculaceae) fennel flower plant (*Nigella sativa).* The black seed of *Nigella sativa* is known by other names, varying from place to place [31].

Others named it *black caraway*, others called it black cumin (Kalonji) or even coriander seeds, and in Ethiopia it is the so-called 'Tekur Azmud.' Unquestionably, black seeds have a beneficial and stabilizing effect on the human body, Black cumin seed is one of the greatest healing herbs of all times. Black cumin seed oil is said to be the universal remedy (a medicine or treatment of disease or Injury except death) [31].

Traditionally, black cumin has been used for immune system support, well-being, digestive health, respiratory issues, kidney and liver support, and heart health. In Asia and the Middle East, black cumin seeds have long been used to treat asthma, bronchitis, rheumatism and other inflammatory diseases [31].

Greek physician, Dioscorides used black cumin seed to treat headaches, nasal congestion, toothache and intestinal parasites. He also reported that they were used as a diuretic to promote menstruation and increase milk production. He described the plant under the name, melanthion, in his 5-volume pharmacology “De MateriaMedica” which was used as a reference for healing with herbs in the Middle Ages [31].

Several studies have shown that over 100 valuable nutrients are found in black seed oil. It contains 21% of protein, 38% of carbohydrates, and 35% of fats and oils from plants. Thymoquinone, nigellone, thaymol, methyl and other fixed oils are among these constituents which are used as antibacterial agent.

Up to 50 percent of thymol, a monocyclic phenolic compound accumulates in the Ethiopian cumin seed variety [33]. Within Ethiopia its main use is as a spice, which is typically ground and mixed with other spices. There is also some use in traditional medicine. Most Ethiopian people use spice preparation as a house keeping Studies also confirm that the application of black cumin seed for medicinal purposes has issues with internal and external therapy [11].

The second cash crop exported next to ginger in Ethiopia is black cumin. The annual production of black cumin seed in Ethiopia is 18000 metric tons in 2014/15 and the national productivity average of black cumin is 0.79 tons per hectare. The overwhelming majority of Ethiopia's exports of black cumin go to Arab countries, which accounted for some 98% of national exports in 2008, along with other mainly Muslim countries.

Cloves (*Syzigium aromaticumL.*) are aromatic spices which belong to the Myrtaceae family. From the Moluccas Islands, actually known as the Spice Island, the tree that produces the miracle of nature originated In Ayurveda; Chinese medicine, and Western herbalism, cloves are used. Some clove components have been shown to be effective for bacterial and fungal infection [22].

The main compounds of clove include eugenol, eugenol aetate, caryophyllene which are used as an antiseptic, antibacterial, analgesic agent in traditional medical practices. Now it is used in pharmaceutical and food products and in beverages as a flavoring agent. The therapeutic benefits of eugenol are well known. In recent times, it has been studied for a variety of promising biological properties [36]. It has been reported to participate in photochemical reactions and to possess insecticidal, antioxidant and anti-inflammatory activities. Several studies have shown that clove has antiviral properties and have inhibitory effect on viruses like Herpes Simplex Virus (HSV) and hepatitis C virus [22].

Clove is known to contain antibacterial properties and is used to cleanse bacteria in several dental creams, tooth pastes, mouth washes, and throat sprays. It is also used to alleviate pain from sore gums and to improve dental health in general [36]. By purifying the blood and helping to protect against different diseases, clove oil strengthen the immune system.

Clove, *Syzygium aromaticum,* Myrteaceae, known in Ethiopia as "Kerunfud" by its vernacular name, is useful as antioxidant activity against hydroxyl radicals and acts as an iron chelator, Antibacterial activity against *Escherichia coli, Listeria monocytogenes, Salmonella enterica, Campylobacter jejuni, Staphylococcus aureus, Bacillus subtilis and Saccharomyces cerevisiae,* Antifungal activity against rye bread spoilage fungi, *Rhizoctonia solani Cladosporium herbarum*, *Penicillium glabrum, P. expansum, Fusarium verticilloides and A. niger* [9].

The antibacterial activity of black seed have been evaluated and reported in Ethiopia as well as out of Ethiopia, however the antibacterial activity of cloves has not been studied extensively in Ethiopia as well as its combined effect with black seed. The present study aims to evaluate the combined effect of black seed and cloves.

1.2. Statement of the Problem

It is well known that multidrug resistant (MDR) bacteria are one of the most significant emerging public health issues. Multidrug-resistant bacteria infections are difficult to manage as few or even no treatment options exist. In certain cases, antibiotics that are more toxic to the patient must be used by health care providers. Globally, there is a growing prevalence of pathogenic bacteria immune to multidrugs. An example is the development of gram-negative bacteria such as *E. coli and Klebsiella pneumoniae* by ESBL (Extended Spectrum Beta Lactamase [5].

ESBL is an enzyme that kills several antibiotics that are clinically essential. It is becoming increasingly prevalent that infections with bacteria expressing ESBLs are difficult to handle. A worrisome trend is that more and more individuals are asymptomatic carriers of bacteria producing ESBL around the world. This puts many of them at risk for potential infections with antibiotic resistance [21].

With the advent of the antibiotic period, the rapid proliferation of multidrug-resistant pathogens has been driven by overuse and inadequate antibiotic consumption and use. Antimicrobial resistance boosts morbidity, mortality, hospitalization time, and the cost of health care. In the 21st century, extended spectrum beta lactamase (ESBL) producing bacteria among gram-positive bacteria, *staphylococcus aureus (MRSA)* and multi drug resistant (MDR) *mycobacterium tuberculosis*, among gram-negative bacteria, have become a major global health issue [10].

In Ethiopia, although this MDR is the core problem, the physicians always prescribe the synthetic drug to treat the patients, the natural remedy does not have acceptance by these physicians because they have not been studied extensively, although a few of them have been studied, they have not got approval from Ethiopian health institute. The present study showed that natural remedy such as clove and black seed which are studied and have been approved for medical use in foreign countries but not in Ethiopia had a great help in protecting against MDR infections.

1.3. Objective of the Study

General objective

To evaluate antibacterial effects of mixtures of black seed (*Nigella Sativa*) and clove (*Syzgium Aromaticum*) crude extracts against some selected pathogenic bacteria

Specific objective

To determine individual antibacterial activity of *N. sativa and S. aromaticum* seed extract

To determine the synergistic antibacterial activity of *N. sativa and S. aromaticum* seed extract

To determine the minimum inhibitory concentration and minimum bactericidal concentration of the synergism and the two plant extract independently

To assess the phytochemical component of each plant extract using standard test

1.4. Significance of the Study

In this study, the antibacterial activity of black seed extracts and cloves have been examined. This study would raise awareness among the society about the potential effects of black seeds and cloves for antibacterial activity and inhibitory effects for other diseases. This research work would provide the awareness and basic knowledge for how

black seeds and cloves can be used for various bacterial infections and control with low coast and cheapest medicinal therapy. The black seed oil and cloves are easily available to treat various bacterial diseases with no side effects. This research work would show to the society that, clove and black seed can be taken in combination to treat infectious disease. This research work would raise awareness that black seed and clove can be taken in a low concentration to treat infectious disease. This study would also reveal to the researchers, that different phytochemicals are available in black seed and clove which are essential for antibacterial activity.

2. Literature Review

2.1. Historical Overview of Medicine from Natural Products

Herbalism, based on the use of plants and plant extracts, is a common herbal or folk medicine practice [26]. The key types of life on earth are herbs/plants, the major component of conventional medicinal materials in the world. Around 350,000 species of existing plants (including seed plants, bryophytes and ferns) are known to exist, of which 287,655 species were reported [49].

Herbal medicine (HM), also known as phytotherapy, phytomedicine or botanical medicine, refers to plants, herbal materials, herbal preparations and finished herbal products containing plant parts or other materials as active ingredients. Seeds, berries, roots, leaves, fruit, bark, flowers, or even the whole plant are the plant components used in herbal therapy.

Before the advent of aspirin derived from Spiraea ulmaria, which was already prescribed for fever and swelling in Egyptian papyri and recommended by the Greek Hippocrates for pain and fever, man was primarily dependent on rudimentary botanical content for medical needs to maintain stamina and heal diseases [50]. While written reports of medicinal plants date back at least 5,000 years to the Sumerians, who identified well-established medicinal uses for plants such as laurel, caraway, and thyme, archeological studies have shown [23] that herbal medicine practice dates back 60,000 years in Iraq and 8,000 years ago in China [49].

Owing to the lack of scientific evidence in the sense of modern medicine, herbal medicine has been challenged by practitioners of clinical medicine over the last century due to the introduction of Western medicine (or "conventional" medicine), despite its long history of successful use. Interestingly, things are changing with time. The use of herbs has been resurgent in recent years due to the side effects of chemical medications, the lack of modern curative therapies for many chronic diseases, and microbial resistance, as well as the extraordinary investment in pharmaceutical research and development (R&D) [49].

For example, only about 1,200 new drugs have been approved by the US Food and Drug Administrator. As a result, over the past 40 years, both in developing and developed countries, the use of herbs and herbal products for health purposes has increased in popularity worldwide. In addition, multinational pharmaceutical firms armed with modern science/technology and ideas have begun to rediscover herbs as a possible source of new drug candidates and have revived their policies in support of the production and discovery of natural product [49].

2.2. Experimental Plants

2.2.1. Black Seed Botanical Description

Black cumin plants are hardy annuals that grow between 8 and 24 inches (20 to 60 cm) in height. Good, deeply divided leaves carry the branched stems, and the plant has an established taproot. Five petals, several stamens, and five or six elongated fused carpels are in the pale blue or white flowers. In a capsule with five or six segments, the black triangular or pyramidal seeds are borne, each ending in an elongated projection. in a variety of soils, the plants can expand and reseed readily, becoming weedy in some regions.

Figure 1. *Black seed flower.*

2.2.2. Black seed Taxonomical Classification

Scientific Classification

Table 1. *Taxonomic Classification of Black Seed.*

Taxonomic classification	Scientific classification of black seed
Domain	Eukarya
Kingdom	Plantae
Subkingdom	Tracheobionta (vascular plants)
Superdivision	Spermatophyta (seed plants)
Phylum	Magnoliophyta (flowering plants)
Class	Magnolioptida (Dicotyledons)
Subclass	Magnoliidae
Order	Renunculales
Family	Renenculaceae (Buttercup family)
Genus	Nigella
Species	N. sativa

2.2.3. Traditional Medicinal Use of Black Seed

Traditionally, seeds of *N. sativa* are widely used for asthma, diabetes, hypertension, fever, inflammation, bronchitis, dizziness, rheumatism, skin disorders, and gastrointestinal disturbances. It is also used as a liver tonic, digestive, antidiarrhoeal, emmenagogue, and to control parasitic infections and boost immune system. Avicenna refers to black seeds in the “The Canon of Medicine”, as seeds stimulate the body's energy and helps recovery from fatigue and dispiritedness [57].

