COMPREHENSIVE STUDY OF ANTIBACTERIAL ACTIVITIES OF MEDICINAL PLANTS AND ESSENTIAL OILS AGAINST *Staphylococcus aureus*

By

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of Bachelor of Science in Microbiology

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Declaration

It is hereby declared that

1. The thesis submitted is my original work while completing my degree at Brac University.

2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.

3. The thesis does not contain material that has been accepted or submitted, for any other degree or diploma at a university or other institution.

4. I have acknowledged all main sources of help.

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Approval

The thesis titled "Comprehensive study of antibacterial activities of medicinal plants and essential oils against *Staphylococcus aureus*" submitted by Tasfia Tawhid (15126006) has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Science in Microbiology on 9 October 2021.

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Ethics Statement

No animals were used in this study.

ABSTRACT

Recently it has been observed that an increasing number of bacteria are building resistance to synthetic antibiotics which hampers controlling infective health conditions. It's familiar to everyone that most antibiotics no longer function; infections are getting worst or impossible to manage. It is time to discover a substitute for antibiotics from organic sources.

Medicinal plants are considered as the abundant bioresource of drugs for conventional medicines, food supplements, and chemical essence for artificial drugs. Nowadays, researchers are progressively focusing their consideration in investigating herbal products due to the higher resistance of microorganisms against the presently used antibiotics and pharmaceutical companies are seeking options for the costly production of synthetic drugs. Medicinal plants can be the method to alternate this condition as most of them are secure with minor side effects.

In the present work, ethanol and aqueous extracts of Neem (*Azadirachta indica*), Tulsi (*Ocimum sanctum*), and Pomegranate (*Punica granatum*) were impinged to microbial sensitive test using disk diffusion method. The microorganism occupied for this research was *Staphylococcus aureus*. The greatest and notable antibacterial activity (zone of inhibition) was noticed with ethanolic extract of Neem (*Azadirachta indica*) extract against *Staphylococcus aureus* (10mm) which is the most effective. No antimicrobial action was found with aqueous extracts of all the three medicinal plants against the chosen bacteria. However, three conventional antibiotics were employed as a positive control against the chosen bacteria which had demonstrated antimicrobial action.

Black cumin oil is familiar to have antibacterial activities and this study was intended to establish the abilities to manage the growth of some skin infection-causing bacteria and also compared the antimicrobial efficacy of Mixed herb oil, Sandalwood oil, and some standard antibiotics. This study of antibacterial activity against chosen pathogen was done by the Disk diffusion method and Agar well diffusion method. In most cases, the inhibition of bacterial growth after one day of incubation demonstrated better results. Mixed herb oil showed the best inhibition against the bacteria chosen for this research whereas Black cumin oil showed maximum and Sandalwood oil showed minimum inhibition against *S.aureus.*

Dedication

I want to dedicate this work to

My Parents

ACKNOWLEDGMENT

I proclaim my submissive gratitude to the Creator of the universe, by whose grace I adapted the strength, patience, and understanding to complete this thesis work.

I am countlessly indebted to my family members for their unconditional support, prayer, and faith in me since my childhood.

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I would like to reveal my gratitude to my supervisor, Mahboob Hossain, Ph.D., Professor, Department of Mathematics and Natural Sciences for his gentle and sincere guidance, supervision, and cooperation. His intellectual vision and constant support lead me to complete my research work through hardship. I will always be grateful to him for giving me the worthy opportunity. I would also like to convey my gratitude to my co-ordinator, Mahbubul Hasan Siddiqee, Assistant professor, Department of Mathematics and Natural Sciences for his humble supervision and cooperation.

Sincerely,

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List of Acronyms

MRSA - Methicillin-resistant *Staphylococcus aureus*

SSTIs - skin and soft-tissue infections

MSSA - Methicillin sensitive *Staphylococcus aureus*

CA-MRSA - Community Acquired-Methicillin resistant *Staphylococcus aureus*

HA-MRSA - Hospital-associated- Methicillin-resistant *Staphylococcus aureus*

MSA - Mannitol Salt Agar

PBP - penicillin-binding protein

TSI - Triple Sugar Iron

MR – Methyl Red

VP - Voges-Proskauer

IMViC - Indole, Methyl Red, Voges-Proskauer, Citrate

MHA - Muller Hinton Agar

NA – Nutrient Agar

TSB - Trypticase Soy Broth

TNTC - Too numerous to count

AI – Activity Index

Chapter 1

Introduction

1.1 Background study

In medical field, *Staphylococcus aureus* (*S. aureus*) is a substantial human disease-causing agent. Hospital-associated MRSA had been widely examined, along with the strong predominance of MRSA (52.3%–76.9%) in mainland China. (Liu, Xu, Yang, Sun, & Ma, 2016; Y.-H. Xiao et al., 2011) Nevertheless, taking into account China's immense area, the data on SSTIs created by community-associated methicillin-resistant *S. aureus* (CA-MRSA) was scanty. Despite the significance of MRSA, methicillin-sensitive *S. aureus* (MSSA) was among the most ordinary formative agents of SSTIs. (Cosgrove et al., 2003; Liu et al., 2016)

S. aureus is commonly resistant to one or more categories of antibiotics, and the ongoing spread of methicillin-resistant *S. aureus* (MRSA) over the past number of decades in both human and animal species has enhanced the risk of developing a resistant infection that makes treatment more complicated and expensive. (Heaton, Gerbig, Sensius, Patel, & Smith, 2020) Based on a study, the MRSA predominance has enhanced from 12% in 1992 to 80.83% in 1999. (Gupta, Mahajan, & Sharma, 2015) Antibiotics are periodically closely related to harmful effects on the host involving hypersensitivity and allergic reactions apart from this problem. (I. Ahmad, Mehmood, & Mohammad, 1998; Gupta et al., 2015) Despite disclosing that some websites promote Neem capsules for MRSA infections, but a literature search showed that no clinical trials have yet been done on the validity of such claims. On the contrary, a few in vitro studies were established which proved the activeness of Neem leaf extract on *Staphylococcus aureus*. (Sarmiento, Maramba, & Gonzales, 2011) Tulsi which is a medicinal plant; has an anti-gonorrheal effect against clinical isolates of beta-lactamase that produces methicillin-resistant *Staphylococcus aureus* and several resistant strains of *Neisseria gonorrhea*. (Eswar, Devaraj, & Agarwal, 2016; Shokeen, Bala, Singh, & Tandon, 2008) Studies have revealed that *Punica granatum* is potent at restraining gram-positive bacterial growth, specifically *Staphylococcus aureus*. (Braga et al., 2005; Holetz et al., 2002; Machado et al., 2003; Prashanth, Asha, & Amit, 2001)

Again, *Staphylococcus aureus* is one such significant stable causative agent exceedingly flexible to antibiotic strain. *Nigella sativa* (black cumin) seed extracts and essential oils are demonstrated to have antimicrobic activity against some pathogens but a less work is done on their effectiveness

against resistant *S. aureus*. The antimicrobic effectiveness of multidrug-resistant hospital strains of *Staphylococcus aureus* has been occupied by *Nigella sativa*. (Salman, Khan, & Shukla, 2016) The greatest impact was noted for sandalwood oil (92.85%) making this the most bio-active extract applied while doing the research. Furthermore, to demonstrate the greatest sensitivity towards *Staphylococcus aureus* the calloused and the substantial embryo extracts were used. (Misra & Dey, 2012)

1.2 Research aim and objectives

The major purpose of the paper is to identify the antibacterial activity of Neem (*Azadirachta indica*), Tulsi (*Ocimum sanctum*), and Pomegranate (*Punica granatum*) towards skin disease-causing pathogen and correlation efficacy with the black cumin oil, mixed herb oil, sandal wood oil, and traditional antibiotics. Considering these, the specific objectives of this study included the following:

1. Determination of the antimicrobial activity of the medicinal plants (three) against *Staphylococcus aureus*.
2. Evaluating the inhibition zone of black cumin oil, mixed herb oil, and sandalwood oil against *Staphylococcus aureus* in contrast of conventional antibiotics.
3. Antimicrobial activity comparison between plant extracts and commercial antibiotics.

1.3 Literature review

In this part, a simple and basic overview of *Staphylococcus aureus* and its morphology, the antibacterial resistance, and natural sources of *Staphylococcus aureus* is mentioned. some plant and oil extraction and several biochemical tests are also reviewed in this section.

1.3.1 *Staphylococcus aureus*

Staphylococcus aureus is generally found within the throat, nares of a wide variety of animal species, and on the human skin layer. *S. aureus* can be spread through animals or animal products (such as raw meats) and person-to-person contact. (Heaton et al., 2020; Kadariya, Smith, & Thapaliya, 2014) *Staphylococcus aureus* normally spreads in developed countries where frequency measurement is 15-40 cases per 100,00 people. (Kaasch et al., 2014; Laupland et al., 2013; Vogel et al., 2016; Zarb et al., 2012) The analysis of the causes of the disease condition of

S. aureus bacterium (SAB) and endocarditis is altered in early decades and *S. aureus* is now becoming the main reason for skin infection in some territories. (Asgeirsson, Thalme, & Weiland, 2018; Federspiel, Stearns, Peppercorn, Chu, & Fowler, 2012; Miro et al., 2005; Murdoch et al., 2009; Selton-Suty et al., 2012; Tleyjeh et al., 2007) *S. aureus* can attain antibiotic resistance demonstratives and thus *S. aureus* isolates repeatedly shows resistance to numerous categories of antibacterial factors. (Kahlmeter et al., 2006) In sense of medical field infections, Methicillin-resistant *Staphylococcus aureus* (MRSA) is still one of the top major multidrug-resistant organisms. (Akpaka, Kissoon, Rutherford, Swanston, & Jayaratne, 2007; Dadashi et al., 2018) and troubles on rising morbidity and mortality. (Dadashi et al., 2018; Jenks, Laurent, McQuarry, & Watkins, 2014) MRSA inflammations destroy ~20,000 confined American sufferers yearly. (Dadashi et al., 2018; M. Rybak et al., 2009)

