



In-vitro and In-vivo studies on antibacterial activity of *Ocimum tenuiflorum* (Kalo tulshi), *Nigella sativa* (Kalo zira) and *Tinospora cordifolia* (Gulancha) extracts against some human pathogenic strains

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Certificate of the Supervisor

The thesis entitled “*In-vitro* and *In-vivo* studies on antibacterial activity of *Ocimum tenuiflorum* (Kalo tulshi), *Nigella sativa* (Kalo zira) and *Tinospora cordifolia* (*Gulancho*) extracts against some human pathogenic strains” has been completed sincerely and satisfactorily by **Mohammad Nazrul Islam** of registration no: 022, session 2012-2013, enrolled in the University of Dhaka, Bangladesh, for the degree of Master of Philosophy (M. Phil) in Nutrition & Food Science. It is an original research work and was supervised by me. It can be submitted to the examination committee for evaluation. The given information is true to best of my knowledge.

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Acronyms

UTI-Urinary Tract Infections	DPPH-2,2-diphenyl-1-picrylhydrazyl
OT- <i>Ocimum tenuiflorum</i>	BHT-Butylated hydroxytoluene
NS- <i>Nigella sativa</i>	CAT-Common Admission Test
MDR- Multi-drug resistant	HRT-Hormone Replacement Therapy
INFS- Institute of Nutrition & Food Sciences	HTLV-human T-lymphotropic virus type 1
HIV- Human Immunodeficiency Virus	KB- Kilobase
GID-Gastro-intestinal Disorders	LPO-Low power objectives
SSTIs- Skin and Soft Tissue Infection	MBC-Minimum Bactericidal Concentration
ATCC-The American Type Culture Collection	MTT- Mean Transit Time
TC- <i>Tinospora cordifolia</i>	OH: Hydroxyl radical
MIC-Minimum inhibitory concentration	TBF-To be fair
PMN-Polymorphonuclear leukocytes	TCE-Trichloroethylene
GMCSF- Granulocyte-macrophage colony-stimulating factor	

Abstract

Background of the study: Plant-derived medicines have been used as part of traditional healthcare for thousands of years. More than 80% of the population in developing countries depend on plants for their medical needs. A study conducted by Bregum F. (2010) showed that 84.1% of respondents in Bangladesh were using medicinal plants for their health ailments. However, only a few of these plants have been investigated for their antimicrobial properties, and the vast majority have not yet been adequately evaluated. Existing allopathic medicines have been observed to cause significant side effects while treating various diseases. In some cases, these side effects can even be life-threatening. Therefore, people are increasingly seeking treatments with fewer or no side effects, often turning to natural medicines to combat diseases. Nowadays, antibiotic resistance has become a serious and devastating public health problem worldwide, requiring immediate action in both developing and developed countries. Although it occurs naturally, the misuse of antibiotics in humans and animals is accelerating the process. A growing number of infections—such as pneumonia, urinary tract infections (UTIs), gastrointestinal (GI) disorders, gonorrhoea, skin and soft tissue infections (SSTIs), and salmonellosis—are becoming harder to treat with existing antibiotics. Antibiotic resistance leads to longer hospital stays, higher medical costs, and increased mortality. It has been shown that inappropriate dosing, incorrect quantities, repeated use of the same antibiotics, and evolving disease patterns contribute to the development of antibiotic resistance. Developing new active ingredients and natural antibiotics to combat resistant bacteria is a time-consuming process. This study is an attempt to develop natural antibiotics to combat bacterial infections and reduce the incidence of antibiotic resistance.

Methodology: It was a laboratory based (*in vitro*) and experimental animal (*in vivo*) study. The *in vitro* and *in vivo* studies were conducted in Microbiology laboratory and Animal House of Institute of Nutrition & Food Science respectively. The ethanolic extract (20, 40 and 80 grams) of *Tinospora cordifolia*, *Ocimum tenuiflorum* and *Nigella sativa* were collected by maceration process. Pathogenic microorganisms such as *Streptococcus pyogenes*, *Staphylococcus aureus*, *Salmonella typhi*, *Escherichia coli*, *Vibrio cholerae* and *Klebsiella pneumoniae* were cultured in Muller Hinton Agar (MHA) media. The antibacterial assay was performed by well diffusion method. The

average diameter of zone of inhibitions were recorded after 24 hours, 48 hours and 72 hours.

For *in vivo* study, 10 μ L/ 0.01ml of *Staphylococcus aureus* broth approximately 10^{14} Colony Forming Units (CFU) were injected to Swiss Albino mice model by subcutaneous. Staphylococcal Skin & Soft Tissue Infections (SSTIs) were observed in and around the sites of injection. After 3 days of inoculation, the extracts were given orally in different dose depended manners like TC20, TC40, TC80, TC160, TC320, Levo 10 and Levo 20 twice daily to the Treatment Groups. After 7 days of treatment, mice blood samples were collected from vein of tail into culture tube. The samples were sent to microbiology laboratory with proper labelling for sero-culture.

Results: *In vitro* antibacterial activities were measured as the maximum zone of inhibition (MZI) in millimeters (mm), and *in vivo* antibacterial activities were assessed by bacterial clearance from plasma. The alcoholic extract of *Tinospora cordifolia* (TC-80) showed the maximum zone of inhibition against *Escherichia coli*, *Streptococcus pyogenes*, *Vibrio cholerae*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Salmonella typhi*, respectively. The study also found that the maximum zone of inhibition was measured in *Nigella sativa* (NS-80) against *Streptococcus pyogenes*, *Staphylococcus aureus*, and *Vibrio cholerae*. Furthermore, the study demonstrated that *Ocimum tenuiflorum* (OT-80) exhibited significant antibacterial activity against *Streptococcus pyogenes*, *Vibrio cholerae*, and *Staphylococcus aureus*. In the *in vivo* study, it was observed that TC-20, TC-40, and TC-80 did not show significant antibacterial effects against *Staphylococcus aureus*, but TC-160 and TC-320 exhibited significant antibacterial activity against *Staphylococcus aureus* after 7 days of administration.

Conclusion: The *in vitro* study revealed that the ethanolic extract of *Tinospora cordifolia* has a significant antibacterial effect against *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus pyogenes*, *Vibrio cholerae*, *Klebsiella pneumoniae*, and *Salmonella typhi*. The *in vivo* study demonstrated that lower concentrations (20, 40, 80) of *Tinospora cordifolia* did not show in bacterial clearance from plasma against *Staphylococcus aureus*, while higher concentrations (160 and 320), administered twice daily, showed significant bacterial clearance from plasma after 7 days of oral administration.

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REVIEW OF LITERATURE

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1.1. Introduction

In most regions of the world, plant-based medicine has been used for the treatment of various diseases for thousands of years (Palombo & Semple, 2001). It has been observed that 80% of the population in developing countries relies on plants for their medical needs (Farnsworth, 1988; Balick et al., 1994). One study conducted by F. Bregum showed that 84.1% of respondents in Bangladesh use medicinal plants for their health care (Bregum, 2010). However, few of these plants have been investigated for their antimicrobial properties; the vast majority have not yet been adequately evaluated (Srinivasan et al., 2001).

One of the plants known for having many medicinal uses in traditional systems of medicine is *Tinospora cordifolia* (Wild) Hook & Thomson (Menispermaceae). It is commonly known as Gulancha in Bangladesh and belongs to the Menispermaceae family. It is widely available in Bangladesh, especially in Sherpur, Tangail, Narshingdi, Rajshahi, and other districts (Rahmatullah, 2010). Ethanolic extracts of this plant have been shown to exhibit significant antibacterial activity against *Staphylococcus aureus* (Jeyachandran et al., 2003). Additionally, ethanolic extracts of *Tinospora cordifolia* demonstrated significant antibacterial activity against *Enterococcus faecalis*, *Staphylococcus aureus*, *Salmonella typhi*, *Salmonella enteritidis*, and *Escherichia coli*. The plant also shows significant effects on fevers, inflammation, skin infections, and urinary tract infections (Jeyachandran, 2003).

Ocimum tenuiflorum (Kalo Tulsi) is an aromatic plant belonging to the family Lamiaceae. It is native throughout the tropics of the Eastern world and is also found in Bangladesh. The flowers are purple and form elongated racemes in close whorls (Staples, 1999). Two main morpho-types are found in India, Nepal, and Bangladesh: the green-leafed type, known as Lakshmi Tulsi, and the purple-leafed type, known as Krishna Tulsi (Warrier, 1995). Isolated extracts of *O. tenuiflorum* have been shown to exhibit significant antibacterial activity against *E. coli*, *S. aureus*, and *Pseudomonas aeruginosa* (Kothari, 2005).

Nigella sativa (black cumin) is an annual flowering plant native to South and Southwest Asia. It has been used medicinally in Asia, the Middle East, and Africa.

Its oil contains an abundance of conjugated linoleic acid (18:2, polyunsaturated omega-6 fatty acid), thymoquinone, nigelline, and tannins. Various crude extracts of *N. sativa* have been tested for antimicrobial activity against different bacterial isolates. It has been shown that *N. sativa* seeds have significant anti-*H. pylori* activity compared to triple therapy (Hannan, 2008).