Black seeds and their oil have a long history of folklore usage in Indian and Arabian civilization as food and medicine. The seeds have been traditionally used in Southeast Asian and the Middle East countries for the

treatment of several diseases and ailments including asthma, bronchitis, rheumatism and related inflammatory diseases. Its many uses have earned Nigella the Arabic approbation 'Habbatul barakah', meaning the seed of blessing. A tincture prepared from the seeds is useful in indigestion, loss of appetite, diarrhoea, dropsy, amenorrhoea and dysmenorrhoea and in the treatment of worms and skin eruptions. Externally the oil is used as an antiseptic and local anesthetic [57].

Figure 2. *Black seed.*

2.2.4. Antibacterial Activity of Black Seed

In a modified paper disc diffusion process, the antibacterial impact of ground black seeds was studied. Simple growth inhibition of *Staphylococcus aureus* was observed at a concentration of 300 mg/mL with distilled water as control, as confirmed by the positive control of Azithromycin. The inhibition obtained was higher with *N. sativa* ground seeds from Hadramout than with *N. sativa* ground seeds from Ethiopia. The positive inhibition may be attributed to the two important active ingredients of *N. sativa*, TQ and melanin [12].

Ethanol and n-hexane extracts of the black seeds recorded remarkable dose dependant antibacterial effects against different gram-positive and gram-negative strains, namely *Bacillus cereus, Bacilllus subtilis, Escherichia coli, Staphylococcus epidermidis, Klebsiella pneumonia and Salmonella typhmurium.* However, no antibacterial activity detected against *Pseudomonas aeruginosa and Enterobacter aerogens.* The black cumin seeds exhibited antibacterial activity against *Salmonella typhi.* Methanol and water extract of the black seed reported remarkable antibacterial efficacy towards *Streptococcus pyogene, Pseudomonas aeruginosa, Klebseilla pneumonia, and Proteus vulgaris,* the greater antibacterial effect was against the gram-positive bacteria [2]. Oil of *Nigella sativa* revealed effective antibacterial activity against considerable number of methicillin resistant and coagulase negative *Staphylococcus aureus,* safety of that oil was examined, and there was no cytotoxic influence on the proliferation of gingival fibroblasts the black seed oil was recommended to be used as an antimicrobial agent in food production to prevent spoilage. Based on the results that showed that this oil at 2.0% concentration was able to inhibit the growth of twenty-four pathogenic, spoilage and lactic acid bacteria [2].

Antibacterial activity of *N. sativa* against and triple therapy in eradication of Helicobacter Pylori in patients with non-ulcer dyspepsia was carried out. It was showed that *N. sativa* seeds possess clinically useful anti H. pylori activity, comparable to triple therapy [54]. Eleven human pathogenic bacteria have been examined for the antibacterial activity of TQ and its biofilm inhibition ability. TQ showed significant bactericidal activity against different pathogenic human bacteria, especially Gram-positive cocci *(Staphylococcus aureus ATCC 25923 and Staphylococcus epidermidis CIP 106510).* TQ stopped cell adhesion to the surface of glass slides.

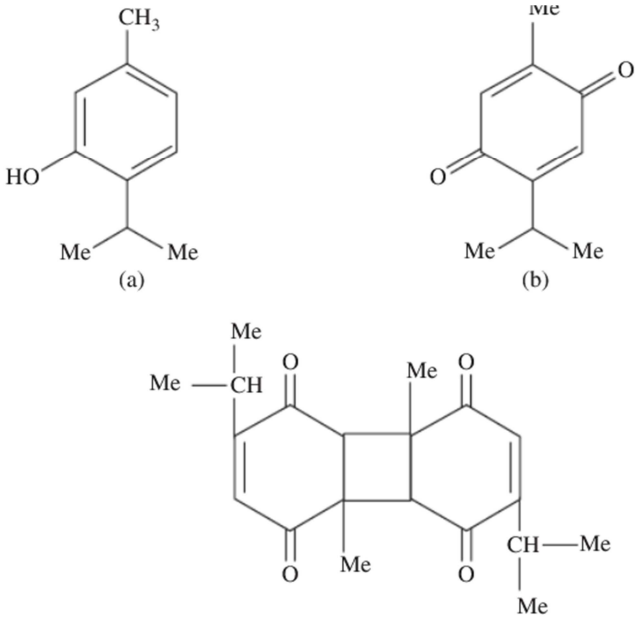


Figure 3. *a) Chemical structure of thymol b) Chemical structure of thymoquinone c) chemical structure of methyl group.*

2.3. Botanical Description of Cloves

The clove tree is an evergreen that grows to a height of around 8 to 12 meters (25 to 40 feet). Tiny, simple, and opposite are its gland-dotted leaves. The trees are normally propagated in shaded areas from seeds that are planted. Flowering starts around the fifth year; up to 34 kg (75 pounds) of dried buds may be yielded annually by a tree. In late summer and again in winter, the buds are hand-picked and are then sun-dried. Cloves range in length from roughly 13 to 19 mm (0.5 to 0.75 inch). The buds contain 14 to 20% essential oil, the main component of which is eugenol, the aromatic oil.

Figure 4. *Clove flower.*

2.3.1. Taxonomic Classification of Cloves

Table 2. *Taxonomic Classification of clove.*

Taxonomic Classification	Scientific Classification of Clove
Kingdom	Plantae, Planta, Vegetal, plants
Subkingdom	Viridiplantae
Infrakingdom	Streptophyta-land plants
Superdivision	Embryophyta
Division	Tracheophyta-vascular plants
Subdivision	Spermatophytina-seed plants
Class	Magnoliopsida
Superorder	Rosane
Order	Myrtales
Family	Myrtaccae
Genus	Syzigium p.Br.ex Gaertn
Species	Syzigium aromaticum

2.3.2. Traditional Medicinal Use of Clove

Traditionally, cloves have been used for centuries in the treatment of vomiting; flatulence; nausea; liver, bowel and stomach disorders; and as a stimulant for the nerves. In tropical Asia, cloves have been documented to relieve different microorganisms as scabies, cholera, malaria, and tuberculosis. As well, in America, clove has been traditionally used in inhibiting food-borne pathogens to treat viruses, worms, candida, and different bacterial and protozoan infections. Moreover, eugenol has been widely used in dentistry because it can penetrate the dental pulp tissue and enter the bloodstream [14].

Cloves are used as a warming and calming agent in conventional Indian and Chinese medicine [13]. Cloves have historically been used for centuries in the treatment of vomiting; flatulence; nausea; diseases of the liver, bladder, and stomach; and as a nerve stimulant. Cloves to cure various microorganisms such as scabies, cholera, malaria, and tuberculosis have been recorded in tropical Asia.

Clove has also been historically used in America to inhibit foodborne pathogens in the treatment of viruses, worms, candida and other bacterial and protozoan infections [17]. In addition, eugenol is commonly used in dentistry because it can penetrate the tissue of the dental pulp and enter the bloodstream [43]. There have been records of anti-carcinogenic activity of sesquiterpenes, isolated from cloves [14].

Figure 5. *Clove seed.*

2.3.3. Antibacterial Activity of Clove

The antimicrobial activities of clove have been proved against several bacterial and fungal strains. Sofia et al. tested the antimicrobial activity of different Indian spice plants as mint, cinnamon, mustard, ginger, garlic and clove [19]. The only sampled that showed complete bactericidal effect against all the food-borne pathogens tested *Escherichia coli (E. coli), Staphylococcus aureus and Bacillus cereus* was the aqueous extract of clove at 3%. At the concentration of 1% clove extract also showed good inhibitory action. In another work published by Dorman and Deans [19] the antibacterial activity of black pepper, geranium, nutmeg, oregano, thyme and clove was tested against 25 strains of Gram positive and Gram negative bacteria.

The oils with the widest spectrum of activity were thyme, oregano and clove respectively. The antibacterial activity of clove, oregano (Origanum vulgare), bay (Pimenta racemosa) and thyme (Thymus vulgaris) essential oil was tested against *E. coli* O157:H7 showing the different grades of inhibition of these essential oils [28].

Likewise formulations containing eugenol and carvacrol encapsulated in a non-ionic surfactant were tested against four strains of two important foodborne pathogens, *E. coli O157:H7 and Listeria monocitogenes,* results reinforces the employment of eugenol to inhibit the growth of these microorganisms in surfaces in contact with food [20].

The essential oils of *Syzygium aromaticum* (clove bud) and *Rosmarinus officinalis L.* (rosemary) were obtained by hydro-distillation. The antimicrobial activity of clove bud oil and rosemary oil was investigated by agar well diffusion method against four multidrug resistant strains namely *Acinetobacter baumannii, Pseudomonas aeruginosa, Staphylococcus aureus and Enterococcus feacalis* as well as two standard strains, *Staphylococcus aureusATCC29213 and Pseudomonas aeruginosaATCC27853* [3].

Both essential oils exhibited inhibitory effects towards all the test organisms, clove essential oil had antibacterial activity little higher than of rosemary oil, MICs ranged from 0.312% (v/v) to 1.25% (v/v) for all tested bacteria while MICs for rosemary oil ranged from 0.312% (v/v) to 5% (v/v). Based on this finding, it may be suggested that these essential oils may be used as natural antibacterial agents to treat infections caused by multidrug resistant bacteria [3].

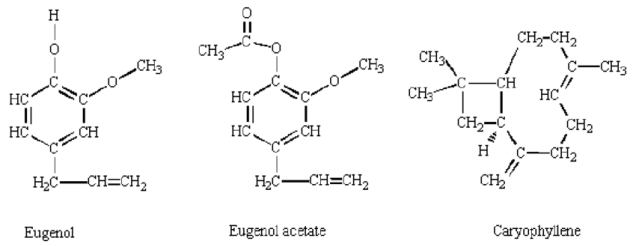


Figure 6. *Chemical structures of the components of clove oil.*

3. Materials and Methods

3.1. Study Area

Gondar town is located in Amhara region, North West Ethiopia, at about 723 kms away from Addis Ababa. It is located, at an altitude of around 2, 225 m (above sea level), 12°35′ 60.000”N of latitude and 37°28’0.120”E of longitude. In particular, this research was carried out in the microbial

Biotechnology laboratory of the Department of Biotechnology at the University of Gondar.

3.2. Study Design

An experimental research design was employed complete randomized design (CRD) with three replications for each treatment.