1.3.2 Morphology of *Staphylococcus aureus*

Staphylococcus aureus is Gram-positive, spherical, or near-spherical in shape, 0.7-1.0μ in diameter, and arranged in pairs or small grape-like clusters. They are not established to be motile, and they do not have the ability to spore. (Cowan, Shaw, & Williams, 1954) They have a low convex elevation, a smooth shiny surface, and an entire edge. When kept at room temperature in diffused daylight colonies increase in size and become colored with a rich cream or gold pigment. The colonies are opaque, butyrous in consistency, and the growth is easily emulsifiable. (Cowan et al., 1954)

1.3.3 History of *Staphylococcus aureus*

Initially, Sir Alexander Ogston explained the staphylococcal disease and its function in boil formation and infestation. (Asgeirsson et al., 2018; Alex Ogston, 1882; Alexander Ogston, 1984) In the last 10 years, a shocking rise in the occurrence of MRSA is examined. (Havaei, Moghadam, Pourmand, & Faghri, 2010) In the early 1960s, at a hospital in Birmingham MRSA was emerged with quick spread, and between 1970–1971 10% of *S. aureus* was identified. (Ayliffe, 1997; Livermore, 2000) Once predominantly a hospital-associated pathogen, the arise of novel strains of MRSA in the 1990s outside of the nosocomial environment led to the recognition of "community-associated MRSA" (CA-MRSA), unlike the historic hospital-associated (HA-MRSA) strains. (Chambers, 2001; Heaton et al., 2020; Papadopoulos et al., 2018; M. J. Rybak & LaPlante, 2005)

Community associated MRSA (CA-MRSA) caused inflammation since 1990. (Papadopoulos et al., 2018; M. J. Rybak & LaPlante, 2005) Recently, the prevalence of MRSA infections has increased and a third epidemiological type was recognized. (Graveland, Duim, Van Duijkeren, Heederik, & Wagenaar, 2011)

1.3.4 Anti-bacterial Resistance of *Staphylococcus aureus*

In consequence of the extensive use of lactam drugs, multidrug-resistant *Staphylococcus aureus* (especially the methicillin-resistant *Staphylococcus aureus*, MRSA) developed into an alarming issue in the past few decades. (Hu, Li, Dai, Cui, & Lin, 2019; Laupland, Church, Mucenski, Sutherland, & Davies, 2003) After the initial case of drug-tolerance strain had been discovered in 1961, MRSA disclosed a marked growth throughout the world. (Bertrand, 2010; Hu et al., 2019) So, the progress of replacement to the existing antibiotics against MRSA inflammations is a matter of concern yet. (Hu et al., 2019; K. A. Lee et al., 2013; Tsuchiya et al., 1996) The manifestation of a foreign and altered penicillin-binding protein (PBP) which is PBP2a is the prime method of methicillin resistance in *S. aureus*. PBP2a has a lower attraction for binding β-lactams such as penicillin, cephalosporins, etc. (Hackbarth, Kocagoz, Kocagoz, & Chambers, 1995) The structural modifications affect penicillin-binding of altered PBPs, the PBP2 gene from two resistant strains was expanded and the sequence was compared with that of a susceptible strain's cloned gene. (Hackbarth et al., 1995) Staphylococcal cassette chromosome mec (SCCmec) – mediated β-lactam resistance leading from the manufacture of an additional penicillin-binding protein (PBP)2a significantly confines the cure options in cases of the hospital- and community-related infections by staphylococci, involving sickness, death, and socio-occupational costs. (Becker et al., 2018; De Kraker, Davey, Grundmann, & Group, 2011; B. Y. Lee et al., 2013) Immunity rates are >60% in mortal *S. aureus* isolates from the overall community and >90% from clinical cases, in spite of the hospital surroundings. (Becker et al., 2018; Köck et al., 2016; Lowy, 2003)

1.3.5 Natural sources of *Staphylococcus aureus*

S. aureus is periodically discovered in large quantity in both aqua and dust, which is connected with swimmer and individuals across the seashore. (Akanbi, Njom, Fri, Otigbu, & Clarke, 2017; Organization, 2003; Papadakis, Mavridou, Richardson, Lampiri, & Marcelou, 1997; Young, 2016) The human epidermis is immediately vulnerable to contagious particles in the timing of bath.

(Akanbi et al., 2017; Henrickson, Wong, Allen, Ford, & Epstein, 2001) and the vulnerability may give rise to the habitation of *S. aureus* with the possibility to attack the body's defense mechanism and induce inflammations. The association between *S. aureus* infection percentage and oceanic disclosure indicates that the entertaining waters are possible origins of community-acquired inflammations. (Akanbi et al., 2017; Charoenca & Fujioka, 1995) Among the swimmers, *S. aureus* and other staphylococci give similar infection rates in the skin, eye, and ear. (Akanbi et al., 2017; Calderon, Mood, & Dufour, 1991; Gabutti, De Donno, Bagordo, & Montagna, 2000; Seyfried, Tobin, Brown, & Ness, 1985)

1.3.6 Biochemical Test

Biochemical tests were conducted in the Microbiology Laboratory for the further configuration of the chosen *S. aureus* isolates following the appropriate method. (Essawi & Srour, 2000) The biochemical tests conducted were indole production test, methyl-red test, Voges-Proskauer test, citrate utilization test, triple sugar iron (TSI) agar test, catalase test, oxidase test, and gram staining. Selected *S. aureus* isolates were developed on the nutrient agar (NA) plates with an incubation period of 24 hours at 37°C.

1.3.7 Gram Staining

The Gram stain is the best commonly used systematic bacterial test. (Gerhardt et al., 1981; Sizemore, Caldwell, & Kendrick, 1990) The procedure is comparatively simple and, in expert hands, gives consistent results. (Sizemore et al., 1990) Christian Gram (as a co-worker of Dr. Friedlander in the municipal hospital of Berlin) tried a progressive method that might be able to differentiate stain Schizomycetes from tissue cells.

1.3.8 Plant extraction

1.3.8.1 Neem **(***Azadirachta indica*)

Azadirachta indica, generally known as Neem is a tall perennial plant whose bark is hard, rough, and flaky. Its leaves are alternate, flowers are small and white. It extends up to 15–20m tall and sometimes reaches up to 35–40m. (Tripathi, Singh, Chauhan, Prasad, & Dubey, 2014) Neem plant has been characterized due to the remarkable bacteriological features in the whole world. Neem oil, leaves, and bark are recognized to develop various clinical issues for the cure of several

infections. (Parthipan et al., 2017) *Azadirachta indica*, commonly recognized as neem, containing azadirachtin which is the major component is widely recognized as flexible medicinal plants showing a broad spectrum of biological activities in the Indian subcontinent such as antiviral, antioxidant, anti-inflammatory, antimutagenic, antimalarial, antiulcer, antibacterial, antifungal, and anticarcinogenic. (Ali, Shahid, Hossain, & Islam, 2019) Neem products have been applied in cosmetology, for example, hair care items, and also have a significant part of the Ayurvedic prescription. Furthermore, Neem derivative goods are also applied to purify the blood, maintain blood sugar levels and boost liver function. In healing skin diseases like eczema and psoriasis, neem leaves have also been applied. (Sohail et al., 2020)

1.3.8.2 Tulsi (*Ocimum sanctum*)

Tulsi (*Ocimum sanctum)* is a several branchy, vertical, firm, and scented plant around 75 cm in height. Tulsi has numerous names such as; Vishnu-Priya Sanskrit, Kala Tulsi in Hindi, and India's Holy Basil in English. (Kulkarni & Adavirao, 2018; Pandey & Madhuri, 2010) The leaves, seeds, and roots of this plant are applied in native herbal treatment. The plant has been historically familiar for its ayurvedic characteristics. (Kulkarni & Adavirao, 2018; Pandey & Madhuri, 2010) Tulsi has two diversities – Black (Krishna Tulsi) and Green (Ram Tulsi). They have identical biochemical and medicative properties. In an animal experiment, there has been an observation that the oral dose of ethanolic extract of tulsi (dose 20mg/kg) of one week has enhanced the amount of adrenaline, non-adrenaline, and decreased dopamine and serotonin levels in mice. (Kulkarni & Adavirao, 2018; Mondal, Mirdha, & Mahapatra, 2009) In another animal study, it is found that due to the exposure to noise stress the alteration in bloodstone amount of corticosterone is prevented by the ethanolic extract of *O. sanctum*. (Das & Vasudevan, 2006; Kulkarni & Adavirao, 2018) For healing of common cold, headache tulsi is used as a traditional medicine. The leaves are great for nerves and to improve memory. (Joshi, Setzer, & Da Silva, 2017; Prajapati, Purohit, Sharma, & Kumar, 2003)

1.3.8.3 Pomegranate (*Punica granatum*)

Punica granatum L., generally familiar as pomegranate, is an important plant in the food sector and is also recognized as an antique, medicative, and divine plant. Traditionally in Ayurvedic, Unani, and Egyptian medicine, its fruit, seed, peel, and roots have been used for a long time. (Akhtar, Ismail, Fraternale, & Sestili, 2015; Šavikin et al., 2018) Nowadays, it is cultured over the Mediterranean region, India, and drier areas of Southeast Asia, America, and tropical Africa. (Šavikin et al., 2018) This fruit is a great source of polyphenols such as ellagic acid and gallic acid. Polyphenols have demonstrated anti-oxidative and anti-swelling properties. (Hosseini, Saedisomeolia, Wood, Yaseri, & Tavasoli, 2016; Yoon & Baek, 2005) The antioxidant property is three times powerful found in pomegranate than many other polyphenols, such as green tea. (Hosseini et al., 2016) In recent investigations, it has been revealed that extracts of pomegranate contain abundant hydrolyzable tannins (such as punicalin, pedunculagin, punicalagin, gallagic acid, and ellagic acid) and anthocyanins (such as delphinidin, cyanidin, and pelargonidin) and possesses anti-tumor properties. (Antuna-Puente, Feve, Fellahi, & Bastard, 2007; Hosseini et al., 2016) A few study has assessed that the inflammation responses of pomegranate extract are indecisive. (Hosseini et al., 2016; Zhang, Gao, Zhang, Liu, & Yu, 2010)