Existing allopathic medicines have been observed to cause numerous side effects while treating various diseases, and in some cases, they can be life-threatening. For this reason, people increasingly seek herbal medicines that claim to have fewer or no side effects. Nowadays, antibiotic resistance has become a serious and devastating public health problem worldwide, requiring immediate action in both developing and developed countries. Antibiotic resistance is one of the biggest threats to global health, affecting people of all ages and in any country. Misuse of antibiotics in humans and animals accelerates the process, even though resistance can also occur naturally. As the effectiveness of medicines used to treat infections such as salmonellosis, gonorrhea, pneumonia, and tuberculosis declines, more infections are becoming increasingly difficult to cure. Antibiotic resistance leads to higher medical expenses, longer hospital stays, and increased mortality (WHO, 2021).

It has been demonstrated that inappropriate dosing, repeated use of the same antibiotics, and changes in bacterial physical and genetic characteristics contribute to the development of antibiotic resistance. Therefore, it is crucial to research and develop new active metabolites and herbal antibiotics to combat resistant bacteria.

The aim of this study was to evaluate the *in vitro* antibacterial activity of *Tinospora cordifolia* (Gulancha), *Nigella sativa* (kalo-zira), and *Ocimum tenuiflorum* (kalo tulsi) against human pathogenic organisms, including *Streptococcus pyogenes*, *Staphylococcus aureus*, *Salmonella typhi*, *Escherichia coli*, *Vibrio cholerae*, and *Klebsiella pneumoniae*, in a dose-dependent manner. After evaluating the *in vitro* activity, the plant extract showing the most significant antibacterial activity against the pathogenic strains was selected for *in vivo* evaluation.

1.2. Objectives

1.2.1. General Objective:

- To evaluate the *in vitro* antibacterial activity of *Ocimum tenuiflorum* (kalo tulsi), *Nigella sativa* (kalo-zira), and *Tinospora cordifolia* (Gulancha) extracts against selected human pathogenic bacterial strains, and the *in vivo* antibacterial activity of *Tinospora cordifolia* (Gulancha) against *Staphylococcus aureus* in a mice model.

1.2.2. Specific Objectives:

- To assess the *in vitro* antibacterial activity of ethanolic extracts of *Ocimum tenuiflorum* (kalo tulsi), *Nigella sativa* (kalo-zira), and *Tinospora cordifolia* (Gulancha) against *Streptococcus pyogenes*, *Staphylococcus aureus*, *Salmonella typhi*, *Escherichia coli*, *Vibrio cholerae*, and *Klebsiella pneumoniae*.
- To determine the maximum zone of inhibition of *Ocimum tenuiflorum* (kalo tulsi), *Nigella sativa* (kalo-zira), and *Tinospora cordifolia* (Gulancha) extracts against the bacterial strains and compare the results with a standard Levofloxacin antibacterial disc.
- To evaluate the *in vivo* bacterial clearance from plasma of *Staphylococcus aureus* following treatment with *Tinospora cordifolia* extract in a dose-dependent manner in a mice model.

2.1. Review of literature

At present, multi-drug resistance (MDR) has become a global public health problem. This may be due to the indiscriminate or repeated use of antimicrobial medications without knowledge of their pharmacological properties. Additionally, the cost of treating disorders with synthetic medications is often high. As a result, new strategies are needed to combat microbial infections (Rajesh et al., 2007). It has been shown that antimicrobial compounds are abundant in medicinal plants. However, only a small percentage of the estimated 250,000–500,000 plant species have been phyto-chemically analyzed (Mahesh and Satish, 2008).

Tinospora cordifolia (locally called Gulancha) has been observed to possess antibacterial, anti-diabetic, anti-spasmodic, anti-inflammatory, anti-arthritis, antioxidant, anti-allergic, anti-stress, anti-malarial, hepato-protective, and immune-modulatory activities. The taxonomic characteristics of *T. cordifolia* are given below:

2.2. Origin and Habitat: The climbing shrub *T. cordifolia* is found in Bangladesh and throughout the Indian subcontinent (Premilla, 2006). It grows well in a variety of soil types where there is sufficient rainfall.

2.3. Common name:

Bengali: Gulancha

Scientific Name: *Tinospora cordifolia* (willd.) Hook.F. & Thomson

English: Gulancha/ Indian Tinospora

Sanskrit: Guduchi (Sharma et.al. 2010)

2.4. Taxonomical classification

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida,

Order: Ranunculaceae

Family: Menispermaceae.

Genus: *Tinospora*; Species: *T. cordifolia* (Ali & Dixit, 2013).

2.5. Distribution:

According to Rahmatullah (2010), *Tinospora cordifolia* is abundant across Bangladesh, particularly in the districts of Sherpur, Tangail, Narshingdi, and Rajshahi. It is also widely distributed in North Africa, West Africa, South Africa, Malaysia, Vietnam, China, Thailand, Philippines, Indonesia, India, Myanmar, and Sri Lanka (Pnedse, 1981; Singh et al., 2003; Mia et al., 2009; Jain et al., 2010). The plant can be found at elevations of up to 1000 feet in both dry and deciduous forests.

2.6. Growth Requirements: The plant grows well in warm environments and thrives during the rainy season, especially from July to August (Shefali & Nilofer, 2013).

2.7. Botanical Description: *Tinospora cordifolia* is a large, deciduous, extensively spreading climbing shrub with several elongated twining branches. Different parts of the plant exhibit distinct morphological features, which are described below:

2.7.1. Root: The roots are aerial, thread-like, long, filiform, and squarish, extending downward from mature branches; sometimes they reach the ground by continually lengthening (Spandana et al., 2013). Microscopic investigations reveal that the basic structure of the aerial roots is tetra- to penta arch. Two distinct zones are observed in the root cortex: an inner parenchymatous zone and an outer thick-walled zone. The dried aerial roots are creamy white to light grey-brown in color, odorless, and bitter. All aerial roots contain starch in the parenchyma.

2.7.2. Stem: The succulent stems are long, fleshy, filiform, and climbing, with aerial roots emerging from the branches. The stems are thin, cylindrical, and somewhat twisted when dry. The outer bark ranges from brown to greyish, and is thin and papery. When sectioned transversely, the stem exhibits a wheel-like shape. Lenticels are rounded and noticeable. Stem powder is creamish brown to dark brown, with a distinct odor and a bitter taste. The stem is traditionally used to treat urinary tract infections, fever, and dyspepsia. Its starch is highly digestible and nutritious (Kirtikar, 2005).

2.7.3. Leaves: The plant has simple, alternate and membranous leaves. The petiole is cylindrical, pulvinate, heart-shaped, and partially rounded, measuring approximately 15 cm in length. Young leaves are bright green, turning yellowish-green and eventually

yellow with age. The lamina is oval-chordate, 10–20 cm long and 8–15 cm wide. Leaves are rough to touch and have a mild, non-distinct odor (Nasreen et al., 2010). They are rich in protein, calcium, and phosphorus (Panda et al., 2014).

2.7.4. Flowers: The flowers are small, unisexual, and greenish-yellow. Male flowers are clustered, while female flowers are solitary. Each flower has six sepals arranged in two sets of three, with the inner sepals larger than the outer ones. There are six free, membranous petals, smaller than the sepals. The plant blooms from March to June (Nasreen et al., 2010; Sinha et al., 2004; Kirtikar, 2005).

2.7.5. Fruit: The fruits are fleshy, single-seeded, and grouped in clusters of one to three. Scars are present on the underside of the sturdy stem that holds the drupelets. The fruit is oval, smooth in texture, and ranges in color from scarlet to orange-red. Fruits are visible during winter (Kirtikar, 2005; Nasreen et al., 2010).

2.7.6. Seed: Seeds are white, curly, and bean-shaped; the embryo naturally assumes a curved form (Singh et al., 2003).

2.7.7. Uses of Plant Parts: The stem is bitter and traditionally used to increase bile secretion, relieve constipation, quench thirst, alleviate burning sensations, induce vomiting, enrich the blood, and treat jaundice (Meshram et al., 2013). Roots and stems are used with other medications as an antidote for snake bites and scorpion stings. Leaf decoctions with honey are used to treat fever.

Figure 1: Leaf of *Tinospora cordifolia*



Figure 2: Stem of *Tinospora cordifolia*



Figure 3: Fruits *Tinospora cordifolia*

2.7.8. History and Traditional Uses:

T. cordifolia is used to treat skin infections, jaundice, diabetes, chronic diarrhoea and dysentery (Misra, 1969), bleeding piles, itching, erysipelas, irritation of stomach, seminal weakness, leprosy, fever, asthma, anorexia and fractured bones (Upadhyay et al, 2010).

2.7.9. Uses in folk and tribal medicine:

T. cordifolia is noted for its usage in tribal or folk medicine in various parts of the world. It is most used by traditional healers living in the Hill tracts of Bangladesh. It is also inhabitants of Jammu (J&K) and Bigwada (Rajasthan) in India and the use a decoction of stem to treat fever (Sinha et al., 2004). Gujjar and Backwal Muslim tribals in Rajouri, Jammu (Tawi) used the herb to treat bone fractures.