3.3. Sampling

Clinical isolates and standard bacterial pathogen which include *Escherichia Coli, Staphylococcus Aureus, salmonella typhi, Klebsella pneumonia* was collected from laboratory of university of Gondar comprehensive and specialized hospital. The bacterial culture was maintained in their appropriate agar slant at 4 degree Celsius until use.

3.4. Collection of Plant Materials

A dried seed sample was obtained from Gondar town's local market Based on the indigenous knowledge of the local traditional medicinal plant healers. A simple random sampling was used to collect the seeds.

3.5. Preparation of Plant Extracts

To dissolve debris and dust particles, the collected plant seed materials was thoroughly washed in flowing tap water and then it was rinsed in distilled water. The seed sample was dried in the laboratory at room temperature in open air for approximately five days and was shielded from sunlight. The fully dried medicinal seeds were grounded, using an electronic blender or mechanical grinder. The fine powder was placed in a sterile bottle at room temperature in a dark spot. The dried and powdered seeds of each plant (100 gm) was extracted with 500 ml of ethanol, chloroform, hexane and acetone [4]. The extracts were filtered through a sterile filter paper of Whatman No. 1 and then, using a rotary evaporator, it was concentrated in a vacuum at 37°C. Each extract was transferred to glass vials and was maintained at 4°C until use [6].

3.6. Inoculum Preparation or Bacterial Suspension and Inoculation of Test Plates

The clinical isolate and standard bacteria were cultivated separately for 24h at 37°C on nutrient agar. This was done by streaking the bacteria comprising the inoculating loop at the top end of the agar plate, going in a horizontal zigzag pattern until 1/3 of the plate has been coated. Then, from an agar plate culture, three to five well-isolated overnight cultured colonies of the same morphological form were chosen.

A sterile bent wire-loop contacted the top of each colony and the growth were transferred into a screw-capped tube containing 5 ml of a suitable broth medium, Tryptic Soy Broth (TSB). The broth cultures (test tubes) were incubated at 37°C for 24 hours without agitation until the turbidity of 0.5 McFarland levels is reached or exceeded.

In order to achieve turbidity optically equivalent to that of 0.5 McFarland turbidity norm 1.5-108 colony-forming units (CFU)/ml, the turbidity of the actively developing broth culture were balanced with sterile saline. There was ample light to visually compare the inoculum tube and the 0.5 McFarland turbidity norm to perform this step properly [59].

Optimally, a small amount of around 0.1 ml of the bacterial suspension was inoculated on the dried surface of the Mueller-Hinton agar plate within 15 minutes after adjustment of the turbidity of the inoculum suspension and spread over the entire sterile agar surface by the sterile cotton swab. In order to ensure an equal distribution of inoculum, this process was repeated by striking two more times, rotating the plate about 60 rev/min each time and finally swabbing the rim of the agar. To allow for any excess surface moisture to be absorbed before applying the crude extracts on the respective well, the lid was left open for 3 to 5 min, but not more than 15 min.

3.7. Antibacterial Activity (Agar-Well Diffusion Method)

The antibacterial activities of the seed extracts (black seed and clove seed extract) were tested against the selected bacterial strains. Suspensions of the bacterial isolates was made in sterile normal saline and adjusted to the 0.5 McFarland's standard. The 40 ml of sterilized Mueller Hinton Agar (MHA) medium was poured in to each sterile large sized petri-plate and allowed to solidify.

The test bacterial cultures was evenly spread over the appropriate media by using sterile cotton Swab. Then a well was made in the medium by using a sterilized cork -borer with 6mm diameter, 4mm deep and about 5.0 cm apart to minimize overlapping of zones. Then 100 µl of each hexane, acetone, ethanol, and chloroform extracts of each seed was transferred into separated wells. After this, the plates were incubated at 37°C for 24-48h.

After incubation, the results were observed and the diameters of inhibition zone around each well were measured. The tested microorganisms were also tested for their sensitivity against the commonly prescribed antibiotics using similar methods used for the determination of antimicrobial activity of *N. sativa and S. aromaticum extracts.* Antibiotic discs (Ciprofloxacin) served as positive controls while dimethayl sulfoxide (DMSO) served as negative control. The result was interpreted as high, moderate and low by comparing the results with what has already been reported by Clinical and Laboratory Standards Institute (CLSI).

3.8. Determination of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Based on the agar well dilution and broth macro-tube dilution methods, the minimum inhibitory concentration (MIC) values of seeds extracted from *Nigella sativa*, *Syzigium aromaticum*, and their mixture were determined. Sterile screw-capped test tubes were placed on a suitable rack in a

number of rows in order to evaluate the MIC and each of them was numbered, including the negative (DMSO) and positive (ciprofloxacin) control test tubes. Each seed extract were diluted to concentrations ranging from 3.125 to 50%. Each test tube contained 10 ml of extract and nutrient broth and 50%, which implies that (5 ml of crude extract and 5 ml of nutrient broth), 25% (2.5 ml of extract and 7.5 ml of nutrient broth), 12.5% (1.25 ml of extract and 8.75 ml of nutrient broth), 6.25% (0.625 ml of extract and 9.375 ml of nutrient broth), and 3.125 (0.3125 ml of extract and 9.6875 ml of nutrient broth) to bring 10 ml.

To each dilution of *N. sativa, S. aromaticum,* and a mixture of both, 100 µl of the bacteria inoculum were carefully dispensed in sterile screw-capped tubes which consist of crude extracts and nutrient broth. Nutrient broth with bacterial inoculation but no any extract (positive control tubes) and nutrient broth only with no bacterial inoculation (negative control tubes) were included for every test microorganism to demonstrate an adequate microbial growth over the course of the incubation period and media sterility, respectively.

Then tubes were incubated aerobically at 37°C for 24 h and examined for bacterial growth. The lowest concentration (highest dilution) of the extract that produced no visible bacterial growth (turbidity) after overnight incubation were recorded as the MIC [59].

To determine the MBC, all the agar wells, macro-test tubes used in the MIC, which did not show any visible growth of bacteria after the incubation period were sub-cultured on to the surface of the freshly prepared Mueller Hinton Agar (MHA) plates and were incubated at 37°C for 24 h. The MBC was recorded as the lowest concentration (highest dilution) of the extract that did not permit any visible bacterial colony growth on the agar plate after the period of incubation [52].

3.9. Phytochemical Screening

The phytochemical investigation of the chloroform, methanol, ethanol, and acetone extracts of black seed and cloves were carried out using standard tests as described below.

Test for terepenoids (Salkowski test)

0.25g of each extract was mixed with 3ml of sulfuric acid then it formed layer showing reddish brown which indicates the presence of terepenoids.

Test for tannin (Ferric chloride test)

Five drops of iron chloride was mixed with each extract which form black color indicating the presence of tannin.

Test for flavonoids

5ml of ammonia was mixed with 5ml of each extract then sulfuric acid was added to form yellow color which indicates the presence of flavonoids.

Test for saponines

0.5ml of each extract was mixed with distilled water and shacked persistence of frothing shows the presence of saponines.

3.10. Data Analysis

All data were analyzed using the SPSS software package version 16.0 for windows. Means and standard deviations of the triplicates analysis were calculated (analyzed) by one-way analysis of variance (ANOVA) to determine the significance differences between the means followed by Duncan's multiple range test ($P \leq 0.05$). The statistically significant difference was defined as $P \leq 0.05$. And the graphs of all MIC and MBC values were sketched using the application of Microsoft Office Excel 2007.

4. Results

In the present study, antibacterial activities of black seed (*N. sativa*) and clove (*S. aromaticum*) against some selected pathogenic bacteria were tested. Clinical isolate and standard pathogenic bacteria species such as *Escherichia coli, Staphylococcus aureus, Klebsella pneumonia* and *Salmonella typhi* from laboratory of University of Gondar comprehensive and specialized hospital were tested for their sensitivity against Black seed and Clove.

4.1. Analysis of Antibacterial Sensitivity Testing

The diameter inhibition zone of *S. aromaticum and N. sativa* against the selected pathogenic bacteria are shown in tables 3 and 4.

Table 3. *Comparison of the efficiency of the solvents on S. aromatium seed extracts.*

Test organism	Extraction solvent	Mean inhibition (mm) ± STD		
		S. aromaticum	+Ve control	-Ve control
			Ciprofloxacin	DMSO
S. aureus (ATCC25923)	Ethanol	(32.0000 ± 3.00000)^{a}	(28.6667±2.88675)^{a}	0.00±0.00
	Acetone	(22.6667 ± 1.15470)^{a}		
	Hexane	(30.0000 ± 2.00000)^{a}		
	Chloroform	(13.0000 ± 1.73205)^{bc}		
S. aureus (clinical isolate)	Ethanol	(23.0000 ± 3.60555)^{bc}	(18.0000±1.00000)^{a}	0.00±0.00
	Acetone	(18.0000 ± 1.73205)^{a}		
	Hexane	(17.3333 ± 2.51661)^{bc}		
	Chloroform	(12.0000 ± 2.00000)^{bc}		
S. typhi (ATCC14028)	Ethanol	(18.0000 ± 1.73205)^{a}	(22.6667±1.15470)^{a}	0.00±0.00
	Acetone	(22.0000 ± 2.30940)^{a}		
	Hexane	(12.6667 ± 1.15470)^{a}		
	Chloroform	(12.6667 ± 3.05505)^{a}		

Test organism	Extraction solvent	Mean inhibition (mm) ± STD		
		S. aromaticum	+Ve control	-Ve control
			Ciprofloxacin	DMSO
S. typhi (clinical isolate)	Ethanol	(20.6667 ± 2.30940)[a]	(26.0000±7.93725)[a]	0.00±0.00
	Acetone	(23.3333 ± 2.88675)[a]		
	Hexane	(22.3333 ± 4.50925)[a]		
	Chloroform	(18.0000 ± 3.60555)[a]		
E. coli (ATCC2592)	Ethanol	(24.3333 ± 2.51661)[a]	(24.6667±3.05505)[a]	0.00±0.00
	Acetone	(18.6667 ± 2.88675)[bc]		
	Hexane	(13.3333 ± 2.30940)[a]		
	Chloroform	(16.6667 ± 2.88675)[a]		
E. coli (clinical isolate)	Ethanol	(24.0000 ± 3.46410)[bc]	(11.33±2.30940)[a]	0.00±0.00
	Acetone	(22.0000 ± 10.00000)[bc]		
	Hexane	(10.6667 ± 1.15470)[a]		
	Chloroform	(27.6667 ± 4.04145)[bc]		
K. pneumoniae (13883)	Ethanol	(25.3333 ± 2.88675)[a]	(32.6667±9.01850)[a]	0.00±0.00
	Acetone	(29.6667 ± 2.51661)[a]		
	Hexane	(20.6667 ± 2.30940)[a]		
	Chloroform	(24.6667 ± 7.50555)[a]		
K. pneumoniae (clinical isolate)	Ethanol	(25.3333 ± 2.88675)[a]	(32.3333±4.50925)[b]	0.00±0.00
	Acetone	(15.3333 ± 1.52753)[a]		
	Hexane	(15.3333 ± 2.51661)[bc]		
	Chloroform	(15.0000 ± 2.00000)[bc]		

*Values were means of triplicate determinations. Values of the same column followed by different letters are significantly different at ($p \leq 0.05$).