1.4 Essential Oil as a treatment agent

Essential oils having different mixtures of organic components are characterized by aromatic plants as secondary metabolites and they are volatile. (Bakkali, Averbeck, Averbeck, & Idaomar, 2008; Bona et al., 2016) They are derived from leaves, flowers, fruits, seeds, roots, buds, stems, and wood. (Bakkali et al., 2008; Başer & Demirci, 2007; Bona et al., 2016) Many essential oils derived from Mediterranean aromatic plants are generally used as a flavoring in food and drinks, and cosmetic and pharmaceutical products. (Bona et al., 2016; Hussain, Anwar, Sherazi, & Przybylski, 2008; Teixeira et al., 2013) The biological constituent is associated with many factors, mainly the geographic location of the plants, their ecotype, soil biological and physical-chemical properties, and seasonal variations. (Bona et al., 2016; A Copetta, Bardi, Bertolone, & Berta, 2011; Andrea Copetta, Lingua, & Berta, 2006; Masotti, Juteau, Bessière, & Viano, 2003; Raut & Karuppayil, 2014) Essential oils are familiar for their antioxidant, antiviral, antibacterial, antifungal, anticancer, and immune-modulatory activities. (Bakkali et al., 2008; Bona et al., 2016)

1.4.1 Black cumin oil

Black cumin (*Nigella sativa*) is a member of the Ranunculaceae family which is an organic plant indigenous to the Mediterranean region. (Thilakarathne, Madushanka, & Navaratne, 2018) A great deal of interest is concentrated on the oil extracted from this seed either solvent extracted or cold pressing as it is used for medicinal activities. It has been conventionally used for treatments related to body functions such as pulmonary health, abdominal and gastrointestinal health, kidney and liver function, and immune system support. (R. Ahmad & Haseeb, 2015; Thilakarathne et al., 2018) The major constituent in the volatile oil of black cumin is pcymene. it has been conventionally applied for the treatments for asthma, cough, bronchitis, headache, rheumatism, fever, influenza, and eczema. (Burits & Bucar, 2000; Thilakarathne et al., 2018)

1.4.2 Mixed herb oil

Several herbs favored edibles, for example, salad dressings, onion flavored vegetable oil, cooking oils and the like have been made in previous. In some of these prior edibles, the herbs were crushed or boiled in vegetable oil, grease, or fat to derive the flavor from the herb. It is familiar that hot oils extract the flavor better than cold oils. (Edalene, 1963) Another purpose of the invention is to provide the process of preparing a plurality of separate and individual containers of herb-flavored edibles from herbs and liquid with the herb-flavored edible in each container having considerably similar flavor, appearance, and physical properties to the edibles in the other containers. The first step in the method is that of forming a mixture comprising herb flakes capable of flavoring oil. The term flakes are meant to comprise leaves, stems, or other particles of herbs that have the capabilities of flavoring oil and float in or be mixed in the oil they are flavoring. (Edalene, 1963)

1.4.3 Sandalwood oil

Sandalwood (*Santalum album*) is one of the long-lived hemi parasitic and financially significant scented plant species. The Sandalwood species characterized as a xylem tapping root hemiparasites belongs to the family Santalaceae, which consist of 18 species. (Fatima et al., 2019; Subasinghe, 2013) Sandalwood has been classified as ‘vulnerable’ by International Union for Conservation of natural resources. (Arunkumar, Dhyani, & Joshi, 2019; Fatima et al., 2019) The sandalwood tree is native to peninsular India. In India, its original allocation is evaluated around the region with

over 90% of the area in Karnataka (5245 km^2) and Tamil Nadu state covering around 3600 km^2. (Fatima et al., 2019; SH Jain, Angadi, & Shankaranarayana, 2003) Apart from India, it is also inaugurated in Australia, Indonesia, Japan, Belgium, China, Cambodia, Fiji, and Madagascar. (Fatima et al., 2019; Purohit, 2018) Sandalwood oil is renowned as an extremely expensive product, which has been applied in perfumes, cosmetics, toiletries, and pharmaceuticals. (Fatima et al., 2019)

Chapter 2

Materials and Methods

2.1 Sample collection

In this research, 8 environmental samples of *Staphylococcus aureus* were used. All these species were collected from several environmental locations of Dhaka city.

Table 2.1.1: **Sample collection - Sample number, Location, and several *Staphylococcus aureus* species.**

Sample no.	Source	Location	Presence of Staphylococcus species
1	Raw fish water	Uttara 11no sector kacha bazar	4/5
2	Raw fish water	Banani kacha bazar	3/6
3	Lake water	Dhanmondi lake	4/6
4	Jheel water	Hateer jheel	2/5
5	Vegetable water	Uttara 11no sector kacha bazar	1/2
6	Vegetable water	Banani kacha bazar	1/2
7	Dish washing scrub	My home in Uttara	1/2
8	Tea water	Local tea stall at Mohakhali	0/1

2.2 Isolation and identification of the reference strains

To isolate and detect *S. aureus* from the collected samples, one selective media was used which was Mannitol salt agar (MSA). The confirmation of *S. aureus*. was guaranteed by the colony morphology and the cultural characteristics of the inoculated samples on this media.

2.2.1 Mannitol salt agar (MSA)

Mannitol Salt Agar is used as a selective and differential medium to identify *Staphylococcus aureus*. Normally, *Staphylococcus aureus* yields yellow colonies with yellow zones as a result of mannitol fermentation. However, several Staphylococci produce small pink or red colonies that indicate no color change to the medium and they cannot ferment mannitol.

• Sample to be tested, was inoculated into 20ml of MSA, taken on a medium-sized Petri plate and incubated at 37°C for 24 hours.

• After the 24 hours incubation, the morphology and cultural characteristics of the colonies on the media were observed to identify and ensure the presence of *S. aureus*.

• Appearance of yellow color on the colony indicated a positive result for the presence of *S. aureus*.

2.3: Biochemical Confirmation of the S. aureus isolates

Biochemical tests were carried out for the further confirmation of the selected *S. aureus* isolates according to the procedures described in the Microbiology Laboratory.

2.3.1: Methyl Red (MR) test

Bacterial isolates were inoculated into 5ml of dextrose phosphate broth (MR-VP broth) and incubated at 37ºC for 48 hours. After 48 hours of incubation, the pH of the medium was checked by the addition of five drops of MR reagent.

2.3.2: Voges-Proskauer (VP) Test

Bacterial isolates were inoculated into 5ml of dextrose phosphate broth and incubated at 37ºC for 48 hours. Following the incubation, 10 drops of Barritt's reagent A were added to each broth culture and were shaken. Instantly, 10 drops of Barritt's reagent B were added and the cultures were shaken again. The cultures were then kept aside for 15 minutes for the reaction to happen. After 15 minutes, the colors of the cultures were reviewed and the results were recorded.

2.3.3: Triple Sugar Iron (TSI) test

A single bacterial colony of each of the isolates was selected from each nutrient agar plate by a needle and stabbed into 6 ml of TSI agar. Caps of the tubes were loosened and incubated at 35°C

overnight and were noted after 18-24 hours for carbohydrate fermentation, CO_2, and H_2S production.

2.3.4: Citrate Utilization Test

A single colony collected from the bacterial isolates was stabbed into the slant of 3ml of Simmon's citrate agar and incubated at 37^{o}C for 24 hours. After 24 hours of incubation, the color change of the media was observed and the results were noted.

2.3.5: Indole Test

The selected isolates were inoculated in 6ml of peptone water broth and incubated overnight at 37^{o}C. After overnight incubation, five drops of Kovac's reagent were added. Then the colors of the cultures were examined and the results were noted. The formation of a rose red ring at the top of the liquid surface gives a positive result. A negative result can have a yellow or brown layer. Usually, *S. aureus* indicates a negative result for the indole production test.

2.3.6: Oxidase test

Two drops of oxidase reagent (p- Amino dimethylaniline oxalate) were added to the filter papers. The filter papers were labeled according to the sample being tested. A loopful of each bacterial isolate to be tested was streaked onto the filter paper. A positive reaction might turn the paper from violet to purple within a few seconds.

2.3.7: Catalase test

A drop of the catalase reagent (hydrogen peroxide) was taken on autoclaved glass slides. The glass slides were labeled according to the sample to be tested. A colony for each of the bacterial isolates was taken from a nutrient agar plate and placed on the reagent drops on the glass slides. An instant bubble formation indicates a positive result.

2.4: Antibiotic disk diffusion

The antibiotic disk diffusion test is done to identify antimicrobial susceptibility in the bacterial isolates and to ensure susceptibility to drugs of choice for infections caused by these bacteria. In

this research, the effectiveness of five different commercially available antibiotics was determined. The list of antibiotics used is as follows:

Table 2.4.1: Sensitivity measurement of five different antibiotics against *S. aureus*

Provided antibiotic disks	Identification number and amount	Resistant	Intermediate	Sensitive
Erythromycin	E (15µg)	≤13	14-22	≥23
Clindamycin	DA (2µg)	≤14	15-20	≥21
Tetracycline	TE (3µg)	≤14	15-18	≥19
Oxacillin	OX (1µg)	≤10	11-12	≥13
Vancomycin	VA (30µg)	≤9	-	≥12

2.5: Inoculation on the MHA plates

• For eight test samples, eight different MHA (Muller-Hinton Agar) plates with proper labeling of the samples were prepared

• Autoclaved cotton swab was dipped into the bacterial suspensions and rotated so that it is completely wet with the suspension.

• The test tubes having the bacterial suspension were vortexed before dipping the cotton swab.

• The swab was then streaked several times on the dried surface of the MHA plate to make a pure lawn assuring the contact of the cotton of the swab with all the edges of the plate.

• The agar plate was being rotated 90 degrees each time it was being streaked, to assure the even distribution of the inoculums

• The plates were next allowed to dry out.

2.6: Placement of the antibiotic disks

• A burnt sterile forcep was used on an MHA plate to insert the antibiotic discs.

• On the surface of the inoculated MHA plate, five different sterile antibiotic discs were placed.

• Each of the discs was slightly pressed with the forceps on the MHA plate so that it sticks properly to the agar surface

• The disks were not placed close to the edge of the plates was as the zones will not be fully round and lead to an error of the test as the measurement cannot be taken properly

• The MHA plates were next inverted and incubated at 37ºC for one day

2.7: Measurement of the zone of inhibition

• Following the incubation, the zone of inhibition for each of the antibiotics was observed on the MHA plate

• The size of zones for each antibiotic were measured cautiously in millimeters (mm) using a ruler

• All the measurements were taken viewing the back of the Petri dish

• The zone size was noted on the recording sheet in a chart

2.8: Preparation of stock sample

When sufficient strains of microorganisms are created, proper preservation is must for use in the future. If the cultures are not properly preserved, their characteristic features may decrease after a specific time.