2.7.10. Chemical constituents of *Tinospora cordifolia*

Different chemical constituents have been isolated from this plant and their structures have been established. The active ingredients found in the shrub are alkaloids, di-terpenoid lactones, glycosides, steroids, sesquiterpenoid, phenolics, aliphatic compounds and polysaccharides. It stem contains different types of alkaloids such as berberine, palmatine, tembetarine, magnoflorine (Singh et al., 2003; Sinha et al., 2004), glycosides such as 18-nor clerodane glucoside, furanoid diterpene glucoside, Tinocordiside, Tinocordifolioside, Cordioside, Cordifolioside, Cordifolioside Syringin, Syringin-apiosylglycoside, Palmatosides, Cordifoliside A, Cordiofolioside B, Cordifoliside C, Cordifoliside D, Cordifoliside E (Singh et al., 2003; Wazir et al., 1995 ; Gangan et al., 1996; Maurya et al., 1997; Ghosal et al., 1997) and steroids like Ecdysterone, Makisterone A, Giloinsterol (Singh et al., 2003) and Tinocordifolin (Maurya et al., 1998).

2.7.11. Medicinal application

The plant *Tinospora cordifolia* is used to treat jaundice (Sangeetha et al., 2013). Plant stems are used to treat dyspepsia, fever, and urinary tract infections (Meshram et al., 2013). It play vital role to modulate the Poly Morpho Nuclear (PMN) leucocytes and macrophages. These phagocytic cells aid in pro-healing activities such as growth factor activation, angiogenesis, and the creation of granulation tissue, which are suppressed the chronic wounds (Purandare & Supe 2007). The powder of root and stem can be administered with milk to treat cancer (Bhatt & Sabnis 1987). It has been discovered that a polyherbal formulation of *T. cordifolia* has a positive effect on HIV (Preeti, 2011). Asthma can also be treated by taking the stem juice with honey and taking it orally (Sinha et al., 2004). *T. cordifolia* is an antioxidant which is used in conjunction

with L-DOPA to treat Parkinson's disease. During the synthesis of dopamine, L-DOPA generates free radicals. As a result, *T. cordifolia* counteracts the drug's adverse effects (Preeti, 2011). The root juice is highly efficient in treating urinary disorders. It reduced blood sugar levels significantly in both normal and Allaxon-induced diabetic mice. The plant extract is used for lowering blood sugar levels (Chattopadhyay, 1999). Gulancha is also used to treat inflamed digestive mucous membrane. It increases mucin synthesis which protects the stomach and duodenum. The clinical efficacy of *T. cordifolia* is proved in obstructive jaundice, hepatic fibrosis, peritonitis, and sepsis. The extract has been demonstrated to help protect against CCl₄ toxicity, most likely through the generation of monocyte colony stimulating factor or granulocyte monocyte stimulating factor (M-CSF/GM-CSF) (Sengupta et al., 2011). Anti-HIV 1 activity has been demonstrated in the leaves extract. As a result, it can be speculated that a biological extract from this plant undoubtedly aid in the prevention and treatment of a variety of viral infections in people (Estari et al., 2012). Giloy juice and Tulsi leaves, has been found to be an effective antidote to *Plasmodium vivax* infections (Vashist et al., 2011). Its crude extract contains a polyclonal B cell mitogen that boosts immunological response in mice. GL-4A, an arabino galactan polysaccharide derived from the stem of *T. cordifolia*, is effective for immunosuppression (Alamgir & Uddin 2010). It increases phagocytic function without influencing the humoral and cell-mediated immune systems. The chemicals also cause large increases in serum IgG antibodies (Sinha et al., 2004). Gulancha also plays a role in tumor suppression. The polysaccharide component of the plant was found to prevent lung metastatic colonies when administered intra-peritoneally in mice (Singh & Saxena, 2017). Its extract act as an immune-stimulant. It has been observed that it has significant impact on HIV symptoms, as evidenced by clinical trials. The plant helps to treat HIV/AIDS (Kalikar et al., 2008).

2.7.12. Nutritional and elemental analysis of *T. cordifolia*

It contains fibre (15.9%), ample protein (4.5 percent-11.2 percent), appropriate carbs (61.66 percent), and low fat (3.1 percent) with high potassium (0.845 percent), chromium (0.006 percent), iron (0.28 percent), and calcium (0.28 percent). It has a nutritional value of 292.54 calories per 100 grams (Imtiyaj et al., 2011). *T. cordifolia* fruit is contained iron, copper, zinc, magnesium potassium, and sodium (Kavya et al., 2015).

2.7.13. Pharmacological activity

Antibacterial Activity: *Tinospora cordifolia* showed notable antimicrobial activity against both Gram-negative and Gram-positive microbes, such as *Klebsiella pneumoniae* (ATCC 15380), *Escherichia coli* (ATCC 25922), *Micrococcus luteus* (ATCC 9341), *Streptococcus pneumoniae* (ATCC 12755), *Staphylococcus aureus* (ATCC 25923), and *Bacillus cereus* (ATCC 11778), even at very low concentrations. It was observed that aqueous extracts exhibited a higher percentage of growth inhibition than solvent extracts. Furthermore, aqueous extracts of *T. cordifolia* showed considerably larger zones of inhibition (20–30 mm). The presence of certain chemical compounds in the plant's leaves is likely responsible for these antimicrobial actions (Upadhyay et al., 2010).

Jeyachandran et al. reported that the stem of *Tinospora cordifolia* was significantly effective against *Escherichia coli*, *Proteus vulgaris*, *Enterobacter faecalis*, *Salmonella typhi* (Gram-negative), and *Staphylococcus aureus* (Jeyachandran et al., 2013). Agarwal et al. found that *T. cordifolia* powder was effective against *Streptococcus mutans* (Agarwal et al., 2019).

Molla et al. reported that the chemical constituents of *T. cordifolia* possess potential antibacterial and antifungal properties. In nutrient broth medium, the minimum inhibitory concentration (MIC) of *T. cordifolia* against *Bacillus megaterium* and *Salmonella typhi*-A was 128 µg/ml. The median lethal concentration (LC₅₀) was reported to be 9.34 µg/ml (Molla et al., 2012).

Another study by Jeyachandran et al. demonstrated the antibacterial activity of ethanolic extracts of *Tinospora cordifolia* stems against *Escherichia coli*, *Proteus vulgaris*, *Enterobacter faecalis*, *Salmonella typhi* (Gram-negative), and *Staphylococcus aureus* using the disc diffusion method. The results indicated that the ethanolic extract of *T. cordifolia* exhibited significant antibacterial activity, particularly against *Salmonella typhi* (Jeyachandran et al., 2003).

A study conducted by Nageswari found that aqueous extracts of *T. cordifolia* exhibited effective antimicrobial activity against *E. faecalis* (28 mm) and *S. typhi* (26 mm) at a concentration of 50 mcg/ml (Nageswari, 2016).

Sandhu et al. reported that *Tinospora cordifolia* is highly effective against *Serratia marcescens*, *E. coli*, *Salmonella typhi*, *Streptococcus thermophilus*, *Fusarium oxysporum*, and *Aspergillus niger*, but showed no effect on *Trichoderma reesei* (Sandhu et al., 2013).

Tembeker et al. demonstrated that *Tinospora cordifolia* possesses antibacterial potential against *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Salmonella typhi*, *Shigella flexneri*, *Salmonella paratyphi* and *Pseudomonas aeruginosa* (Tembeker et al., 2009).

Sharma et al. demonstrated that aqueous extracts of *T. cordifolia* have significant antibacterial effects against pathogenic bacteria such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Salmonella typhi* (Sharma, A et al., 2010).

Radha et al. reported that *Tinospora cordifolia* exhibits noteworthy antibacterial activity against three Gram-negative and two Gram-positive bacteria, namely *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Staphylococcus aureus* and *Bacillus subtilis*, using a standard gentamicin disc (10 mcg/disc) in the agar well-diffusion assay (Radha et al., 2014).

Nagaprashanti et al. found that the *in vitro* antibacterial activity of hydroalcoholic extracts of *Tinospora cordifolia* stem was effective against *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Salmonella typhi*. The zone of inhibition was 12 mm for *Staphylococcus aureus*, 10 mm for *Klebsiella pneumoniae*, and 7 mm for *Salmonella typhi* (Nagaprashanti et al., 2012).

The extract of *T. cordifolia* possesses a wide spectrum of antioxidant and antibacterial activity against bacterial and fungal infections (Prasad & Chauhan, 2019).

Taechowisan demonstrated that crude extracts of *Tinospora cordifolia* (Gulancha) exhibit excellent antibacterial activity against *Bacillus cereus* and *Staphylococcus aureus* (Taechowisan, 2019).

Singh et al. investigated the antibacterial efficacy of silver nanoparticles synthesized from *T. cordifolia* against multidrug-resistant microorganisms. The zone of inhibition ranged from 10 ± 0.58 to 21 ± 0.25 mm. The MIC of AgNPs against *Pseudomonas*

aeruginosa ranged from 6.25 to 200 µg/ml, indicating strong antibacterial potential (Singh et al., 2014).

Shanthi and Nelson studied the antibacterial activity of *T. cordifolia* against *E. coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, and *Pseudomonas aeruginosa* and found that the ethanol extract of the leaves exhibited better inhibitory effects than aqueous and chloroform extracts (Shanthi & Nelson, 2013).