The mean inhibition zone of all *S. aromaticum* crude extract against the entire selected pathogenic bacteria exhibited the highest and the moderate inhibition zone. There was no statistical difference between the mean inhibition zone of *S. aromaticum* hexane and chloroform extract against *S. typhi (ATCC14028)*. The inhibition zone of ciprofloxacin is greater than *S. aromaticum* extracts while it exhibited lower inhibition zone against *E. coli* clinical isolate. The mean inhibition zone of DMSO was zero and it didn't show any effect against the pathogens.

Table 4. *Comparison of the efficiency of the solvents on N. sativa seed extracts.*

Test organism	Extraction solvent	Mean inhibition (mm)±STD		
		N. sativa extract	+ve Control	-ve Control
			Ciprofloxacin	DMSO
S. aureus (ATCC25923)	Ethanol	(19.0000 ± 6.00000)[a]	(28.6667±9.01850)[a]	0.00±0.00
	Acetone	(26.0000 ± 7.21110)[a]		
	Hexane	(21.3333 ± 7.02377[a]		
	Chloroform	(21.0000 ± 2.64575)[a]		
S. aureus (clinical isolate)	Ethanol	(20.3333 ± 3.51188)[a]	(18.0000±1.00000)[a]	0.00±0.00
	Acetone	(30.6667 ± 2.30940)[a]		
	Hexane	(18.6667 ± 2.88675)[bc]		
	Chloroform	(8.0000 ± 2.64575)[a]		
S. typhi (ATCC14028)	Ethanol	(18.3333 ± 5.13160)[a]	(27.0000±1.73205)[a]	0.00±0.00
	Acetone	(14.6667 ± 2.51661)[a]		
	Hexane	(13.6667 ± 2.08167)[bc]		
	Chloroform	(11.6667 ± 1.52753)[bc]		
S. typhi (clinical isolate)	Ethanol	(11.6667 ± 2.88675)[a]	(29.3333±4.61880)[b]	0.00±0.00
	Acetone	(12.6667 ± 4.61880)[a]		
	Hexane	(12.0000 ± 3.46410)[a]		
	Chloroform	(13.0000 ± 5.19615)[a]		
E. coli (ATCC2592)	Ethanol	(24.0000 ± 7.54983)[a]	(33.0000±3.60555)[a]	0.00±0.00
	Acetone	(11.6667 ± 2.88675)[a]		
	Hexane	(10.6667 ± 1.15470)[a]		
	Chloroform	(14.3333 ± 1.52753)[a]		
E. coli (clinical isolate)	Ethanol	(11.6667 ± 2.88675)[a]	(24.0000±7.21110)[a]	0.00±0.00
	Acetone	(12.6667 ± 4.61880)[a]		
	Hexane	(10.6667 ± 1.15470)[a]		
	Chloroform	(15.3333 ± 3.51188)[a]		
K. pneumoni (13883)	Ethanol	(8.6667 ± 1.15470)[bc]	(32.3333±4.50925)[b]	0.00±0.00
	Acetone	(16.6667 ± 6.11010)[a]		
	Hexane	(12.0000 ± 3.46410)[a]		
	Chloroform	(12.6667 ± 3.05505)[a]		

Test organism	Extraction solvent	Mean inhibition (mm)±STD		
		N. sativa extract	+ve Control	-ve Control
			Ciprofloxacin	DMSO
K. pneumoniae (clinical isolate)	Ethanol	(14.6667 ± 3.05505)[a]	(16.6667±2.88675)[a]	0.00±0.00
	Acetone	(13.3333 ± 2.30940)[a]		
	Hexane	(13.3333 ± 2.30940)[a]		
	Chloroform	(12.6667 ± 1.15470)[a]		

*Values were means of triplicate determinations. Values of the same column followed by different letters are significantly different at (p≤0.05).

There was no statistical difference between the mean inhibition zone of *N. sativa* hexane and chloroform extracts against *S. aureus (ATCC25923)* but acetone extract was significantly (P= 0.025) greater than the other extracts. The mean inhibition zone of the chloroform extract against *S. aureus (clinical isolate)* was significantly (P= 0.035) less than the other extracts as well as the weakest antibacterial activity since it is less than (10mm). The mean inhibition zone of ethanol extract against *S. typhi (ATCC14028)* was significantly (P= 0.025) greater than the other extracts. All the extracts Against *S. typhi (clinical isolate), E. coli (clinical isolate)*, *and K. pneumoniae (clinical isolate)* had modarate activity since they range from (10-15 mm). The mean inhibition zone of ethanol extract against *E. coli (ATCC2592*) is significantly (P= 0.03) stronger than the other extracts since it is above (15mm) but ethanol extract against *K. pneumoniae (13883)* is the weakest one when compared to the other extracts.

Table 5. *Comparison of the efficiency of the solvents on combined activity of S. aromaticum and N. sativa seed extracts.*

Test organism	Extraction solvent	Mean inhibition±STD		
		Combined extract	+ve Control	-ve Control
			Ciprofloxacin	DMSO
S. aureus (ATCC25923)	Ethanol	(33.0000 ± 5.00000)[a]	(34.6667±2.30940)[a]	0.00±0.00
	Acetone	(28.0000 ± 2.64575)[a]		
	Hexane	(31.3333 ± 2.30940)[a]		
	Chloroform	(22.6667 ± 4.61880)[a]		
S. aureus (clinical isolate)	Ethanol	(27.3333 ±4.50925)[a]	(29.30940±2.88675)[b]	0.00±0.00
	Acetone	(24.6667 ± 7.50555)[a]		
	Hexane	(22.0000 ± 5.00000)[a]		
	Chloroform	(26.3333 ± 4.04145)[bc]		
S. typhi (ATCC14028)	Ethanol	(28.0000 ± 3.46410)[a]	(28.6667±4.04145)[b]	0.00±0.00
	Acetone	(23.3333 ± 2.30940)[a]		
	Hexane	(17.6667 ± 2.51661)[bc]		
	Chloroform	(28.3333 ± 2.88675)[a]		
S. typhi (clinical isolate)	Ethanol	(28.6667 ± 2.30940)[a]	(30.3333±2.88675)[b]	0.00±0.00
	Acetone	(23.6667 ± 2.88675)[a]		
	Hexane	(12.6667 ± 4.61880)[a]		
	Chloroform	(23.3333 ± 5.77350)[a]		
E. coli (ATCC2592)	Ethanol	(27.3333 ± 1.15470)[a]	(29.0000±7.21110)[a]	0.00±0.00
	Acetone	(13.3333 ± 2.30940)[a]		
	Hexane	(21.6667 ± 2.88675)[a]		
	Chloroform	(24.0000 ± 4.00000)[a]		
E. coli (clinical isolate)	Ethanol	(27.0000 ± 5.00000)[a]	(28.6667±2.88675)[a]	0.00±0.00
	Acetone	(24.6667 ± 3.21455)[a]		
	Hexane	(11.0000 ± 2.64575)[a]		
	Chloroform	(29.0000 ± 3.60555)[a]		
K. pneumoniae (13883)	Ethanol	(26.3333 ± 4.04145)[bc]	(32.6667±1.15470)[a]	0.00±0.00
	Acetone	(30.0000 ± 5.29150)[a]		
	Hexane	(26.3333 ± 1.52753)[a]		
	Chloroform	(28.6667 ± 4.61880)[a]		
K. pneumoniae (clinical isolate)	Ethanol	(29.3333 ± 2.30940)[a]	(31.3333±2.30940)[b]	0.00±0.00
	Acetone	(22.3333 ± 4.50925)[a]		
	Hexane	(19.0000 ± 3.60555)[a]		
	Chloroform	(29.6667 ± 1.15470)[a]		

*Values were means of triplicate determinations. Values of the same column followed by different letters are significantly different at (p≤0.05).

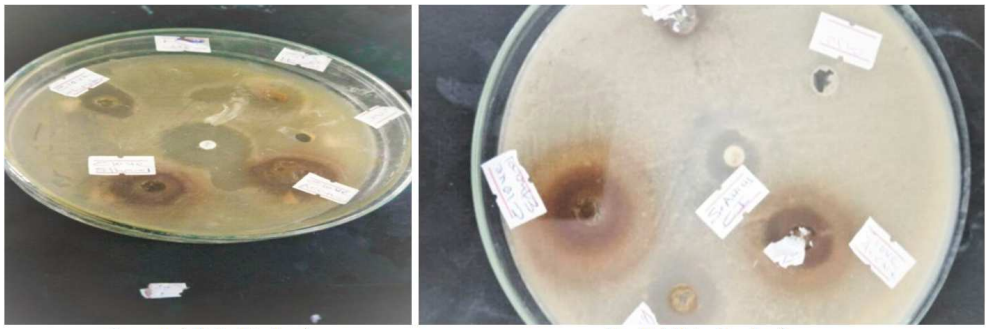


Figure 7. *Antibacterial activity of Clove.*

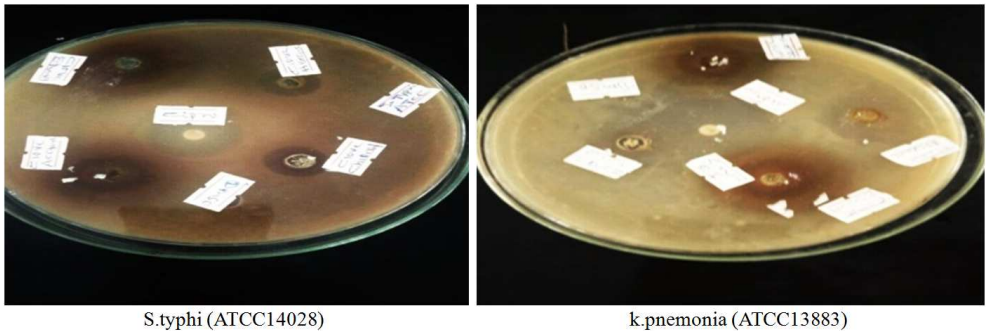


Figure 8. *Antibacterial activity Black seed.*

4.2. Evaluation of Synergistic Effect of N. sativa and S. aromaticum Seed Crude Extracts Against Tested Bacteria

The synergistic antibacterial activity of *N. sativa* and *S. aromaticum* are shown in table 5.