In this research, two procedures have been employed for maintaining the organisms in viable conditions over a long period**.**

2.8.1: Long term preservation

The media Trypticase Soy broth (TSB) was prepared in a sterile cryovial. For long-term preservation, 500µl of bacterial culture was grown in Trypticase Soy Broth at 37ºC for 6 hours. After the incubation period, the cryovial was stored at -20ºC, and 500µl of sterile glycerol was added to the broth culture.

2.8.2: Short term preservation

- Stock: 3 ml of T_1N_1 media was prepared in sterile vials. Colonies from the bacterial samples to be preserved were touched by a needle from nutrient agar plates and stabbed onto the butt of the vials. Then the vials were incubated at 37ºC for 6 hours. Following the incubation period, 200µl of paraffin oil was added to the surface of the medium contained in each of the vials. All the vials were labeled carefully and preserved at room temperature.
- Subculture: The chosen colonies from nutrient agar were re-cultured onto fresh NA plates for further stocking and biochemical tests.

Chapter 3

Results

3.1: Confirmation of *Staphylococcus aureus*

Ten different environmental samples were gathered from several locations of Dhaka city. The samples were enriched in LB broth and after enrichment, the culture broth was subjected to a two folds dilution. The samples from the appropriate dilutions were next spread plated on the Nutrient agar to determine the heterotrophic count. For the separation of staphylococcal species, one selective Media- Mannitol Salt agar was used. From 8 samples, 30 isolates have been selected and culture on the selective media ensured the presence of 15 staphylococcal isolates. Colonies showing typical morphological characteristics of Staphylococcus were next taken for biochemical tests. A series of biochemical tests- IMVIC, TSI test, Gram staining, etc. confirmed the presence of *S. aureus*. Through the biochemical tests, 8 *S. aureus* isolates were confirmed from the 10 staphylococcus isolates

Table 3.1.1: Total Aerobic (TA) count of the 8 samples collected on nutrient agar

Sample number and name	Dilution*		CFU/ml
	10^{-1}	10^{-2}	10^{-3}
1.Raw fish water	TNTC	75	$7.5X10^4$
2. Raw fish water	TNTC	70	$7.0X10^4$
3.Dhanmondi lake water	TNTC	63	$6.3X10^4$
4.Hatir jheel water	TNTC	69	$6.9X10^4$
5.Vegetable water	TNTC	72	$7.2X10^4$
6.Vegetable water	TNTC	69	$6.9X10^4$
7.Dish washing scrub	TNTC	52	$5.2X10^4$
8.Tea water	TNTC	59	$5.9X10^4$

TNTC= Too Numerous to Count * 100 µl sample was added on each plate

Table 3.1.2: Colony characteristics of 10 Staphylococcus isolates on selective media

Isolate no.	Shape	Elevation	Margin	Consistency	Colony (MSA)
1	Circular	Convex	Entire	Smooth	Pink to slight yellow
2	Circular	Convex	Entire	Smooth	Pink to yellowish
3	Circular	Convex	Entire	Creamy	Pink to slight yellow
4	Circular	Convex	Entire	Creamy	Pink to slight yellow
5	Circular	Convex	Entire	Smooth	Slight yellow
6	Circular	Convex	Entire	Smooth	Slight yellow
7	Circular	Convex	Entire	Creamy	Pink to yellowish
8	Circular	Convex	Entire	Smooth	Pink to yellowish
9	Circular	Convex	Entire	Smooth	Pink to slight yellow
10	Circular	Convex	Entire	Smooth	Pink to slight yellow

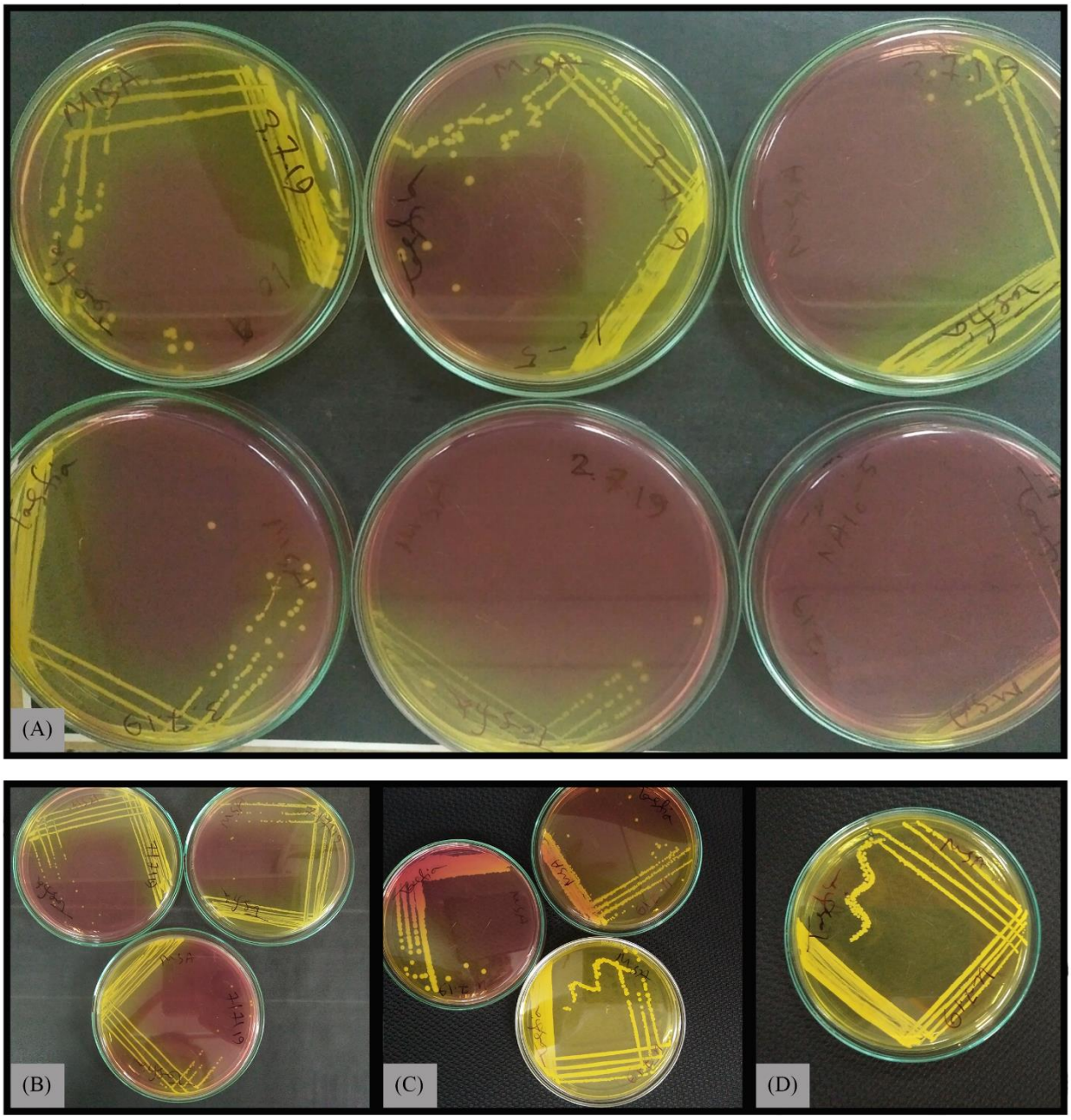

Fig 3.1.1: Growth of *Staphylococcus aureus* on Mannitol Salt Agar (MSA) media.

3.2: Biochemical Identification:

Biochemical tests were performed for the 10 selected staphylococcal isolates. Among these, 8 of the isolates gave standard results for the *S. aureus*. Standard results of the biochemical tests of the isolates are as follows:

Table 3.2.1: **Biochemical Identification Results**

Isolate no.	**Methyl Red test**	**Voges Proskauer test**	**Citrate utilization test**	**Indole test**	**Oxidase test**	**Catalase test**	**Triple Sugar Iron (TSI) test**			
							Slant	Butt	H2	Gas
1	+	+	+	+	_	+	A	A	_	_
2	+	+	+	+	_	+	A	A	_	_
3	+	+	_	+	_	+	A	A	_	_
4	+	+	_	+	_	+	A	A	_	_
5	+	+	_	+	_	+	A	A	_	_
6	+	+	_	+	_	+	A	A	_	_
7	**+**	**+**	**_**	**+**	**_**	**+**	A	A	**_**	**_**
8	+	+	_	+	_	+	A	A	_	_

KEY: + = positive, - = negative, A= acidic condition (Yellow). The Methyl Red test for 8 isolates showed positive results. Voges Proskauer test for 8 isolates showed a positive result. Citrate utilization test, isolate no 1 & 2 showed positive result and rest of the isolates showed negative result. Indole test for 8 isolates showed positive results. Oxidase test for 8 isolates showed negative results. Catalase test for 8 isolates showed positive results. TSI test for 8 isolates showed acid formation in slant and butt, no H2S and Gas production.

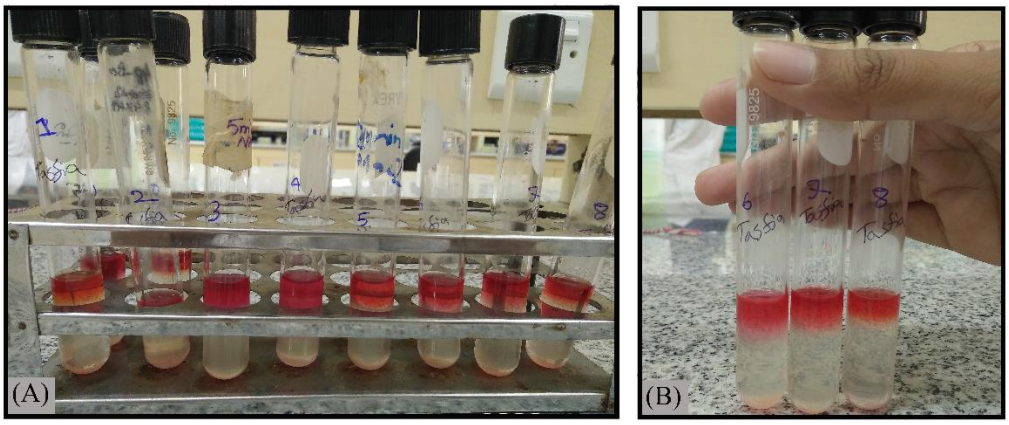

Fig 3.2.1: Methyl Red (MR) test of isolated *Staphylococcus aureus*. (A) MR test result of ten isolated *S. aureus* of different sources. (B) Red color formation indicates a positive result.