Another study by Singh et al. (2015) showed that extracts of *Tinospora cordifolia* have potential effects against uropathogens such as *Escherichia coli* and *Staphylococcus aureus* using the disc diffusion method. The efficacy followed the order: ethanolic (highest) > methanolic (moderate) > aqueous (lowest) (Singh et al., 2015).

Kumari demonstrated that hydroalcoholic stem extracts prepared by maceration exhibited *in vitro* antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Enterobacter faecalis*, and *Salmonella typhi* (Kumari, 2013).

Toxicology:

The toxicity of *Tinospora cordifolia* in humans is largely unknown. Whole plant extracts administered to rats at doses of 1,000 mg/kg and to rabbits at oral doses up to 1.6 g/kg did not produce any adverse effects. However, 40% of mice died after receiving 500 mg/kg body weight of *Tinospora* stem extract (Neeraja & Margaret, 2013).

2.2. *Nigella sativa* (Kalo-zira Seed)

Nigella sativa is a medicinal plant belongs to Ranunculaceae family. It's also referred as black cumin. It has been observed that *Nigella sativa* having wide range of health benefits.



Fig 4: *Nigella sativa* Flower



Fig 5 : *Nigella sativa* seeds

2.2.1. Classification of *Nigella sativa*

Kingdom: Plantae

Class: Tracheophytes

Order: Ranunculales

Family: Ranunculaceae

Genus: *Nigella*

Species: *N. sativa*

2.2.2. Chemical constituents of *N. sativa*: *N. Sativa* seeds contain around 32% to 40% oil (Gharby S et al., 2015). It contains linoleic acid, oleic acid, palmitic acid, and *trans*-anethole, and other minor constituents, such as nigellicine, nigellidine, nigellimine, and nigellimine N-oxide. Aromatics include thymoquinone, dihydrothymoquinone, *p*-ymene, carvacrol, α -thujene, thymol, α -pinene, β -pinene and *trans*-anethole ([wikipedia.org/wiki/Nigella_sativa](https://en.wikipedia.org/wiki/Nigella_sativa))

It has been used for medicinal purposes for centuries in Asia, Middle East, and Africa (Zohary and Hopf, 2000). It is used for different health ailments like respiratory, digestive, circulatory and immune system as well as general well-being (Khan et al., 2003). Its oil has been used in treatment of dermatological diseases e.g. eczema and boils (Zohary and Hopf, 2000). Bioactive compound of *N. sativa* are thymoquinone (volatile oil of these seeds) and Melanine (fixed oil) (Roy et al. 2006; Adel, 2006).

2.2.3. Antibacterial Activity of *Nigella sativa*: Several studies have suggested that *Nigella sativa* seed oil possesses significant antibacterial activity (Hanafy & Hatem, 1991; Morsi, 2000; Roy et al., 2006).

A study conducted by Mohammad Tariq Salman et al. (2016) demonstrated that *Nigella sativa* (black cumin) seed extract was effective against 38 multidrug-resistant strains of *Staphylococcus aureus* out of 40 isolates.

Another study by Lorina Badger Emeka et al. (2015) showed that *Nigella sativa* oil exhibited significant antibacterial activity against multidrug-resistant isolates of *Staphylococcus aureus* at varying concentrations using the well diffusion method. The oil demonstrated the dose-dependent antibacterial activity. Out of 19 isolates, 8 (42%) were sensitive to the undiluted oil sample, while 4 (21%) showed sensitivity at concentrations of 200 mg/ml, 400 mg/ml, and 800 mg/ml.

Grzegorz Gawron et al. (2019) reported that the antibacterial activity of *N. sativa* seed extract against *Staphylococcus aureus* is attributed to the presence of thymoquinone, which is effective at concentrations of 4–16 µg/ml.

Hanafy and Hatem (1991) demonstrated that filter paper discs impregnated with diethyl ether extracts of *Nigella sativa* seeds (25–400 µg/disc) produced concentration-dependent inhibition of Gram-positive bacteria, particularly *Staphylococcus aureus*. Gram-negative bacteria such as *Pseudomonas aeruginosa* and *Escherichia coli* were also affected, but *Salmonella typhi* showed resistance.

R.A. Usman et al. (2017) evaluated the antibacterial activity of black seed extracts against *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*. The seeds were extracted using methanol and petroleum ether via the percolation technique,

and antibacterial activity was assessed using the agar well diffusion method. Methanolic extracts at 100 mg/ml showed notable activity against *S. aureus* (19 mm) and *P. aeruginosa* (12 mm). Petroleum ether extracts showed similar activity against *S. aureus* and *P. aeruginosa* (10 mm). However, *E. coli* was resistant at all tested concentrations.

Sewara J. Mohammed et al. (2019) reported that black cumin seed oil exhibited considerable antibacterial activity against selected Gram-positive bacteria, with the highest inhibition observed for *Bacillus subtilis* and the lowest for *Bacillus cereus*. The extract also showed strong activity against multidrug-resistant bacteria (MRSA). However, no antibacterial activity was observed against Gram-negative bacteria.

Shah S.B. et al. (2018) found that methanolic extracts of *Nigella sativa* exhibited maximum activity against *Staphylococcus aureus* (20 mm zone of inhibition). Ethanolic extracts showed optimal activity against *Proteus vulgaris* and *Pseudomonas aeruginosa* (21 mm), while aqueous extracts showed the highest activity against *Staphylococcus aureus* (16 mm) and the lowest against *Proteus vulgaris* (4 mm).

Abdul Hannan et al. (2008) observed that all tested MRSA strains were sensitive to *Nigella sativa* extract at a concentration of 4 mg/disc, with MIC values ranging from 0.2 to 0.5 mg/ml.

Nadya Nazim Uddin Putriet et al. (2022) demonstrated that ethanolic extracts of black cumin seeds exhibited antibacterial activity against *Klebsiella pneumoniae* (inhibition zones: 4.5–12.8 mm) and *Pseudomonas aeruginosa* (7.5–15.6 mm), compared to positive controls (24.3 mm and 20.7 mm, respectively), while no inhibition was observed in negative controls.

H. Zuridah et al. (2008) reported that *N. sativa* seed extracts at concentrations of 25 mg/20 µL, 50 mg/20 µL, and 100 mg/20 µL produced increasing zones of inhibition in *Staphylococcus aureus* (14, 17, and 25 mm) and *Pseudomonas aeruginosa* (8, 9, and 10 mm), respectively.

Ayse Ruveyda Ugur et al. (2016) reported that the MIC values of *Nigella sativa* oil against standard strains were as follows: *Staphylococcus aureus* ATCC 29213 (0.5

µg/ml), *Enterococcus faecalis* ATCC 29212 (2 µg/ml), *Escherichia coli* ATCC 25922 (64 µg/ml), and *Pseudomonas aeruginosa* ATCC 27853 (64 µg/ml). For clinical isolates of *Staphylococci*, MIC values ranged from <0.25 µg/ml to 1.0 µg/ml. Among 45 MRSA strains, 41 had MICs <0.25 µg/ml, two had 0.5 µg/ml, and two had 1 µg/ml. Among 77 MRCoNS strains, 53 had MICs <0.25 µg/ml, 19 had 0.25 µg/ml, two had 0.5 µg/ml, and three had 1 µg/ml.

2.3.1. *Ocimum tenuiflorum* (Kalo Tulsi)

Ocimum tenuiflorum, commonly known as Holy Basil, is an aromatic plant native to the tropical regions of Asia and Africa. It belongs to the Lamiaceae family and is predominantly cultivated in the Indian subcontinent (Juyal & Ghildiyal, 2017). Due to its significant medicinal properties, it is often referred to as the “Queen of plants” or the “mother medicine of nature” (Singh et al., 2010).

It is an erect, highly branched plant that typically reaches a height of 30–60 cm at maturity. The plant has an acrid and bitter taste and thrives well in moist soil. Its leaves are approximately 5 cm long and possess toothed margins.



Fig 6: *Ocimum tenuiflorum*

2.3.2. Classification of *Ocimum tenuiflorum*:

Kingdom: Plantae

Division: Magnoliophyte

Class: Magnoliopsida

Order: Lamiales

Family: Lamiaceae

Genus: *Ocimum*

Species: *O. tenuiflorum*

2.3.3. Other Names

English name: Holy basil

Hindi: Tulsi

Sanskrit: Tulsi

Ancient literature indicates that *Ocimum tenuiflorum* (Tulsi) has been used as a therapeutically effective medicinal plant for over 2,500 years (Chang et al., 2016). The earliest references to the use of Tulsi are found in the *Rigveda*, dating from approximately 3500–1600 BC (Pattanayak et al., 2010). Different parts of *Ocimum tenuiflorum* contain a wide variety of chemical constituents or bioactive compounds (Jiang et al., 2014, 2015; Mandave et al., 2014; Sun et al., 2014), as well as secondary metabolites (Singh et al., 2010; Wu et al., 2016).