Figure 9. *Combined antibacterial activity of black seed and clove against S. typhi (ATCC14028) and S. typhi (clinical isolate).*

The inhibition zone of all combined extract against *S. aureus (ATCC25923), S. aureus (Clinical isolate)* and *S. typhi (ATCC14028)* exhibited the highest inhibition zone. The inhibition zone of combined hexane extract against *S. typhi* (Clinical isolate) exhibited moderate inhibition zone since it is less than 15mm. All combined extract against *E. coli (ATCC2592)* and *E. coli (clinical isolate)* exhibited the highest inhibition zone except combined acetone and hexane extract. All the combined extract mean inhibition zone against *K. pneumoniae (13883)* and *K. pneumoniae (clinical isolate)* exhibited strong activity since they are all greater than (15mm).

Figure 10. *Combined antibacterial activity of black seed and clove against K. pneumoniae (13883) and K. pneumoniae (clinical isolate).*

Table 6. *Comparison of antibacterial activity of the extracts alone and in combination.*

Test organism	Solvent for extraction	Mean inhibition zone of clove extracts (mm)	Mean inhibition zone of black seed extracts (mm)	Mean inhibition zone of combined extracts (mm)
S. aureus (ATCC25923)	Ethanol	(32.0000±3.00000)[a]	(19.0000±6.00000)[a]	(33.0000±5.00000)[a]
	Acetone	(22.66 ± 1.15470)[a]	(26.000± 7.21110)[a]	(28.0000±2.64575)[a]
	Hexane	(30.000 ± 2.00000)[a]	(21.333± 7.02377)[a]	(31.3333±2.30940)[a]
	Chloroform	(13.000 ± 1.73205)[b]	(21.0000±2.64575)[a]	(22.6667±4.61880)[a]
S. aureus (clinical isolate)	Ethanol	(23.0000±3.60555)[b]	(20.333 ± 3.51188)[a]	(27.3333±4.50925)[a]
	Acetone	(18.0000±1.73205)[a]	(30.666 ± 2.30940)[a]	(24.6667±7.50555)[a]
	Hexane	(17.333 ± 2.51661)[b]	(18.666 ± 2.88675)[b]	(22.0000±5.00000)[a]
	Chloroform	(12.000 ± 2.00000)[b]	(8.0000 ± 2.64575)[a]	(26.3333±4.04145)[b]
S. typhi (ATCC14028)	Ethanol	(18.0000±1.73205)[a]	(18.333± 5.13160)[a]	(28.0000±3.46410)[a]
	Acetone	(22.000± 2.30940)[a]	(14.666± 2.51661)[a]	(23.3333±2.30940)[b]
	Hexane	(12.6667±1.15470)[a]	(13.6667±2.08167)[b]	(17.6667±2.51661)[a]
	Chloroform	(12.6667±3.05505)[a]	(11.6667±1.52753)[b]	(28.3333±2.88675)[a]
S. typh (clinical isolate)	Ethanol	(20.6667±2.30940)[a]	(11.6667±2.88675)[a]	(28.6667±2.30940)[a]
	Acetone	(23.333±2.88675)[a]	(12.666 ± 4.61880)[a]	(23.6667±2.88675)[a]
	Hexane	(22.333±4.50925)[a]	(12.0000±3.46410)[a]	(12.6667±4.61880)[a]
	Chloroform	(18.000 ± 3.60555)[a]	(13.0000±5.19615)[a]	(23.3333±5.77350)[a]
E. coli (ATCC2592)	Ethanol	(24.3333±2.51661)[a]	(24.0000±7.54983)[a]	(27.3333±1.15470)[a]
	Acetone	(18.666 ± 2.88675)[b]	(11.6667±2.88675)[a]	(13.3333±2.30940)[a]
	Hexane	(13.333± 2.30940)[a]	(10.666 ± 1.15470)[a]	(21.6667±2.88675)[a]
	Chloroform	(16.6667±2.88675)[a]	(14.333 ± 1.52753)[a]	(24.0000±4.00000)[a]
K. pneumonia (13883)	Ethanol	(24.0000±3.46410)[b]	(11.6667±2.88675)[a]	(27.0000±5.00000)[a]
	Acetone	(22.0000±10.00000)[b]	(12.666± 4.61880)[a]	(24.6667±3.21455)[a]
	Hexane	(10.6667±1.15470)[a]	(10.666 ± 1.15470)[a]	(11.0000±2.64575)[a]
	Chloroform	(27.6667± 4.04145)[b]	(15.3333±3.51188)[a]	(29.0000±3.60555)[a]
K. pneumonia (clinical isolate)	Ethanol	(25.3333 ± 2.88675)[a]	(8.6667± 1.15470)[b]	(26.3333±4.04145)[b]
	Acetone	(29.6667 ± 2.51661)[a]	(16.6667±6.11010)[a]	(30.0000±5.29150)[a]
	Hexane	(20.6667 ± 2.30940)[a]	(12.0000±3.46410)[a]	(26.3333±1.52753)[a]
	Chloroform	(24.6667 ± 7.50555)[a]	(12.6667±3.05505)[a]	(28.6667±4.61880)[a]
	Ethanol	(25.3333 ± 2.88675)[a]	(14.6667±3.05505)[a]	(29.3333±2.30940)[a]
	Acetone	(15.3333 ± 1.52753)[a]	(13.3333±2.30940)[a]	(22.3333±4.50925)[a]
	Hexane	(15.3333 ± 2.51661)[b]	(13.3333±2.30940)[a]	(19.0000±3.60555)[a]
	Chloroform	(15.0000 ± 2.00000)[b]	(12.6667±1.15470)[a]	(20.6667±1.15470)[a]

*Values were means of triplicate determinations. Values of the same column followed by the letter "b" are significantly different at ($p \leq 0.05$).

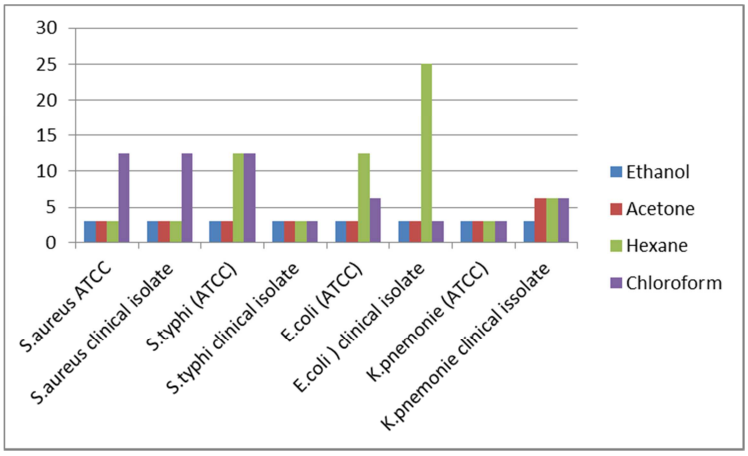


Figure 11. *Minimum inhibitory concentration of clove (S. aromaticum) against pathogenic bacteria.*

The mean inhibition zone of combined ethanol extract against *S. aureus (ATCC25923*) is significantly (P=0.011) greater than the other combined extract. The mean inhibition zone of all the combined extracts against the *S. aureus (clinical isolate)* and *S. typhi (ATCC14028)* are greater than the mean inhibition of the extracts alone. On the other hand the combined hexane extraction against *S. typhi (clinical isolate)* is significantly (P=0.042) less than the clove extract alone but greater than black seed hexane extract alone. The mean inhibition zone of combined acetone extract against *E.*

coli (ATCC2592) is significantly (0.010) less than the clove acetone extracts alone but it is greater than black seed acetone extract alone. The combined activity of all extracts against *E. coli (clinical isolate), K. pneumonia (13883), K. pneumonia (clinical isolate)* is greater than clove and black seed all solvent extracts alone.

4.3. Determination of Minimum Inhibition Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

4.3.1. Determination of Minimum Inhibition Concentration (MIC) of S. aromaticum

As shown in figure 9, the minimum inhibitory concentration of (MIC) *S. aromaticum* chloroform extract against *S. aureus (ATCC25923)* exhibited 12.5% while the other extracts exhibited 3.125%. The same is true for *S, aureus (clinical isolate)*. Ethanol and acetone extract against *S. typhi (ATCC14028)* had MIC of 3.125% while the other extracts exhibited 12.5%. The MIC of all *S. aromaticum* extract against *S. typhi (clinical isolate)* exhibited 3.125%. The MIC of ethanol and acetone extract against *E. coli (ATCC2592)* was 3.125% while hexane and chloroform was 12.5% and 6.25% respectively. The MIC of all *S. aromaticum* extract against *E. coli (clinical isolate)* was 3.125% except hexane extract. The MIC of all *S. aromaticum* extract against *K. pnemonie (ATCC13883)* was 3.125%. The MIC of all *S. aromaticum* extract against *K. pnemonie (clinical isolate)* was 6.25% except ethanol extract.

4.3.2. Determination of Minimum Inhibitory Concentration (MIC) of N. sativa

The minimum inhibitory concentrations of (MIC) all *N. sativa* seed extract against the tested pathogenic bacteria exhibits 3.125%%, 6.25% and 12.5% except chloroform and ethanol extracts. *N. sativa* chloroform extract against *S. aureus (clinical isolate)* exhibits MIC of 25%. The same is true for Ethanol extract against *K. pneumonie (ATCC13883)*.