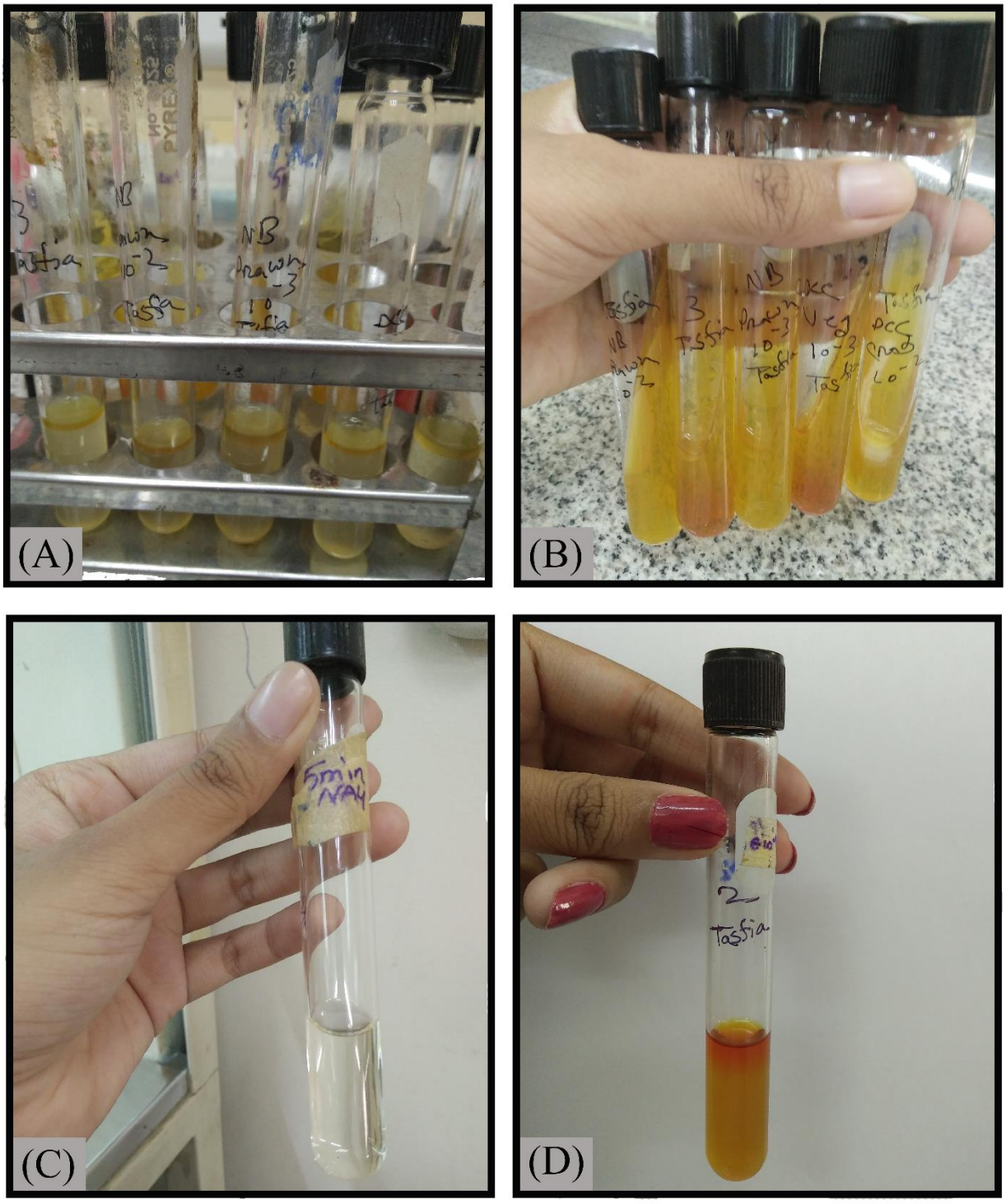

Fig 3.2.2: (A) Voges Proskauer (VP) test represents the formation of acetoin which showed a positive result. (B) Triple sugar iron (TSI) test 1,3,5 undergoes acid formation showing yellow color slant and butt and 2,4 remains alkaline showing red color butt and slant. (C) Indole test showed negative result and (D) Indole test showed positive result by forming a red rose ring.

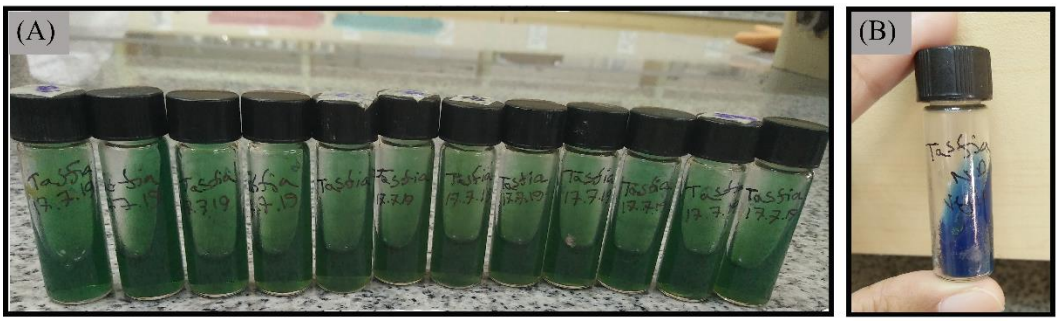

Fig 3.2.3: Citrate Utilization test of isolated *Staphylococcus aureus*. (A) Citrate Utilization test of twelve isolated *S. aureus* of different sources which showed the negative result of original green media and (B) The vial showed positive result by turning the media into blue color.

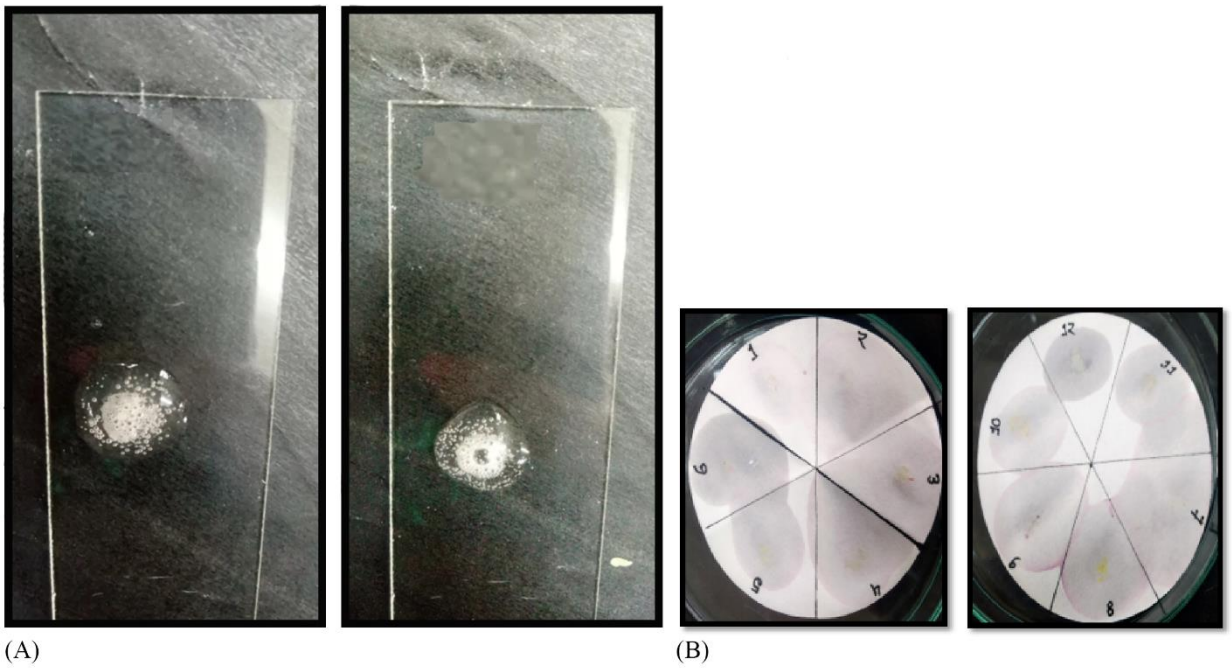

Fig 3.2.4: (A) Catalase test result of isolated *Staphylococcus aureus* showed a positive result. (B) Oxidase test results of isolated *Staphylococcus aureus* showed negative results (1,2,5,10,11,12) and the rest of them showed a positive result.

3.3: Morphology confirmation of the isolates through gram staining:

A colony of the bacterial isolate was touched with a burnt loop and placed on a sterile glass slide. A drop of saline was taken by the loop and then the bacterial colony was smeared on the glass slide. The glass slide was heat fixed and the smear was enabled to dry. A drop of crystal violet, the primary stain was added to the smear and after one minute, the crystal violet was thoroughly

washed off the glass slide. A drop of the mordant, iodine was next added and then after one minute, the grams of iodine were thoroughly washed off the slide. A drop of 70% ethanol was added as the decolorizing agent and washed off after 15 seconds. Safranin, the counter stain was added and after 60 seconds it was washed off the glass slide. The slide was enabled to dry off completely, after which it was observed under the microscope. The morphology of the confirmed isolates from the biochemical test was monitored through gram staining. *S. aureus* is usually gram-positive organism and would give purple color on staining as seen under the microscope.