The leaves contain a high concentration of essential oils, including toluene, camphene, octane, benzene, citronellal, sabinene, limonene, ledol, dimethylbenzene, ethyl-2-methylbutyrate, eugenol, terpinolene, β -elemene, isocaryophyllene, isoeugenol, α -amorphene, α -guaiene, α -humulene, α -terpineol, borneol, nerolidol, carvacrol, geraniol, humulene oxide, elemol, tetradecanal, (E,Z)-farnesol, cis-sesquisabinene hydrate, α -bisabolol, selin-11-en-4- α -ol, α -muurolene, and 14-hydroxy- α -humulene (Singh N et al., 2012).

Alcoholic extracts of the leaves and aerial parts contain compounds such as luteolin, orientin, ursolic acid, apigenin-7-O-glucuronide, luteolin-7-O-glucuronide, isoorientin, aesculin, triacontanol ferulate, vanillic acid, gallic acid, cirsilineol, aesculetin, chloro-

genic acid, stigmasterol, caffeic acid, 4-hydroxybenzoic acid, vicianin-2, protocatechuic acid, phenylpropane glycosides, and β -stigmasterol.

The seeds are a rich source of fixed oils, including oleic acid, stearic acid, palmitic acid, linoleic acid, and linolenic acid (Bhowmik et al., 2008). *O. tenuiflorum* also contains monoterpenes and sesquiterpenes, along with compounds such as rosmarinic acid, thymol, linalool, and methyl chavicol (Dhar et al., 1968).

The whole plant of *Ocimum tenuiflorum* has been reported to possess a wide range of therapeutic properties (Singh et al., 2010). Traditionally, it has been used to treat common colds, headaches, malaria, inflammation, and heart diseases (Pattanayak et al., 2010). The essential oils extracted from the leaves have been shown to be effective as expectorants, analgesics, anti-emetics, and antipyretics. Additionally, the plant exhibits hypoglycemic, hepato-protective, hypotensive, hypo-lipidemic, and immune-modulatory effects (Singh et al., 2010).

Several studies have demonstrated the pharmacological effects of *Ocimum tenuiflorum* using different extraction methods, including steam distillation, benzene extraction, and petroleum ether extraction. Prakash and Gupta (2005) reported that the plant exerts pharmacological effects on the central nervous system, immune system, cardiovascular system, reproductive system, as well as the gastrointestinal and urinary systems.

2.3.4. Antibacterial Activity of *Ocimum tenuiflorum*: It has been observed that *Ocimum tenuiflorum* is effective against skin and soft tissue infections (SSTIs), particularly those caused by *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli* (Dryden, 2009, 2010).

Tulsi contains approximately 54 bioactive compounds. Among these, camphor, eucalyptol, and eugenol have demonstrated significant antibacterial activity against *Staphylococcus aureus* (including MRSA), *Pseudomonas aeruginosa*, and *Escherichia coli* (Yamani et al., 2016).

Joshi and Rao investigated the antibacterial activity of *Ocimum tenuiflorum* against pathogens such as *Escherichia coli*, *Staphylococcus aureus*, *Bacillus anthracis*, *Bacillus subtilis*, *Salmonella typhi*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, and

Mycobacterium tuberculosis. They reported significant antibacterial activity against *E. coli*, *Klebsiella aerogenes*, *Proteus mirabilis*, *Salmonella typhi*, *Shigella dysenteriae*, *Vibrio* spp., *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Rao (1977) further stated that eugenol is the most therapeutically effective constituent of Tulsi (Rao & Grover, 1977).

Jabeen et al. (2008) demonstrated the antibacterial effects of *Ocimum tenuiflorum* against *Pasteurella multocida*, *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Salmonella typhi*, *Salmonella paratyphi A*, and *Klebsiella* spp.

Kumar et al. (2010) conducted *in vitro* studies on Tulsi against specific pathogens and observed inhibitory effects over a defined period. The essential oil of Tulsi leaves showed significant inhibitory activity against *Escherichia coli*, *Bacillus subtilis*, *Bacillus anthracis*, *Staphylococcus aureus*, *Proteus vulgaris*, and *Pseudomonas aeruginosa*.

Aqueous and alcoholic extracts of *Ocimum tenuiflorum* leaves have also demonstrated antibacterial activity against various enteric pathogens, including *Escherichia coli*, *Salmonella typhi*, *Shigella dysenteriae*, *Vibrio cholerae*, and *Staphylococcus aureus* (Tiwari et al., 2011).

Mallikarjun (2016) reported that *Ocimum tenuiflorum* is effective against resistant strains such as *Neisseria gonorrhoeae*. The fixed oil exhibited strong antibacterial activity against *Staphylococcus aureus*, while the essential oil showed activity against both Gram-positive and Gram-negative bacteria. Ethanolic extracts inhibited methicillin-resistant *Staphylococcus aureus* (MRSA) as well as methicillin-sensitive *Staphylococcus aureus* (MSSA).

Mittal et al. (2018) reported that the antibacterial activity of Tulsi leaf extract against *Klebsiella pneumoniae* and *Staphylococcus aureus* was highest at 100% concentration, followed by 75% and 50%. The maximum zones of inhibition were 21 mm and 11 mm against *Klebsiella pneumoniae*, and 18 mm and 15 mm against *Staphylococcus aureus*, respectively, indicating potent antibacterial properties.

The antibacterial activity of *Ocimum tenuiflorum* was also evaluated using a liquid inhibition test. *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Salmonella typhi* were found to be susceptible to the extracts, as evidenced by reductions in optical density ranging from 0.20 to 0.85. The chloroform extract was the most effective, particularly against *Pseudomonas aeruginosa*. The extracts were effective against both gram-positive and gram-negative bacteria (Mishra & Mishra, 2011).

Rathnayaka (2013) reported significantly higher antibacterial activity of *Ocimum tenuiflorum* extract against *Staphylococcus aureus*.

Singh et al. (2010) demonstrated that *Ocimum tenuiflorum* exhibits strong antibacterial activity against *Klebsiella* spp., *Escherichia coli*, *Staphylococcus aureus*, and *Vibrio cholerae*.

Sadul et al. (2009) observed that *Ocimum tenuiflorum* was effective against *Escherichia coli*, with antibacterial activity increasing with higher concentrations and longer contact time.

2.4.1. *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive, round-shaped bacterium belonging to the phylum Bacillota. It is commonly found in the upper respiratory tract and on the skin. It is a catalase-positive, facultative anaerobe and an opportunistic pathogen.

Staphylococcus aureus is a major cause of various infections, including skin infections such as abscesses, respiratory infections such as sinusitis, and food poisoning. Pathogenic strains promote infection by producing virulence factors, including potent protein toxins, and by expressing cell-surface proteins that bind to and inactivate antibodies. It is one of the leading pathogens associated with deaths due to antimicrobial resistance, particularly with the emergence of antibiotic-resistant strains such as methicillin-resistant *Staphylococcus aureus* (MRSA).

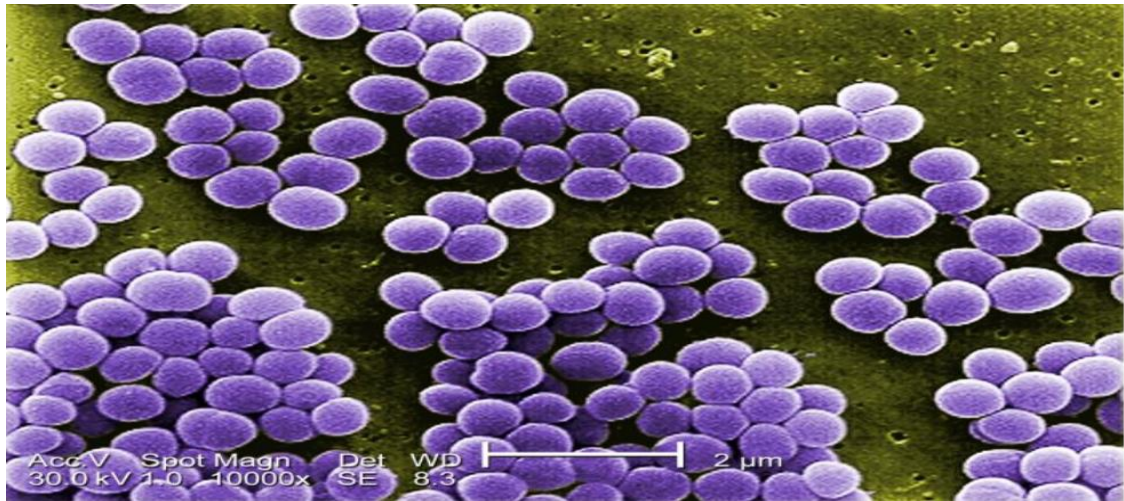


Fig 7: *Staphylococcus aureus*

S. aureus also cause different skin infections like pimples, impetigo, boils, cellulitis, folliculitis, carbuncles, scalded skin syndrome, and abscesses, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome, bacteraemia and sepsis.

2.4.2. Classification of *S. aureus*:

Domain: Bacteria

Phylum: Bacillota

Class: Bacilli

Order: Bacillales

Family: Staphylococcaceae

Genus: *Staphylococcus*

Species: *S. aureus*

In 1880, Scottish surgeon Alexander Ogston found that *Staphylococcus* could lead to wound infections. He named it *Staphylococcus* due to its clustered appearance. In 1884, German researcher Friedrich Julius Rosenbach identified *Staphylococcus aureus* and distinguished it from *Staphylococcus albus*. In the early 1930s, physicians began using a more efficient test to identify the presence of *S. aureus* infection through coagulase testing. They later discovered that penicillin could effectively treat *S. aureus* infections.