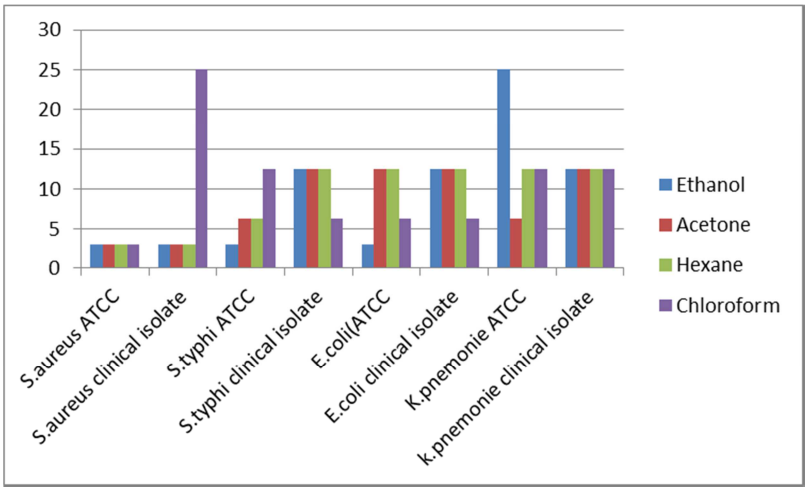


Figure 12. *The minimum inhibitory concentration of N. sativa seed extract against pathogenic bacteria.*

Figure 13. *The minimum inhibitory concentration of N. sativa and S. aromaticum against K. pnemonie (ATCC13883) and S. aurues (ATCC25923).*

4.3.3. Determination of Minimum Inhibitory Concentration (MIC) of Combined Extract

The minimum inhibitory concentrations of all *N. sativa* and *S. aromaticum* combined extract against *S. aureus (ATCC25923)* was 3.125% except chloroform extract. The MIC of the combine ethanol and chloroform extract against *S. aureus (clinical isolate)* exhibited 3.125% while the other extracts were 6.25%. The same is true for the combined extract against *S. typhi (ATCC14028).* The MIC of the combined ethanol and acetone extract against *S. typhi (clinical isolate)* was 3.125% while hexane and chloroform was 12.5% and 6.25% respectively. The MIC of combined hexane and chloroform extract against *E. coli (ATCC2592)* was 6.25% while combined ethanol and acetone extracts were 3.125% and 12.5%. The MIC of combined ethanol and chloroform extract against *E. coli (clinical isolate)* was 3.125% while acetone and hexane was 6.25% and 12.5% respectively. The MIC of all combined extract against *K. pnemonie (ATCC13883)* was 3.125%. Combined Ethanol and chloroform extract against *K. pnemonie (clinical isolate)* exhibited MIC of 3.125% while hexane and acetone was 6.25%.

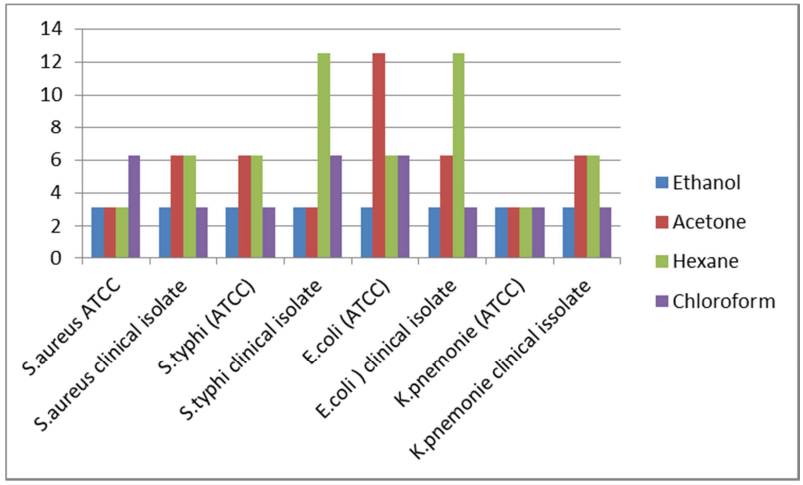


Figure 14. *Minimum inhibitory concentration combined black seed and clove extract.*

Figure 15. *Minimum inhibitory concentration of combined extract against S. aurues (clinical isolate) and S. aurues (ATCC25923).*

4.3.4. Determination of the Minimum Bactericidal Concentration (MBC) of S. aromaticum

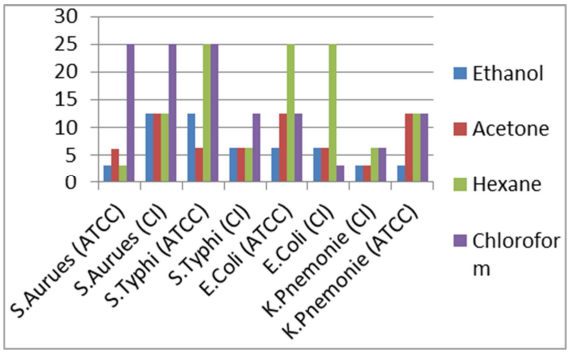


Figure 16. *Minimum bactericidal concentration (MBC) of S. aromaticum.*

The minimum bactericidal concentration of ethanol and hexane extract against *S. aureus (ATCC25923)* was 3.125% whereas the chloroform *S. aromaticum* extract was 25%. All *S. aromaticum* extract against *S, areus (clinical isolate)* exhibit (MBC) of 12.5 except chloroform extract. The (MBC) of All *S. aromaticum* extract against *S. typhi (ATCC14028)* was in between (6.25%-25%). Except chloroform extract against *S. typhi (clinical isolate)* All the *S. aromaticum* extract exhibit MBC of 6.25%. All *S, aromaticum* extract against *E. coli (ATCC2592) E. coli (clinical isolate), K. pneumonie (ATCC) and K. pneumonie (clinical isolate)* was in between (3.125%-25%).

4.3.5. Determination of the Minimum Bactericidal Concentration (MBC) of N. sativa

The minimum bactericidal concentrations of all *N. sativa* seed extracts against *S. aureus (ATCC25923)* were 3.125% and 6.25%. *N. sativa* acetone extract exhibit the lowest (MBC) against *S. aureus* (clinical isolate). The MBC of *N. sativa* hexane and acetone extract against *S. typhi (ATCC14028)* exhibited MBC of 12.5% whereas ethanol and chloroform exhibited 6.25% and 25% respectivel. All *N. sativa* extract against *S. typhi* (clinical isolate) exhibit MBC of 25%. The MBC of acetone and hexane extract against *E. coli (ATCC2592*) was 25% whereas the ethanol and chloroform were 6.25% and 12.5% respectively. The MBC of all extract against *E. coli (clinical isolate)* were 25% except chloroform extract. Hexane and chloroform extract exhibited 25% of MBC against *K. pneumoniae (clinical isolate)* while ethanol and acetone exhibit MBC of 50% and 12% respectively. All *N, sativa* extracts against *K. pneumonie (ATCC13883)* exhibit MBC of 25% except ethanol extract.

4.3.6. Determination of Minimum Bactericidal Concentration (MBC) of Combined Extracts

The minimum bactericidal concentration of all combined black seed and clove extract against *S. aureus (ATCC25923)* was 3.125% except chloroform extract. The MBC of all combined extract against *S. aurues (clinical isolate)* was 3.125% except hexane extract. The MBC of combined hexane extract against *S. typhi (ATCC2592)* was 12.5% whereas ethanol and chloroform combined clove and black seed extract exhibited 3.125%. The MBC of combined ethanol and acetone extract against *S. typhi (clinical isolate)* was 3.125% whereas the hexane and the chloroform were 25% and 6.25% respectively. The MBC of combined ethanol and chloroform extract against *E. coli (ATCC2592)* was 3.125% whereas combined acetone and hexane exhibited 25% and 6.25% respectively. The MBC of

combined hexane extract against *E. coli (clinical isolate)* was 25% while the other combined extracts exhibited 3.125%. The MBC of all combined extract against *K. pneumoniae (clinical isolate)* was 3.125%. Combined Ethanol and chloroform extract against *K. pneumoniae (ATCC13883)* exhibited MBC of 3.125%.

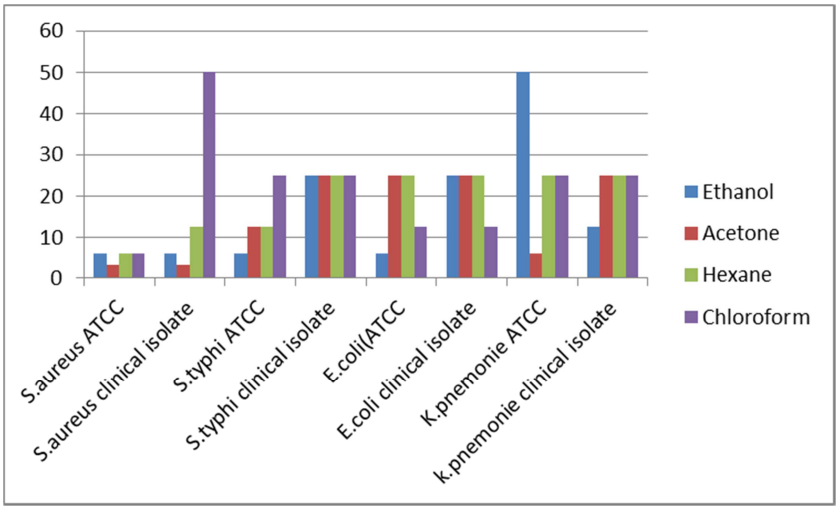


Figure 17. *Minimum bactericidal concentration (MBC) of N. sativa.*

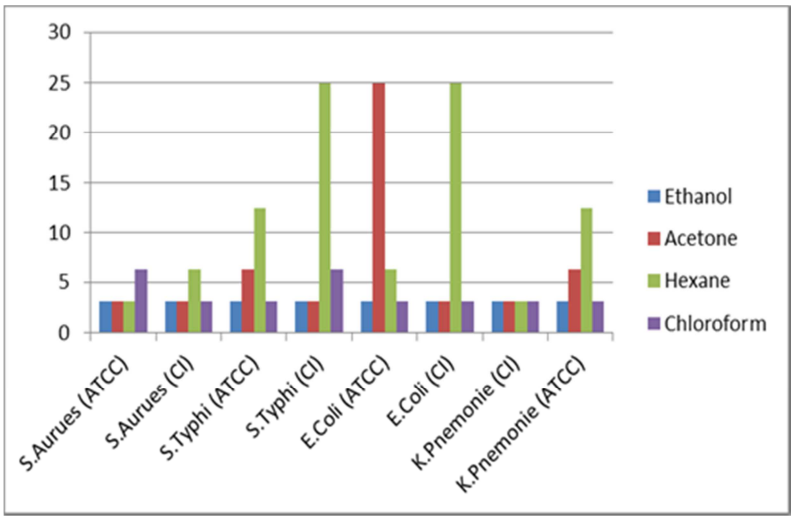


Figure 18. *Minimum bactericidal concentration (MBC) of combined clove and black seed extract.*

MBC of black seed against *K. pnemonie (ATCC13883)*

Figure 19. *MBC of clove against S. typhi (clinical isolate).*

Figure 17 minimum bactericidal concentration (MBC) of *N. sativa* and *S. aromaticum*.