Table 3.3.1: **Morphological properties of the isolates from gram staining**

Sl no.	Isolates	Shape under microscope	Color after staining	Arrangement	Inference
1	Raw fish water	Cocci	Purple	Cluster	Gram-positive
2	Raw fish water	Cocci	Purple	Cluster	Gram-positive
3	Dhanmondi lake water	Cocci	Purple	Cluster	Gram-positive
4	Hatir jheel water	Cocci	Purple	Cluster	Gram-positive
5	Vegetable water	Cocci	Purple	Free and cluster	Gram-positive
6	Vegetable water	Cocci	Purple	Free and cluster	Gram-positive
7	Dish washing scrub	Rod	Purple	Free	Gram-positive
8	Tea water	Rod	Purple	Free	Gram-positive

After performing all the biochemical and morphological tests, 8 isolates were chosen- 1,2,3,4,5,6which gave similar results when compared with the standard results. The research work from here was next performed with these 6 isolates and their antibacterial resistance pattern were checked, observed, and compared.

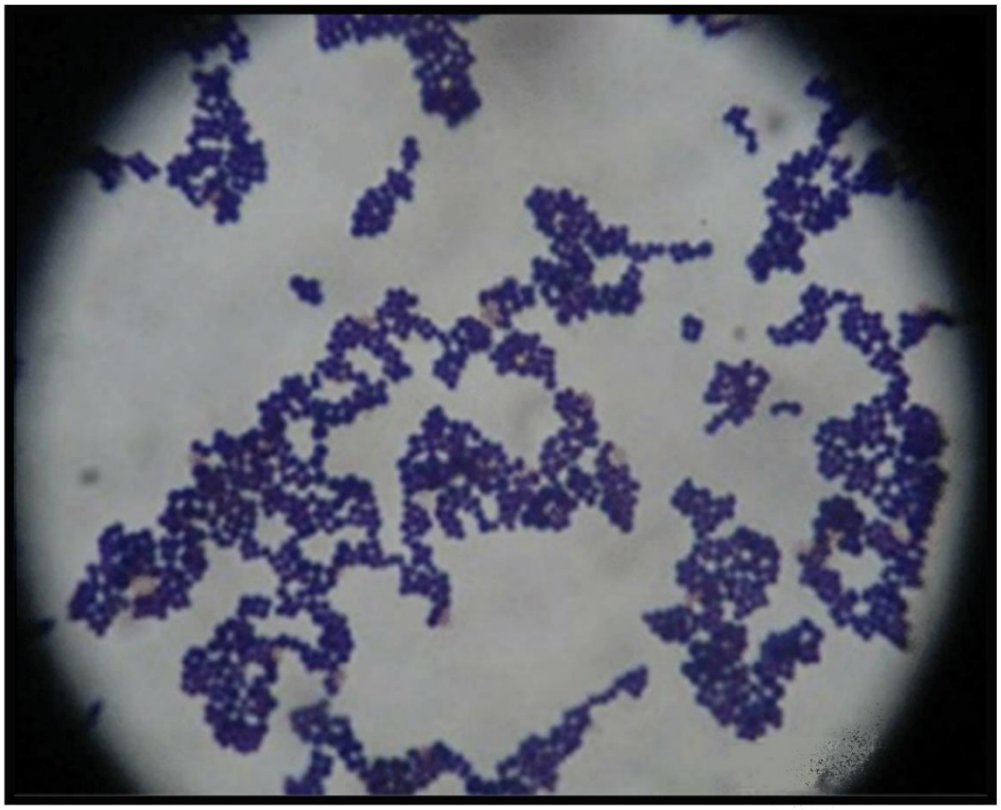

Fig 3.3.1: Gram staining result of *Staphylococcus aureus* showing positive result.

3.4: Selective antimicrobial activity test using antibiogram method

The confirmed isolates were chosen to perform the standard disc diffusion test. The test was conducted with five commercial antibiotics- Erythromycin (E 15), Tetracycline (TE 3), Oxacillin (OX 1), Clindamycin (DA 2), and Vancomycin (VA 30). The size of the zone of inhibition determines the resistance and sensitivity of the organisms to the respective antibiotics. The antibiotic disk diffusion test carried out for each of the isolates showed the following results.

Table 3.4.1: **Antibiotic susceptibility test of isolated *Staphylococcus aureus* (Raw fish water from Uttara 11no sector kacha bazar)**

Antibiotic disks	Zone of inhibition in diameter(mm)
E 15	26 (Sensitive)
TE 3	30 (Sensitive)
OX 1	15 (Sensitive)
DA 2	24 (Sensitive)
VA 30	22 (Sensitive)

Table 3.4.2: **Antibiotic susceptibility test of isolated *Staphylococcus aureus* (raw fish water from Banani kacha bazar)**

Antibiotic disks	Zone of inhibition in diameter(mm)
E 15	21 (Intermediate)
TE 3	22 (Sensitive))
OX 1	20 (Sensitive))
DA 2	12 (Resistant)
VA 30	7 (Resistant)

Table 3.4.3: **Antibiotic susceptibility test of isolated *Staphylococcus aureus* from Dhanmondi lake water**

Antibiotic disks	Zone of inhibition in diameter(mm)
E 15	0(Resistant)
TE 3	20(Sensitive)
OX 1	16(Intermediate)
DA 2	22(Sensitive)
VA 30	8(Resistant)

Table 3.4.4: **Antibiotic susceptibility test of isolated *Staphylococcus aureus* from Hatir jheel water**

Antibiotic disks	Zone of inhibition in diameter(mm)
E 15	8 (Resistant)
TE 3	18 (Intermediate)
OX 1	14 (Sensitive)
DA 2	14 (Intermediate)
VA 30	12 (Sensitive)

Table 3.4.5: **Antibiotic susceptibility test of isolated *Staphylococcus aureus* (Vegetable water from Uttara 11no sector kacha bazar)**

Antibiotic disks	Zone of inhibition in diameter (mm)
E 15	10(Resistant)
TE 3	20(Intermediate)
OX 1	0(Resistant)
DA 2	0(Resistant)
VA 30	4(Resistant)

Table 3.4.6: **Antibiotic susceptibility test of isolated *Staphylococcus aureus* (Vegetable water from Banani kacha azar)**

Antibiotic disks	Zone of inhibition in diameter(mm)
E 15	20(Intermediate)
TE 3	4(Resistant))
OX 1	0(Resistant)
DA 2	0(Resistant)
VA 30	0(Resistant)

3.5 Plant extracts obtained from different solvents

The table below outlined the number of crude extracts obtained from three different medicinal plant samples using two different solvents: ethanol and distilled water.

Table 3.5.1: **Number of plant extracts obtained using ethanol**

Plant sample	Type of solvent	The volume of solvent(ml)	Amount of powder(gm)	Amount of crude extract(gm)
Neem	Ethanol	100	10	0.49
Tulsi	Ethanol	100	10	0.69
Pomegranate	Ethanol	100	10	0.54

Table 3.5.2: **Number of plant extracts obtained using distilled water**

Plant sample	Type of solvent	The volume of solvent(ml)	Amount of powder(gm)	Amount of crude extract(gm)
Neem	Distilled water	100	10	0.41
Tulsi	Distilled water	100	10	0.62
Pomegranate	Distilled water	100	10	2.10

3.6: Observation of antibacterial activity of ethanolic, methanolic, and aqueous extracts of selected plants with allopathic antibiotics:

In this research, *Staphylococcus aureus* was used for comparing the antimicrobial properties of ethanolic and aqueous extracts of the three medicinal plants: Neem, Tulsi, and pomegranate. During the antimicrobial assay, a positive control in the form of the antibiotic disc was used on the petri plate as well as different extracts of each of the three medicinal plants to assess and compare the activity of the extracts with that of the antibiotic disc against the bacteria.

Table 3.6.1: **Zone of inhibition produced by antibiotic, ethanol, and aqueous extract of neem against *Staphylococcus aureus***

Name of bacteria	**Name of antibiotic**	**Zone of inhibition(mm)**		
Staphylococcus aureus		Antibiotic disk	Ethanolic extract	Aqueous extract
	Erythromycin	26	10*	0
	Vancomycin	25		
	Clindamycin	30		

*****The Highest zone of inhibition by the extract

Table 3.6.2: **Zone of inhibition produced by antibiotic, ethanol, and aqueous extract of Tulsi against *Staphylococcus aureus***

Name of bacteria	**Name of antibiotic**	**Zone of inhibition(mm)**		
Staphylococcus aureus		Antibiotic disk	Ethanolic extract	Aqueous extract
	Erythromycin	26	2	0
	Vancomycin	25		
	Clindamycin	30		

Table 3.6.3: **Zone of inhibition produced by antibiotic, ethanol, and aqueous extract of pomegranate against *Staphylococcus aureus***

Name of bacteria	**Name of antibiotic**	**Zone of inhibition(mm)**		
Staphylococcus aureus		Antibiotic disk	Ethanolic extract	Aqueous extract
	Erythromycin	26	8	0
	Vancomycin	25		
	Clindamycin	30		