However, by the late 1940s, resistance to penicillin had become common among this bacterium, leading to outbreaks of resistant strains (Orent W., 2006).

Staphylococcus aureus is also known as "golden staph." It is non-motile and does not form spores. *S. aureus* appears as *Staphylococci* (grape-like clusters) when viewed under a microscope and forms large, round, golden-yellow colonies, often with hemolysis, when grown on blood agar plates. It reproduces asexually by binary fission. Complete separation of daughter cells is mediated by autolysin; in its absence or inhibition, the daughter cells remain attached, forming clusters.

S. aureus is catalase-positive, meaning it produces the enzyme catalase, which converts hydrogen peroxide (H_2O_2) into water and oxygen. Catalase activity tests are sometimes used to distinguish staphylococci from enterococci and streptococci. Previously, *S. aureus* was differentiated from other staphylococci using the coagulase test; however, not all strains are coagulase-positive.

Most *S. aureus* infections are skin-related. Common conditions include cellulitis, impetigo, folliculitis, small benign boils, and more severe invasive soft-tissue infections. These infections are especially common in individuals with atopic dermatitis (eczema). The bacteria typically colonize active areas such as the scalp, hair, and armpits. If left untreated, lesions may worsen. New-borns are particularly susceptible to a severe condition known as staphylococcal scalded skin syndrome (SSSS). *S. aureus* is believed to exploit defects in the skin barrier of individuals with atopic dermatitis, worsening symptoms by inducing cytokine production.

2.4.3. Enzymes: *S. aureus* produces several enzymes, including coagulase (both bound and free), which converts fibrinogen to fibrin and promotes clot formation. De-oxy-ribonuclease (DNase) helps protect the bacteria from neutrophil extracellular traps by breaking down DNA. Additionally, *S. aureus* produces beta-lactamase (for antibiotic resistance), lipase (for lipid digestion), and staphylokinase (which promotes fibrin breakdown and dissemination).

2.4.4. Toxins: Different strains of *S. aureus* secrete various exotoxins, which fall into three main categories. Many of these toxins are associated with specific diseases.

2.4.5. Super antigens: Super antigens can cause toxic shock syndrome (TSS). This group includes approximately 25 staphylococcal enterotoxins (SEs), such as enterotoxin type B and toxic shock syndrome toxin-1 (TSST-1), which is associated with tampon related TSS. Symptoms of TSS include skin peeling, low blood pressure, erythematous rash, shock, and multiple organ failure. The pathophysiology involves a lack of antibodies to TSST-1. Some strains of *S. aureus* also produce enterotoxins that cause gastroenteritis. This condition is self-limiting and characterized by vomiting and diarrhoea occurring 1–6 hours after ingestion of the toxin, with recovery typically within 8–24 hours. Symptoms include nausea, vomiting, diarrhoea, and severe abdominal pain.

2.4.6. Exfoliative toxins: Exfoliative toxins are exotoxins associated with staphylococcal scalded skin syndrome (SSSS), primarily affecting new-borns and young children. Outbreaks may occur in hospital nurseries. These toxins cause skin peeling through their protease activity.

2.4.7. Other toxins: Staphylococcal toxins that act on cell membranes include alpha, beta, and delta toxins, as well as several bi-component toxins. Certain strains of *S. aureus* carry bacteriophages, such as prophage Φ -PVL, which produce Panton–Valentine leukocidin (PVL), increasing virulence. PVL is associated with severe necrotizing pneumonia, particularly in children. The genes encoding PVL are carried on bacteriophages commonly found in community-associated MRSA strains.

2.5.1. *Streptococcus pyogenes*

Gram-positive cocci (singular: coccus), or spherical bacteria belonging to the *Streptococcaceae* family, are members of the order *Lactobacillales* (lactic acid bacteria) within the phylum *Bacillota*. In addition to being facultative anaerobes (able to grow both aerobically and anaerobically), most streptococci are oxidase- and catalase-negative.



Fig 8: *Streptococcus pyogenes*

2.5.2. Classification of *Streptococcus pyogenes*

Domain: Bacteria

Phylum: Bacillota

Class: Bacilli

Order: Lactobacillales

Family: Streptococcaceae

Genus: Streptococcus

Species: *S. pyogenes*

In addition to streptococcal pharyngitis (sore throat), certain *Streptococcus* species are responsible for many cases of bacterial pneumonia, endocarditis, erysipelas, and necrotizing fasciitis (the “flesh-eating” bacterial infection). However, many streptococcal species are non-pathogenic and form part of the normal human microbiota of the mouth, skin, intestine, and upper respiratory tract.

Species of *Streptococcus* are classified based on their hemolytic properties. Alpha-hemolytic species cause oxidation of iron in hemoglobin within red blood cells, producing a greenish discoloration on blood agar. Beta-hemolytic species cause complete lysis of red blood cells, which appears as clear zones surrounding bacterial colonies on blood agar. Gamma-hemolytic species cause no hemolysis.

Beta-hemolytic streptococci are further classified using the Lancefield grouping system, a serological classification based on specific carbohydrates present on the bacterial cell wall. The 21 identified serotypes are designated as Lancefield groups A to W (excluding I and J). This classification system was developed by Rebecca Lancefield, a scientist at Rockefeller University.

In medical settings, the most important groups include the alpha-hemolytic streptococci, such as *S. pneumoniae* and the *Streptococcus viridians* group, as well as the beta-hemolytic streptococci of Lancefield groups A and B (commonly referred to as “group A strep” and “group B strep”).

2.5.3. *Salmonella typhi*

Salmonella typhi (*S. typhi*) bacteria infect the intestinal tract and the bloodstream. The disease is referred to as typhoid fever. *S. Paratyphi* A, B, and C bacteria cause a similar illness, which is included under the typhoid category. However, paratyphoid fever is generally milder and shorter in duration than typhoid fever. *S. typhi* and *S. paratyphi* originate from the same sources and are common in many developing countries where sewage and water treatment systems are inadequate. Most reported cases occur among travellers returning from those areas.



Fig 9: *Salmonella typhi*

2.5.4. Scientific Classification of *S.typhi*:

Domain: Bacteria

Phylum: Pseudomonadota

Class: Gammaproteobacteria

Order: Enterobacterales
Family: Enterobacteriaceae
Genus: *Salmonella*
Species: *S. typhi*

Salmonella typhi is a Gram-negative, facultative anaerobe (capable of growing in the absence of oxygen) that belongs to serogroup D within subspecies I of the genus *Salmonella*, and it is represented by a specific antigenic formula. The characteristics of *S. typhi* are both geno-typically and phenotypically similar to those of other members of the genus *Salmonella*. However, it displays distinctly different reactions in a number of biochemical tests that are normally used for the characterization of *Salmonella spp.*

Salmonella typhi is transmitted through the ingestion of food or water contaminated with the excreta of infected individuals and can survive the acidic gastric pH barrier in the stomach before adhering to the small intestine (Parry et al., 2002). The infectious dose of *Salmonella typhi* in healthy individual's ranges between 1,000 and 1 million organisms, but this can vary depending on the host's defence mechanisms.

2.6.1. *Escherichia coli*

It is also known as *E. coli*. It is a Gram-negative, facultative anaerobic, rod-shaped, coliform bacterium belonging to the genus *Escherichia* and it is commonly found in the lower intestine of warm-blooded organisms.

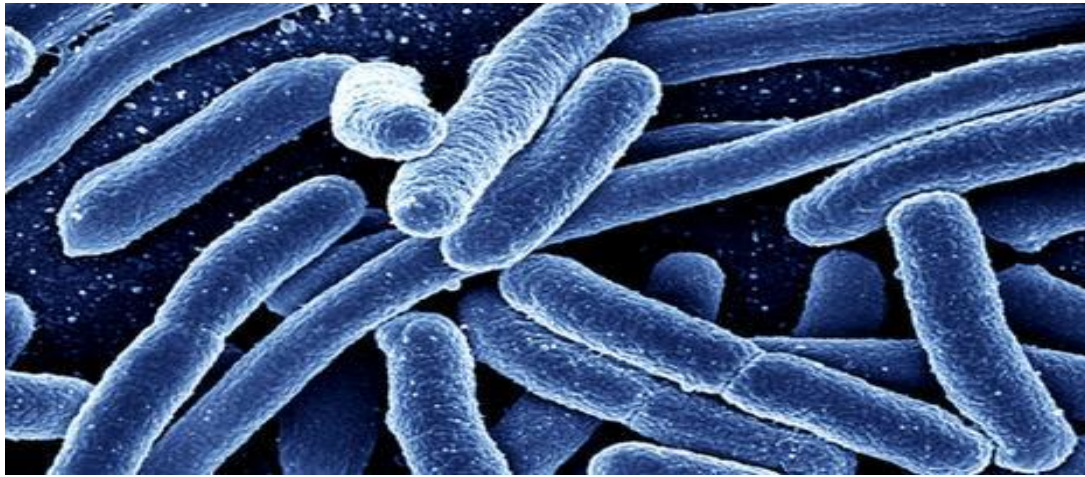


Fig 10: *E. coli*

2.6.2. Scientific Classification of *E. coli*:

Domain: Bacteria

Phylum: Pseudomonadota

Class: Gammaproteobacteria

Order: Enterobacterales

Family: Enterobacteriaceae

Genus: *Escherichia*

Species: *E.coli*

Most *E. coli* strains are harmless; however, some serotypes can cause serious food poisoning in their hosts and are occasionally responsible for food contamination. The harmless strains are part of the normal gut micro-biota and can benefit their hosts by producing vitamin K2 and preventing the colonization of the intestine by pathogenic bacteria, thereby maintaining a mutualistic relationship.