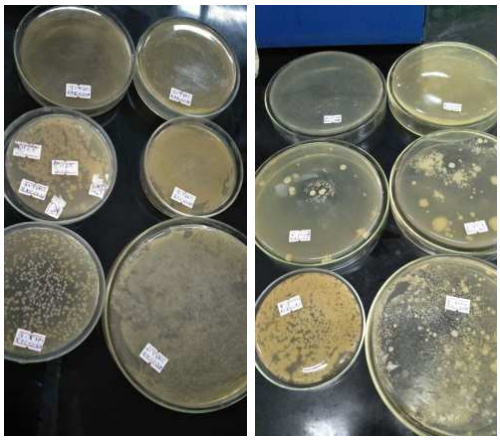


Figure 20. *Minimum bactericidal concentration (MBC) combined clove and black seed extract against S. aurues (ATCC25923) and K. pnemonie (ATCC13883).*

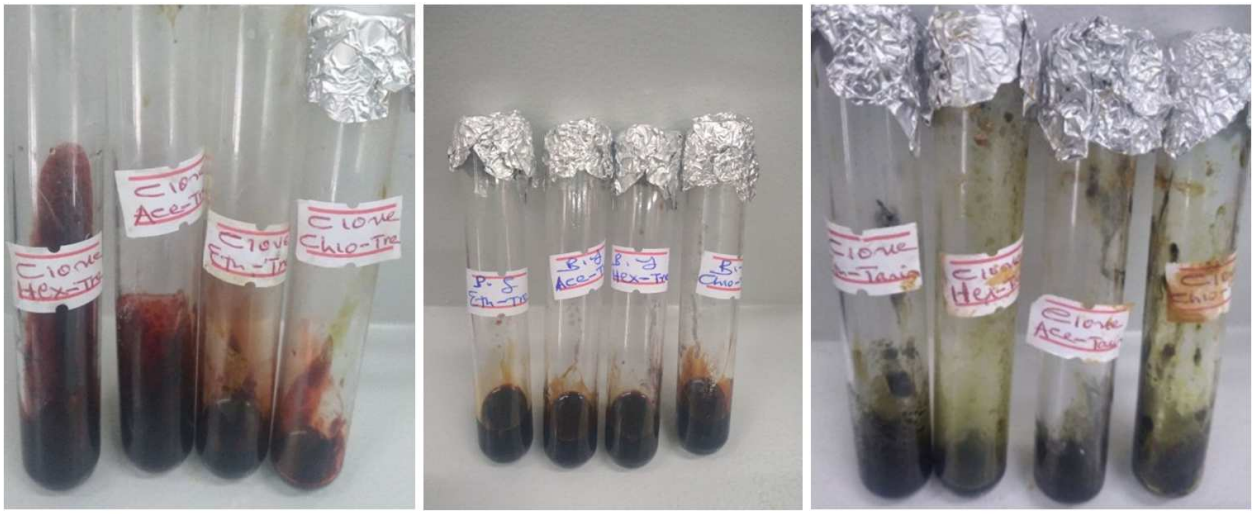
4.4. Phytochemical Analysis Result

Table 7. *Phytochemical screening of N. sativa by standard test.*

	Phytochemicals			
	Terepenoids	**Tanins**	**Flavonoid**	**Saponins**
N. sativa extracts				
Ethanol	+	+	+	+
Acetone	+	+	-	+
Hexane	+	+	+	+
Chloroform	+	+	+	+
S. aromaticum extracts				
Ethanol	+	+	+	+
Acetone	+	+	-	+
Hexane	+	+	+	+
Chloroform	+	+	+	+

(+) = presence (-) = Absence.

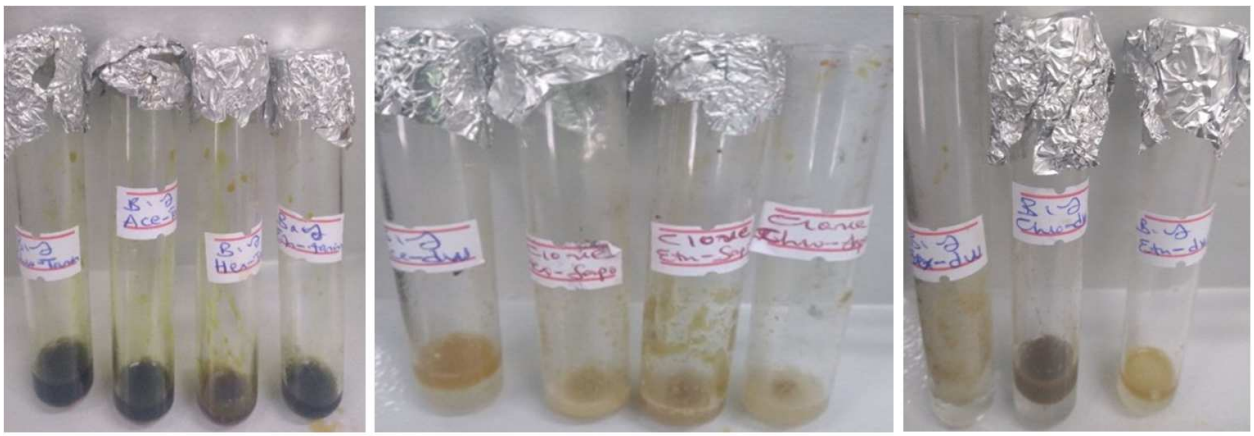
On the table 7 above all the extract of *N. sativa* and *S. aromaticum* contained trepenoids, tanins, flavonoids and saponins except acetone extract of both plants which did not show the presence of flavonoids.



Phytochemical screening of trepenoids from Clove extracts.

phytochemical screening of trepenoids from Black seed extracts

Phytochemical screening of tannin from Clove extracts



phytochemical screening of tannin from from black seed extracts

Phytochemical screening of saponins from clove extracts

Phytochemical screening of saponins from black seed extracts

Figure 21. *Phytochemical screening.*

5. Discussion

In this study, the combined and the separate seed crude extracts showed great activity against most microorganisms tested except *K. pnuemonia (standard)* and *S. aureus (clinical isolate)* for which the separate activity of the *N. sativa* plant showed a relatively lowest antibacterial effect on these pathogenic bacteria. It is interesting to note that the mixture and the separate crude extracts of these plants showed very interesting antibacterial activity against clinical bacteria namely; *K. pneumoniae, S. aureus, S. typhi and E. coli (clinical isolate)* and their standard derivatives where modern antibiotic therapy has limited effect.

The in vitro antibacterial activity of the combined crude seed extracts of *N. sativa and S. aromaticum* also showed excellent antibacterial activity against *S. aureus (ATCC25923), K. pneumonia (clinical isolate), E. coli (ATCC2592), S. typhi (ATCC14028), E. coli (clinical isolate), K. pneumoni (ATCC1338), S. aureus (clinical isolate) and S. tyhpi (clinical isolate).* A study carried out by [2] claimed that ethanol and hexane extracts of the black seed recorded remarkable dose of antibacterial effects against *E. coli, k. pneumonia and S. typhi.* A study carried out by [7] showed that ethanol extract of *N. sativa* was reported to be very effective against *S. aureus*.

A study carried out by [53] showed that antibacterial activity of black seed oil against *E. coli* (clinical isolate) exhibited moderate zone of inhibition which is line with the current study. Similarly the inhibition zone of black seed oil against *S. aureus* (clinical isolate) showed moderate inhibition zone which is lower than the current study. This might because of the black seed oil has not been altered by any chemicals.

A study carried out by [32] showed that ethanol extract of black seed against *E. coli* (clinical isolate) exhibited moderate inhibition zone which is in line with the current study. This study also reveals that ethanol extract of black seed against *S. aureus* (clinical isolate) exhibits lower inhibition zone which is in disagreement with the current study. The researchers in this study also revealed that chloroform extract of black seed against both *E. coli* (ATCC) and *S. aurues* (ATCC) exhibit moderate inhibition zone which is in agreement in this study. The same is true for hexane and acetone extract.

An active principle isolated from seeds of *N. sativa* called thaymoquinone showed a broad spectrum activity against different gram positive and gram negative bacteria such as *S. aureus (ATCC25923), E. coli (ATCC25922)* and *S. typhi (14028)* according to the study carried out by [58]. A study carried out by [56] showed both essential oil and acetone extract of *N. sativa* were found to be effective against *Escherichia coli* and *Salmonella typhi* at all the tested concentrations. A number of reports have been published on the action of *N. sativa* extracts or its oil on different bacterial isolate.

The current result of antibacterial activity of *N. sativa* agrees with the evidence mentioned above. It was found that the ethanol extract of clove was potentially active against *S. aureus* with zones of inhibition ranging from 13.4 to 26.3 mm according to the study carried out by [30]. Phenolic compounds present in clove extract have been reported to possess strong inhibitory effect against a vast range of microorganisms.

The clove hexane extracts inhibit the growth of bacterial species such as *E. coli,* and *Staphylococcus aureus* according to studies carried out by [47]. A study carried out by [48] reveals that clove oil against *S. typhi* (ATCC), *K. pnemonie* (ATCC), *E. coli* (ATCC) and *S. aureus* exhibited highest inhibition zone Except *K. pnemonie* which is line with the current study. The inhibition zone of *K. pnemonie* (ATCC) in disagreement with the current study.

A study carried out by [55] revealed that clove chloroform, ethanol and acetone extract exhibit moderate inhibition zone against *E. coli* (ATCC). Clove Chloroform, acetone and ethanol extract exhibit the highest inhibition zone against *S. aureus (ATCC)* in the same research above. The researchers in the above study also revealed that clove ethanol extract against *K. pnemonie* (ATCC) exhibited moderate inhibition zone while acetone extract exhibited the lowest inhibition zone which is lower than the current study. This might be because of the bacterial strain used is different. Garba, L. et al. [24] showed that ethanol extract of clove exhibited the highest inhibition zone against *E. coli* (clinical isolate) as well as for *K. pnemonie* which is in line with the current study. In accordance with the evidence mentioned above the antibacterial activity of *S. aromaticum* has been confirmed on this current study.

The inhibition zone of crude seed extracts of *S. aromaticum* against most tested pathogenic bacteria were significantly ($p \leq 0.05$) greater than the inhibition zone of *N. sativa*, but less than the inhibition zone of their synergism. This might be because of the main bioactive compound of clove which is essential for antibacterial activity higher than black seed bioactive compound i.e. eugnol (80%) and TQ (30%). On the other hand, the inhibition zone of the synergistic antibacterial effect of mixture of *S. aromaticum* and *N. sativa* crude seed extracts against all tested pathogenic bacteria was significantly ($p \leq 0.05$) far greater than the mean inhibition zone of the antibacterial agents of *S. aromaticum* and *N. sativa* alone. However, it is necessary to underline that the crude seed extracts of *S. aromaticum and N. sativa* separately have also good potential of a broad spectrum of activity against both clinical isolate and standard bacteria.