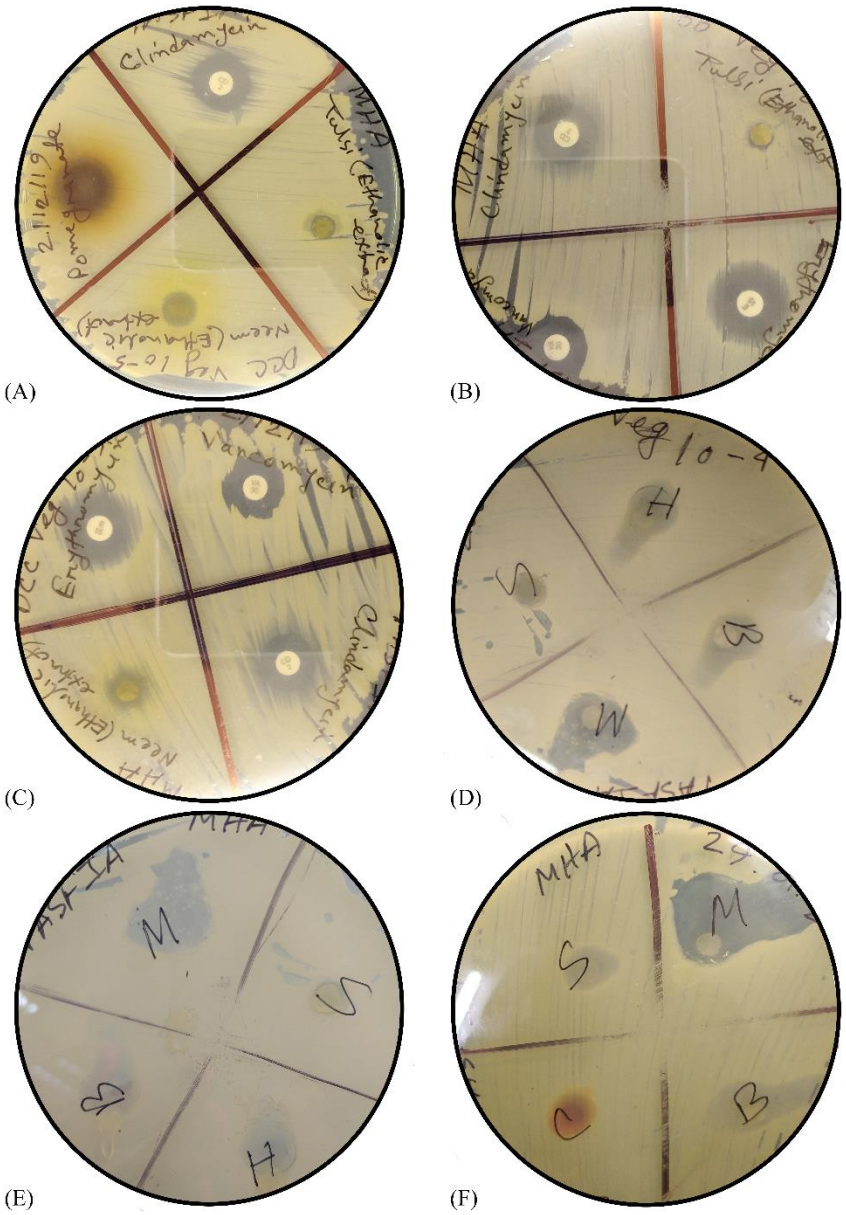

Fig 3.6.1: Antibiotic susceptibility test result of the confirmed *S. aureus* isolates compared with the medicinal plants and essential oils. (A) Antibiotic susceptibility of Neem, Tulsi, and Pomegranate against the conventional antibiotic Clindamycin. (B) Antibiotic susceptibility of Tulsi against the conventional antibiotic Erythromycin, Vancomycin, and Clindamycin. (C) Antibiotic susceptibility of Neem against the conventional antibiotic Erythromycin, Vancomycin, and Clindamycin. (D-F) Antibiotic susceptibility of Black Cumin Oil, Mixed Herb Oil against the conventional antibiotic Erythromycin, Vancomycin, and Clindamycin.

3.7: Comparative study of antibacterial activity by showing activity index

Activity index (AI) values are the estimated measure of the potency of antimicrobial activity of plant extracts by quantitatively comparing them to the respective standard antibiotics. In this study, the AI values are calculated for the ethanolic extract of three different medicinal plants against three different antibiotics, named: Erythromycin, Vancomycin and Clindamycin Using the formula,

$$\text{Activity Index (AI)} = \frac{Zone\ of\ inhibition\ of\ extract}{Zone\ of\ inhibition\ of\ antibiotic}$$

AI values as shown in the following graphs have been calculated for the ethanolic extracts of all the three medicinal plants against the selected bacteria.

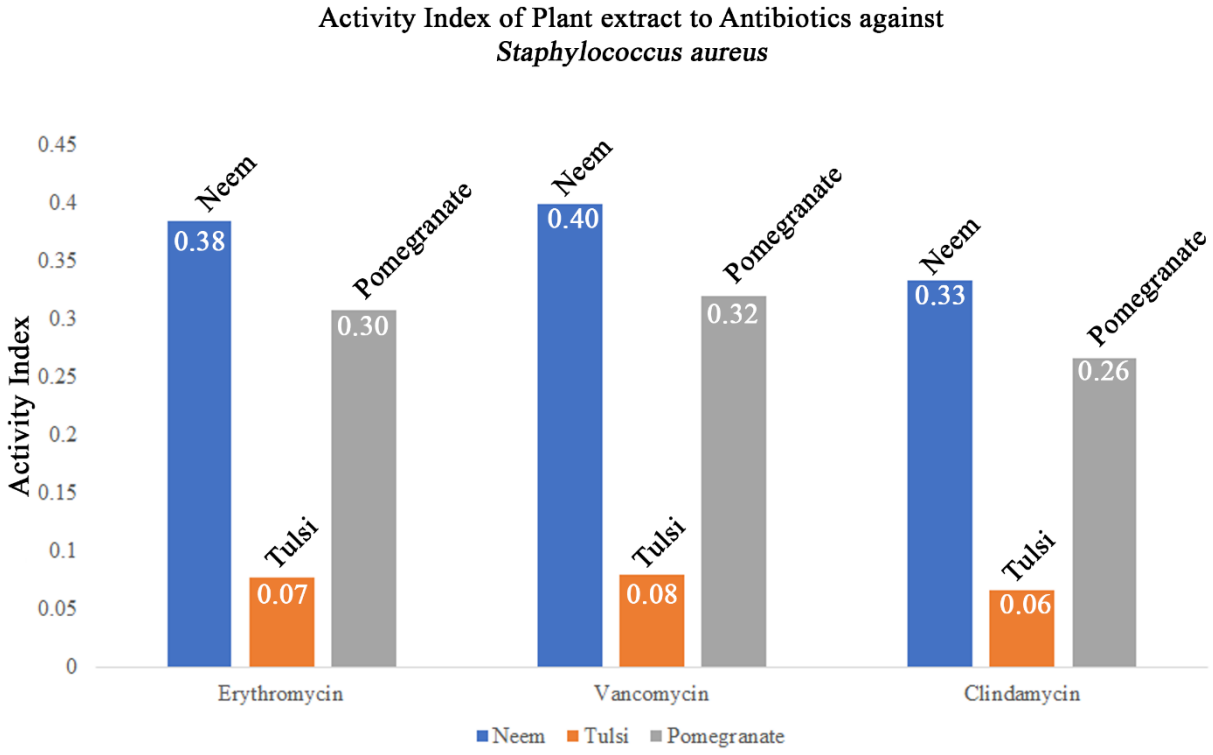

Fig 3.7.1: Activity Index of Neem to antibiotics against *Staphylococcus aureus* in which the value 0.38 denotes effective result against Erythromycin. Activity Index of Neem to antibiotics against *Staphylococcus aureus* in which the value 0.33 denotes effective result against Clindamycin. Activity Index of Neem to antibiotics against *Staphylococcus aureus* in which the value 0.50 denotes effective result against Vancomycin.

3.8: Observation of antibacterial activity of Black cumin oil, Mixed herb oil, and sandalwood oil with allopathic antibiotics

A similar quantity of three types of oil was taken and serially diluted in saline. Then the same amount of diluted suspension was taken and spread on Muller Hinton Agar (MHA) containing a similar amount of bacterial lawn. After incubation, fewer colonies emerged on the agar plate containing oils than saline which determines the antimicrobial activity of these oils against the selected pathogen. Pathogens were incubated with oils for 24 hours. The number of colonies from each plate was essential to determine the inhibition rate.

Table 3.8.1: Total viable colony count of *Staphylococcus aureus* in MHA and MHA with oils

Pathogen	Dilution	MHA 24 hours incubation CFU/100µl	MHA and Black cumin oil 24 hours incubation CFU/100µl	MHA and Mixed herb oil 24 hours incubation CFU/100µl	MHA and Sandalwood oil 24 hours incubation CFU/100µl	Inhibition of Percentage
Staphylococcus aureus (2.8×10^7 per ml)	10^{-1}	TNTC	0	0	2	0.99%
	10^{-2}	TNTC	0	0	0	0
	10^{-3}	TNTC	0	0	0	0
	10^{-4}	280	0	0	0	0

3.9: Comparison among three types of oil and conventional Antibiotics

Comparing antimicrobial activity of three types of oil and conventional antibiotics was identified and the zone of inhibition was observed and the diameter (mm) was calculated as follows:

Table 3.9.1: Zone of inhibition in response to three types of oil and conventional antibiotic disks

		Zone of inhibition(mm)			
Name of organism	**Name of Antibiotics**	**Antibiotic disk**	**Black cumin oil 50µl**	**Mixed herb oil 50µl**	**Sandalwood oil 50µl**
Staphylococcus aureus	Erythromycin	26	18	24	14
	Vancomycin	25			
	Clindamycin	30			

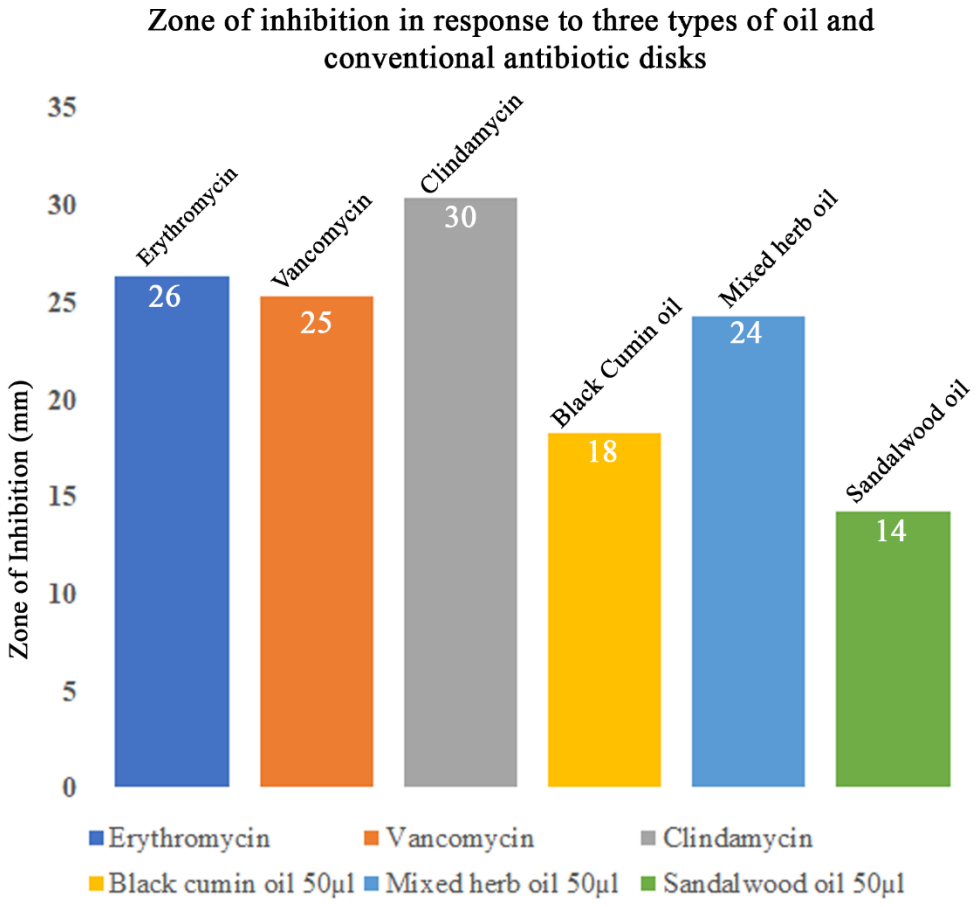

Fig 3.9.1: Zone of inhibition in response to three types of oil and conventional antibiotic disks. In which Mixed herb oil showed the best antimicrobial activity (24mm) against *S. aureus*. Black cumin oil also showed better antimicrobial activity (18mm) against *S. aureus*. Sandalwood oil also showed good antimicrobial activity (14mm) against *S. aureus*.