E. coli is expelled into the environment in faecal matter. The bacterium grows rapidly in fresh faecal matter under aerobic conditions for up to three days, but its numbers decline gradually thereafter. *E. coli* and other facultative anaerobes constitute about 0.1% of the gut micro biota, and the faeco–oral route is the primary means by which pathogenic strains cause disease.

The cells can survive outside the body for a limited period, making them useful indicator organisms for testing environmental samples for faecal contamination. However, a growing body of research has identified environmentally persistent *E. coli* strains that can survive for many days and even grow outside a host.

2.7.1. *Vibrio cholerae*

Vibrio cholerae is a species of Gram-negative, facultative anaerobic, comma-shaped bacteria. These bacteria naturally inhabit brackish or saltwater environments, where they readily attach to the chitin-containing shells of crabs, shrimp, and other shellfish. Some strains of *V. cholerae* are pathogenic to humans and cause the deadly disease cholera, which can result from the consumption of undercooked or raw marine organisms.

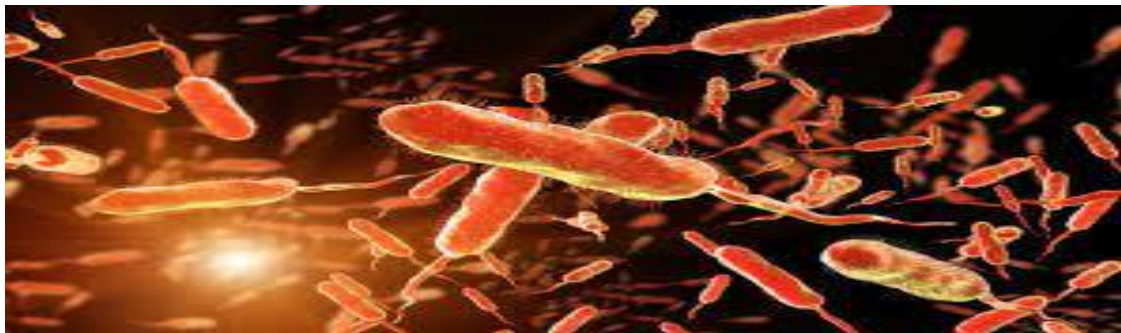


Fig 11: *Vibrio Cholera*

2.7.2. Scientific Classification of *Vibrio Cholera*:

Domain: Bacteria

Phylum: Pseudomonadota

Class: Gammaproteobacteria

Order: Vibrionales

Family: Vibrionaceae

Genus: *Vibrio*

Species: *V. Cholerae*

Vibrio cholerae was initially characterized by *Félix-Archimède Pouchet* in 1849 as a type of protozoan. *Filippo Pacini* later accurately recognized it as a bacterium. The link between the bacterium and cholera was established by *Robert Koch* in 1884. In 1959, *Sambhu Nath De* isolated the cholera toxin and demonstrated that it is responsible for causing cholera.

The bacterium possesses a flagellum at one end and multiple pili across its cell membrane. Cholera outbreaks are primarily caused by two serogroups: O1 and O139. Transmission mainly occurs through the consumption of contaminated water; therefore, it is closely associated with sanitation and hygiene practices. After ingestion, the bacterium can penetrate the intestinal lining and lead to diarrhea and vomiting within a few hours to 2–3 days. Common treatment methods include oral rehydration solution and antibiotics such as fluoroquinolones and tetracyclines.

Vibrio cholerae contains two circular DNA molecules. One of these is associated with the production of cholera toxin (CT), which leads to excessive, watery diarrhoea, often referred to as “rice-water stool.” However, the genes responsible for cholera toxin production are actually carried by CTX ϕ (CTXphi), a temperate bacteriophage. The toxin is produced only when this phage integrates into the bacterial DNA.

Vibrio cholerae is the etiological agent of cholera, an epidemic disease of major public health importance due to its rapid spread in areas with poor sanitation and hygiene, and its severe consequences when access to healthcare is limited.

2.8.1. *Klebsiella pneumoniae*

This bacterium is characterized as a Gram-negative, non-motile, encapsulated, lactose-fermenting, facultative anaerobic rod. On MacConkey agar, it appears as a mucoid lactose fermenter. Although it is part of the normal flora of the mouth, skin, and intestines, it has the potential to cause severe lung damage in both humans and animals if aspirated, particularly affecting the alveoli and leading to the production of sputum that is bloody, brownish, or yellow and jelly-like in appearance. In clinical contexts, it is recognized as the most significant species within the genus *Klebsiella* of the family enterobacteriaceae.

Other species, such as *K. oxytoca* and *K. rhinoscleromatis*, have also been identified in clinical specimens from humans. In recent years, *Klebsiella* species have emerged as important pathogens associated with nosocomial infections. These bacteria are naturally found in soil, and approximately 30% of strains have the ability to fix nitrogen under anaerobic conditions.

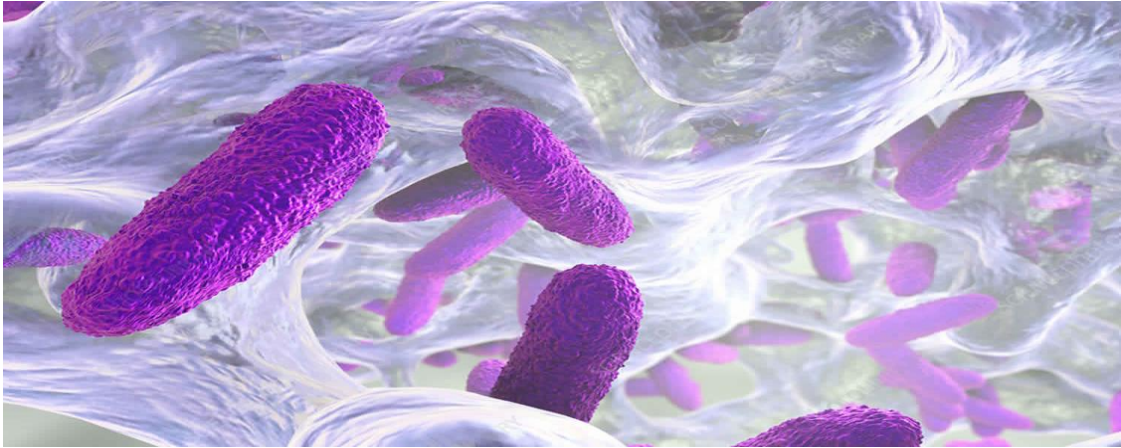


Fig 12: *Klebsiella pneumoniae*

2.8.2. Classification of *Klebsiella pneumoniae*

Domain: Bacteria

Phylum: Pseudomonadota

Class: Gammaproteobacteria

Order: Enterobacterales

Family: Enterobacteriaceae

Genus: *Klebsiella*

Species: *K. pneumoniae*

The genus *Klebsiella* is named in honour of the German microbiologist *Edwin Klebs* (1834–1913). It is also referred to as Friedländer's bacillus, in recognition of *Carl Friedländer*, who suggested that this bacterium is the causative agent of pneumonia, particularly in immunocompromised individuals, including those with chronic illnesses or alcohol dependence. Pneumonia acquired in the community due to *Klebsiella pneumoniae* may be referred to as Friedländer's pneumonia.

Additionally, *Klebsiella* species are capable of causing infections in the urinary tract, lower biliary tract, and surgical wounds. The spectrum of clinical conditions associated with this bacterium includes pneumonia, thrombophlebitis, urinary tract infections, cholecystitis, diarrhea, upper respiratory tract infections, wound infections, osteomyelitis, meningitis, bacteremia, and sepsis.

The most common condition caused by *Klebsiella* outside hospital settings is pneumonia, typically presenting as bronchopneumonia or bronchitis. These patients have an increased tendency to develop complications such as lung abscesses, cavitation, empyema, and pleural adhesions. Clinical features of *Klebsiella pneumoniae* include fever, nausea, tachycardia, vomiting, and a productive cough.

K. pneumoniae must enter the respiratory tract to cause pneumonia or the bloodstream to cause a systemic infection. It can spread through person-to-person contact, such as via contaminated hands of healthcare personnel or through patient-to-patient transmission, and less commonly through environmental contamination. However, transmission directly from the environment to patients remains controversial and requires further investigation. The bacteria are not spread through the air.

Patients in healthcare settings may also be exposed to *K. pneumoniae* when using ventilators, intravenous catheters, or through open wounds. These medical devices and conditions can facilitate the entry of the bacterium into the body and lead to infection.