In addition to this, the results of crude seed extracts of S. aromaticum *and N. sativa* were compared with the common commercial antibiotic discs (Ciprofloxacin). The *S. aromaticum* extracts are significantly greater than ciprofloxacin while some of the extracts are significantly less than ciprofloxacin the same is true for *N. sativa* extracts. The zone of inhibition varied among suggesting that the varying degree of efficacy and different phyto constituents of the extracts on the target organism. This difference in

antibacterial effects may be due bacterial strains differences used in the study.

According to this study, MIC and MBC of *S. aromaticum, N. sativa* and mixture of them against most clinical isolated pathogenic bacteria were 3.125% and 6.25% as well as for those standard pathogenic bacteria, the MIC and the MBC were (6.25 and 3.125%). A study carried out by [48] reveals that clove oil can inhibit *E. coli (*ATCC) and *S. aureus* (ATCC) at the lowest concentration which is in line with the current study. The same is true for MBC. A study carried out by [27] showed that the MIC and MBC of black seed extracts exhibit the lowest inhibition zone which is confirmed on this study.

The results of phytochemical screening all showed favorable results except acetone *extract of S. aromaticum and N. sativa.* A study carried out by [35] claim that ethanol, acetone and chloroform extract of clove do not contain saponins, flavonoids and tanins which is in disagreement with the current study. This might be because of the types of seeds and geographical area they were collected. This study also claim that clove ethanol, acetone and chloroform extract contain trepenoids which is in line with the current study. A study carried out by [32] ethanol extract of black seed contains tanins, flavonoids and saponins. This study also revealed that chloroform extract of black seed contains trepenoids and flavonoids which are in agreement with the current study. According to the study carried out by [32] hexane, acetone and chloroform black seed extracts do not contain tanins and saponins which is in disagreement with the current study. In accordance with the evidence above the presence of tanins, trepenoids, flavonoids and saponins has been confirmed. This indicates that the secondary metabolite of clove and black seed are the main reason for their antibacterial activity.

This finding was largely favor the claim of the local society or community to use the combination of *S. aromaticum and N sativa* rather than using *S. aromaticum and N. sativa* separately for the treatment of different pathogenic bacteria infections and it opens a door to consider and acknowledge the traditional medical practices for the treatment of different infectious ailments using natural resources such as *S. aromaticum and N. sativa.*

6. Conclusion and Recommendation

6.1. Conclusion

In conclusion of this study, antibacterial activities of black seed and clove from Gondar local town were assessed. The result showed potential antibacterial effects of black seed and clove against bacterial strains tested and were effective against *Klebsella pneumonia, Slmonella typhi, Escherichia coli and Staphylococcus aureus* both clinical isolate and standard. In addition on this study combined antibacterial effects of black seed and clove have been assessed. The result showed favorable antibacterial effect against *Klebsella pneumonia, Slmonella typhi, Escherichia coli and Staphylococcus aureus* clinical and standard.

The result showed that low concentration of black seed and clove extracts can inhibit the growth of bacterial strains tested and also low concentration of black seed and clove extracts can kill bacterial pathogens such as *Klebsella pneumonia, Salmonell typhi, Escherichia coli and Staphylococcus aureus,* clinical and standard.

The study also demonstrated that black seed extract contains phytochemicals such as Tanins, trepenoids, flavonoids and saponis as well as clove extract contains the phytochemicals mentioned above. The present study provides clear evidence that the synergistic effect of balck seed and clove has an antibacterial effect against bacteria pathogens and can cure diseases caused by bacterial species such as *Klebsella pneumonia, Salmonella typhi, Escherichia coli and Staphylococcus aureus.* Even though we showed potent in vitro antibacterial black seed and clove extracts for certain bacteria, it may not be translated in vivo.

6.2. Recommendation

The results of this study were found to be promising to be applied to address practical problems of bacterial diseases treatment by using combined extract of black seed and clove. Hence, the researcher has suggested the following recommendations to be considered.

Further studies should be done on the black seed and clove extract for the in vivo using a different plant laboratories and human volunteer for the trail effect of the antimicrobial agent of the black seed and clove extract.

Further studies are required to indicate the active antibacterial components of clove and black seed to study its effect on other bacterial in a preliminary step to introduce black seed's and clove extract or its active components into local and systemic antimicrobial pharmaceutical preparations.

Pharmaceutical industries should take black seed and clove as a valuable agent that can be produce antibiotics.

Governments and agencies should conserve black seed and clove as they have values more than Modern medicine.

Instead of disc diffusion method, cold percolation method, soxhlet extraction, subfraction, might exhibit better antibacterial activity.

Further investigations are necessary to evaluate ant mycobacterial, antiviral, and antiparasitic activity. Moreover, other parts of the plant such as leaves and roots need to be studied to evaluate the studied plant extracts as a potential antimicrobial agent.

List of Abbreviation

ANOVA- One Way Analysis Of Variance
ESBL- Extended Spectrum Beta Lactamase
HM- Herbal Medicine
MBC- Minimum Bactericidal Concentration
MDR- Multi Drug Resistance Bacteria
MHA- Mueller Hinton Agar
MIC- Minimum Inhibitory Concentration
MRSA- Staphylococcus Aureus

N. sativa- Nigella Sativa
S. Aromaticum- Syzigium Aromaticum
TQ- Thymoquinone
TSB- Tryptic Soy Broth
WHO- World Health Organization
ATCC- American type culture collection
CI- clinical isolate
PDS-plant derived substance
MDR-multi drug resisstance
CLSI-clinical and laboratory standard institute
DMSO- dimethyl sulfo oxide

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Last but not least, my deepest gratitude goes to my beloved family for their moral and financial support. I would like to declare this thesis work to my beloved mother Fatuma Mohammed. Your prayer and strength is what makes me to go on in this harsh world. Long live to you umii!

Appendix

Appendix 1. Preparation of Reagents

Appendix 1.1. Turbidity Standard for Inoculums Preparation

0.5 McFarland turbidity standard are used to standardize the approximate number of bacteria in a liquid suspension visually comparing to the turbidity of the test with the turbidity of McFarland.

Procedure

1. Prepare a 1% solution of anhydrous barium chloride ($BaCl_2$).
2. Prepare a 1% solution of sulfuric acid (H_2SO_4).
3. Combine and completely mix the barium chloride and sulfuric acid solutions to form a turbid suspension and $BaSO_4$ in a specific proportion for each McFarland turbidity standard as shown in Table 8.
4. Place the resulting mixture in a foil-covered screw –cap tube.
5. Store the McFarland standard at room temperature (25°C) when not in use. McFarland standard density solution will precipitate and clump over time, and it needs vigorous
6. Vortexing before each use. Mark the tube to indicate the level of liquid, and check before use to be sure that evaporation has not occurred.

Table 8. *McFarland turbidity standard no.*

McFarland turbidity standard no.	0.5	1	2	3	4
1% barium chloride (ml)	0.05	0.1	0.2	0.3	0.4
1% sulfuric acid (ml)	9.95	9.9	9.8	9.7	9.6
Approx. cell density (1×1^8 CFU/ml)	1.5	3	6	9	12

Appendix 1.2. Preparation of Mueller-Hinton Agar

Mueller-Hinton agar (MHA) is the best medium to use for routine antimicrobial susceptibility testing using Kirby-Bauer disc diffusion method for nonfastidious bacteria (both aerobe and facultative anaerobe). Use of media other than Mueller-Hinton agar may result in erroneous results.

Procedure

1. Suspend 38 g of medium (or the components listed above) in 1 liter of purified water.
2. Mix thoroughly.
3. Heat with frequent agitation and boil for 1 minute to completely dissolve the components.
4. Autoclave at 121°C for 15 minutes.
5. Cool to 45°C
6. Pour cooled Mueller Hinton Agar into sterile petri dishes on a level, horizontal surface to give uniform depth.

Note: The plates must be poured to a depth of 4 mm (approximately 25 ml of liquid agar for 100-mm plates and 60 ml of liquid agar for 150-mm plates, but in any case to a measured depth of 4 mm). Plates that are too shallow will produce false susceptible results as the antimicrobial compound will diffuse further than it should, creating larger zones of inhibition. Conversely, plates poured to a depth >4 mm will result in false resistant results.

7. Allow to solidify at room temperature.
8. Check prepared Mueller Hinton Agar to ensure the final pH is 7.3 ±1 at 25°C. Note: If the pH is <7.2 certain drugs will appear to lose potency (aminoglycosides, quinolones, macrolides), while other agents may appear to have excessive activity (tetracycline). If the pH is >7.4, the opposite results may occur.
9. Prepared media can be stored at 4 to 8°C. Mueller-Hinton agar is stable for approximately 70 days from the date of preparation.

Data Analysis

Calculation of P value for efficacy of clove extracts against *S. aureus (ATCC25923)* value using one sample test.

Table 9. *P value calculation using one sample t test.*

	T	Df	Sig. (2tailed)	Mean Difference	95%ConfidenceIntervof the Difference	
					Lower	Upper
Ethanol	18.475	2	.003	32.00000	24.5476	39.4524
Acetone	34.000	2	.001	22.66667	19.7982	25.5351
Hexane	25.981	2	.001	30.00000	25.0317	34.9683
Chloroform	13.000	2	.006	13.00000	8.6973	17.3027
Ciprofloxacin	17.200	2	.003	13.00000	21.4956	35.8378

Appendix 2. Corresponding Figures

Appendix 2.1. Seed Drying

Figure 22. *Black seed and clove drying at room temperature.*

Appendix 2.2. Extracts After Filter

Figure 23. *Extracts after filter.*

Appendix 2.3. Crude Extracts

Figure 24. *Crude extracts.*

Appendix 2.4. Grown Bacteria and Turbidity Check

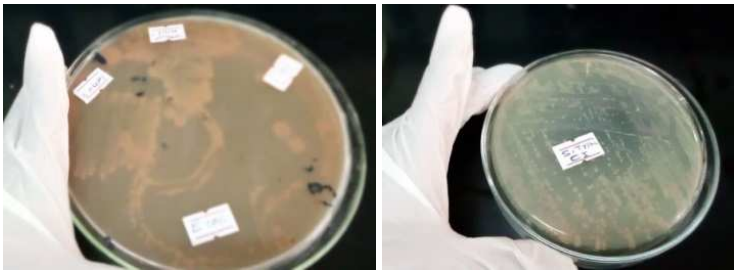

Figure 25. *Grown bacteria.*

Figure 26. *Turbidity.*

Appendix 2.5. Laboratory Procedure and Activities

Figure 27. *Laboratory work.*

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