4. Discussion

The objective of the research was to prepare three different types of extracts from Neem, Tulsi, and pomegranate using two different solvents: ethanol and distilled water to determine the antimicrobial activity of the various extracts of the three medicinal plants against *Staphylococcus aureus*. Nowadays, researchers are progressively turning their focus to investigating herbal products to combat the increasing occurrences of microbial drug resistance.

Many researchers have revealed the antioxidant activity of the *Azadirachta indica*. Antioxidant activity of the flavonoids (orientin and vicenin) in vivo was declared in a major depletion in the radiation-induced lipid peroxidation in mouse liver. (Pandey & Madhuri, 2010) The inhibition zone and their comparison by the ethanolic extracts of all the three medicinal plants showed that the highest zone of inhibition (10mm) (Table 3.6.1) was determined by Neem against *Staphylococcus aureus*. On the other hand, the inhibition zone and their comparison by the ethanolic extracts of all the three medicinal plants showed that the best zone of inhibition (8mm) (Table 3.6.3) was done by pomegranate against *Staphylococcus aureus*. Different bio-actives for example asteroids, sugars, triterpenoids, alkaloids, reducing sugars, tannins, flavonoids, sesquiterpene lactones are accountable for the in-built antimicrobial properties. (Ali et al., 2019; Tesso, Nisha, & Kumsa, 2015; Vijayaram et al., 2016) Although the ethanolic extracts of all three medicinal plants have shown antimicrobial activity, the aqueous extract of these medicinal plants did not show any antimicrobial activity. This might be due to the appearance of some of the chemical elements in these plant extracts whose solubility may vary in different solvents. In this research, ethanolic extract of Neem sample had shown significantly better antibacterial activities contrary to aqueous extract, where they considered that, it may be due to organic properties of ethanol and also for its high ability to dissolve more organic and active antibacterial compositions. However, in the common households of Bangladeshi people, Neem and Tulsi are not generally eaten in raw form, rather they are usually eaten in a mixture with water. Normally it is not eaten in a mixture with ethanol or methanol. Neem leaves are typically not eaten but used externally on the body as a homemade herbal remedy for skin allergies by either boiling the leaves in water to make an aqueous extract or made into a semi-solid paste using water by mortar and pestle. In order to reduce toxins from the body, enhance body immunity and improve gut health the Neem leaf powder is used. Also, it can produce the bioconversion of nanoparticles from their equivalent at comparatively smaller amounts because of the native availability of the Neem plant. (Sohail et al.,

2020) So, the in vitro antimicrobial effect shown in this study may consider that these plant extracts may not be as effective against any of these organisms in vivo. The concentrations of the extracts can be changed, or different extraction methods can be performed to produce different substantial results. So, by considering the overall outcomes of the antimicrobial effects of the three medicinal plant extracts of this study, it can be noted that further thorough experimental in-vitro study has to be done which may lead to clinical trials, giving rise to production of herbal medicines using these medicinal plant extracts.

A comparison of the antibacterial activity of the medicinal plant extracts and allopathic antibiotics was done in this study by calculating the activity index values. The AI values are the approximate potency of antimicrobial activity of plant extracts by quantitatively comparing them to the respective standard antibiotics. High AI values indicate that the extracts have excellent activity against the bacteria in comparison with the standard antibiotics.

From this study, the activity index value "0.40" obtained from the ethanolic extract of Neem to Vancomycin for *Staphylococcus aureus* was the remarkable one.

However, the AI values were very low which were acquired for the antibiotics that were highly effective against the test bacteria and this indicated that the antibiotics can be more effective than the natural plant extracts unless the microorganisms develop resistance to them. This difference in the efficacy of bacterial growth inhibition by the natural plant extracts and the allopathic antibiotics can be there for the different mechanisms of interactions of these antimicrobial agents on the bacteria.

The emergence of antibiotic-resistant organisms is rising firmly and undoubtedly becoming overall consideration for researchers. The requirement of replacement to antibiotics is increased to restrain a large number of antibiotic-resistant strains. The latest antibiotics should discover or current antibiotics need to be altered so that they may acquire a wide spectrum of activities. Application of antibacterial activity of organic products like different extracts of plants, essential oils, herbs might be a great replacement to restrain these microorganisms.

The objective of the research was to assess the antibacterial properties of black cumin oil and to figure out which elements helps in this issue. In the food and pharmaceutical industry, Black cumin oil is a revealed medicinal plant. Both Indian and Ethiopian black cumin oils contain rich fatty acids. Healthier properties of the oil will be able to make interesting pathways in future research. (Thilakarathne et al., 2018) The outcomes of the recent work show the revolutionary cleaning

properties of *N. sativa*, demonstrates that the application of black cumin seeds for the cure of several infections is beneficial and satisfactory. (Burits & Bucar, 2000) In this study, the significance of antimicrobial and anti-inflammatory reactions is analyzed and the safeness and contagiousness of the oil are reviewed.

Another essential oil, the mixed herb oil showed best antimicrobial activity (24mm) (Table 3.9.1) against *Staphylococcus aureus*. In this segment, three oils have been involved and three broad-spectrum antibiotics are used. Undoubtedly three oils proved themselves as a great replacement for conventional antibiotics.

Essential oils are condensed fickle liquids removed from plants. These are extensively applied in detoxification, preparing foods, and also in drug therapies specifically. Both Gram-negative and Gram-positive bacteria are killed by essential oils which have antibacterial activity. (Bouhdid, Abrini, Zhiri, Espuny, & Manresa, 2009) Sandalwood oil also showed good antibacterial activity against skin-causing bacteria *Staphylococcus aureus*. To treat diarrhea and gastritis arisen due to digestive problems, Sandalwood is used. (Purohit, 2018) Another species of Sandalwood which is *Santalum spicatum*, gave promising antibacterial activity against *S. aureus* . (S. Xiao, Cui, Shi, & Zhang, 2020) So, these are the major drawbacks of this study.

Conclusion and Recommendation

5. Conclusion

Overall, the organic solvent extracts of Neem, Tulsi, and pomegranate were the most effective antibacterial agent compared to allopathic antibiotics against *Staphylococcus aureus* and have also demonstrated good antibacterial activity against the other test bacteria of this research.

The potential value of these medicinal plant extracts is on the rise when compared to that of allopathic antibiotics as certain bacteria have established resistance against allopathic antibiotics but are vulnerable to the natural plant extracts. It is expected that the outcomes of this research may inspire other researchers to design clinical trials and to come up with a less expensive antibacterial agent that may be effective for people from developing countries like Bangladesh.

In addition, the potency of Black cumin oil, Mixed herb oil, and Sandalwood oil to be applied as organic antimicrobic agent is recommended as antimicrobial activity against *Staphylococcus aureus.* The antibiotics would never be up for employment in the first place. But the medicinal plants and the essential oils used in this research are efficient against the selected bacteria.

6. Recommendation

This research can be further developed by adopting some measures such as:

1. *In Silico* prediction such as molecular docking of the molecules of these potential medicinal plants found in this research work with *S. aureus* along with molecular dynamics and molecular mechanics can be applied for further in-depth analysis.

2. Besides medicinal plants and essential oils, other crops (such as. jute) can be considered as a potential resource of antibiotics against *S. aureus*.

3. Different lantibiotics, which are ribosomal synthesized antimicrobial peptides can be investigated from different sources of the environment to determine potential antibiotics of *S. aureus*.

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APPENDIXES

Media composition: The composition of the media used in the present study has been given below. Unless otherwise mentioned, all the media were autoclaved at 121OC for 15 min.

1. Nutrient Agar (Himedia, India)

Ingredients	Amount (g/L)
Peptic digest of animal tissue	5.0
Beef extract	1.50
Sodium chloride	5.0
Yeast extract	1.50
Agar	1.50

2. Nutrient Broth (Oxoid, England)

Ingredients	Amount (g/L)
Lab-lemcopowder	1.0
Yeast extract	2.0
Peptone	5.0
Sodiumchloride	5.0

3. T_1N_1 soft agar

Ingredients	Amount (g/L)
Tryptone	0.6
Sodiumchloride	0.3
Agar	0.42

4. Simmon's citrate agar (Oxoid, England)

Ingredients	Amount (g/L)
Magnesiumsulfate	0.2
Ammonium dihydrogenphosphate	0.2
Ammoniumphosphate	0.8
Sodiumcitrate	2.0
Sodiumchloride	5.0
Agar	15.0
Bactobromthymolblue	0.08

5. MR-VP broth

Ingredients	Amount(g/L)
Peptone	7
Dextrose	5
Potassiumphosphate	5

6. Triple sugar iron agar (Himedia, India)

Ingredients	Amount(g/L)
Peptic digest of animal tissue	10.0
Sodium chloride	5.0
Lactose	10.0
Sucrose	10.0
Ferrous sulfate	0.20
Sodium thiosulfate	0.30
Casein enzymatic hydrolysate	10.0

7. Mannitol Salt agar (Oxoid, England)

Ingredients	Amount(g/L)
Peptone	10.0
Mannitol	10.0
Lab-lemco powder	1.0
Sodium chloride	75.0
Phenol red	0.025
Agar	15.0