3.1. Materials and Methods

The dried stems of *Tinospora cordifolia*, leaves of *Ocimum tenuiflorum*, and seeds of *Nigella sativa* were collected from a local market, and their taxonomic identities were confirmed by a taxonomist from the Department of Pharmacy, Jahangirnagar University (JU). After obtaining the taxonomic certificate from JU, the plant materials were processed into fine powders. The powders were stored in airtight containers and used as test drugs.

The maceration process was followed to prepare the extracts. Pathogenic microorganisms were collected from the Microbiology Laboratory of INFS. The antibacterial activity of *Tinospora cordifolia* stem, *Ocimum tenuiflorum* leaves, and *Nigella sativa* seeds was evaluated using the well diffusion method against *Streptococcus pyogenes*, *Staphylococcus aureus*, *Salmonella typhi*, *Escherichia coli*, *Vibrio cholerae*, and *Klebsiella pneumoniae*.

3.2. Material required for the study

- Ethanol 96.4%
- Whatman filter paper No.1 (6mm)
- Petri dish
- Muller Hinton Agar media
- Nutrient Agar Media
- Rotary Evaporator
- UV Spectrophotometer
- Autoclave
- Incubator
- Culture vials
- Disposable single use Syringes
- Mice gavage needle
- Pathogenic strains
- Incubator
- Sterile glass rod

- Conical flask

3.3. Preparation of stock solution

20 g, 40 g, and 80 g of fine powders of *Tinospora cordifolia*, *Ocimum tenuiflorum*, and *Nigella sativa* were placed in separate conical flasks containing 400 mL of 100% ethanol, respectively, and allowed to stand for a period of 7 days with frequent agitation. The mixtures were filtered with Whatman filter paper No. 1. The filtrates were subsequently sent to the Food Chemistry Laboratory for rotary evaporation.

The extracts were concentrated using a BUCHI rotary evaporator at 78°C. A volume of 300 µL of the concentrated extract was placed into wells to evaluate the antibacterial activity of *Tinospora cordifolia*, *Ocimum tenuiflorum*, and *Nigella sativa* against *Streptococcus pyogenes*, *Staphylococcus aureus*, *Salmonella typhi*, *Escherichia coli*, *Vibrio cholerae*, and *Klebsiella pneumoniae*. The Petri dishes were incubated at 37°C, and the results were observed after 24, 48, and 72 hours.

3.4. Preparation of Media

Thirty-eight grams (38 grams) of Mueller–Hinton agar medium was dissolved in 1 liter of distilled water. The medium was heated to ensure complete dissolution and then sterilized by autoclaving. After sterilization, the medium was poured into sterile Petri dishes, allowed to cool, and stored in a refrigerator.

3.5. Preparation of Bacterial Culture

Fresh (young) cultures of *Streptococcus pyogenes*, *Staphylococcus aureus*, *Salmonella typhi*, *Escherichia coli*, *Vibrio cholerae*, and *Klebsiella pneumoniae* were prepared in broth. The prepared agar plates were de-moisturized in a dry heat chamber for 2 minutes. After de-moisturization, the petri dishes were inoculated with the bacterial cultures using sterile cotton swabs and then incubated at 37°C for 12 hours.

3.6. Maceration:

In this process, finely powdered material was placed in a container with a tight stopper. The solvent was added, and the mixture was allowed to stand for a period of 7 days

with frequent agitation to facilitate the dissolution of soluble constituents. After maceration, the mixture was filtered through Whatman No. 1 filter paper.

3.7. Test Microorganisms

The following microorganisms were used for antibacterial testing:

- *Streptococcus pyogenes*
- *Staphylococcus aureus*
- *Salmonella typhi*
- *Escherichia coli*
- *Vibrio cholerae*
- *Klebsiella pneumoniae*

3.8. Determination of Zone of Inhibition

The antibacterial activity of the extracts was evaluated *in vitro* using the well diffusion assay. A 0.2 ml diluted bacterial culture of each strain was poured into sterile 9 cm Petri plates containing 20 ml of Mueller–Hinton agar (MHA) medium. The inoculum was evenly spread over the agar surface using a sterile glass L-rod.

Wells were created in the agar, and 300 µl of each extract was introduced into the respective wells. The plates were allowed to dry and then incubated at 37°C for 24 hours.

Negative control for *in vitro* study was conducted by using Ethanol, Distilled water and blank in separate Petri plates.

All experiments were performed in triplicate, and the average diameter of the zones of inhibition was measured and expressed in millimetres (mm).

3.9. Mueller–Hinton Agar Medium

Mueller–Hinton agar is a standard microbiological growth medium commonly used for antibiotic susceptibility testing, particularly in disc diffusion and well diffusion methods.

It typically contains:

- 2.0 g beef extract
- 17.5 g casein hydrolysate
- 1.5 g starch
- 17.0 g agar
- 1 litre of distilled water.
- P^H adjusted to neutral at 25 °C. (Hendriksen,2002)

3.10. Preparation of Mueller Hinton agar

- Suspend 38g of Mueller Hinton agar powder (CM0337B) in 1L of distilled water.
- Mix and dissolve them completely.
- Sterilize by autoclaving at 121°C for 15 minutes.
- Pour the liquid into the petri dish and wait for the medium to solidify.

3.11. Principle of MHA

Mueller–Hinton agar medium consists of beef extract, acid hydrolysate of casein, starch, and agar. Beef extract and acid hydrolysate of casein serve as sources of nitrogen, carbon, vitamins, amino acids, sulphur, and other essential nutrients required for bacterial growth.

Starch is included in the medium to absorb toxic metabolites produced by bacteria during growth. During hydrolysis, starch releases dextrose, which provides an additional source of energy for microbial metabolism.

Agar acts as the solidifying agent, giving the medium its firm structure suitable for microbial culture and antibiotic susceptibility testing.

3.12. Uses of MHA

- ❖ Mueller–Hinton agar (MHA) is widely used for antimicrobial susceptibility testing. It is the standard medium for the Bauer–Kirby disc diffusion method, and its performance is specified by the National Committee for Clinical Laboratory Standards (NCCLS).
- ❖ It can also be used for the cultivation of *Neisseria* species.
- ❖ MHA is recommended in the FDA Bacteriological Analytical Manual for food testing and is commonly used for procedures involving aerobic and facultative anaerobic bacteria.

3.13. Limitations

- ❖ This medium is acclaimed for susceptibility testing of pure cultures only.
- ❖ Inoculum density may affect the zone size. Heavy inoculum may result in smaller zones or too less inoculum may result in bigger zones.
- ❖ Fastidious organisms may not grow on this medium and may require supplementation of blood.

3.14. MHA is used for antibiotic susceptibility testing

- ❖ It is a non-selective, non-differential medium, meaning that almost all organisms can grow on it.
- ❖ It contains starch, which absorbs toxins released by bacteria so that they do not interfere with the antibiotic. It also mediates the rate of diffusion of antibiotics through the agar.
- ❖ It is a loose agar, which allows better diffusion of antibiotics than most other media. Better diffusion leads to the formation of a more accurate zone of inhibition.
- ❖ MHA shows acceptable batch-to-batch reproducibility for susceptibility testing.
- ❖ MHA contains low levels of sulphonamide, trimethoprim, and tetracycline inhibitors (i.e., the concentrations of thymidine and thymine are low in MHA).

- ❖ When Mueller-Hinton Agar is used to assess the sensitivity of bacterial isolates to these antimicrobials, the amounts of para-aminobenzoic acid (PABA) and thymine/thymidine are minimized, which significantly reduces the inactivation of sulphonamides and trimethoprim.

3.15. Study design: Laboratory based (*in vitro*) and experimental animal (*in vivo*) study.

3.16. Standard of Antibiotic Disc: Levofloxacin 5mg

3.17. Bias on was minimized by: Negative control of *in vitro* was observed by Using Alcohol, demineralized sterile water and blank.

3.18. Animals: Male and female Swiss albino mice, with an average weight of 35 g, were collected from Jahangirnagar University. The animals were housed under standard laboratory conditions (relative humidity 55–65%, room temperature $23 \pm 2^{\circ}\text{C}$, and a 12-hour light–dark cycle) in the INFS animal house. The animals were fed a standard diet and provided with water *ad libitum*. They were divided into different groups for the experiment. Ethical approval was obtained from the Animal Ethical Committee of the Department of Biological Sciences, University of Dhaka.

3.19. Experimental Design: Animals were divided into 6 groups.

Group A: This group (n = 6) served as the control. They were not treated throughout the experiment but were given free access to normal food pellets and water *ad libitum*.

Group M1: This group (n = 6) consisted of pathogenic strain-induced mice treated with TC extract at a dose of 20 mg/kg body weight/day, administered twice daily for 7 days.

Group M2: This group (n = 6) consisted of pathogenic strain-induced mice treated with TC extract at a dose of 40 mg/kg body weight/day, administered twice daily for 7 days.

Group M3: This group (n = 6) consisted of pathogenic strain-induced mice treated with TC extract at a dose of 80 mg/kg body weight/day, administered twice daily for 7 days.

Group M4: This group (n = 6) consisted of pathogenic strain-induced mice treated with TC extract at a dose of 160 mg/kg body weight/day, administered twice daily for 7 days.

Group M5: This group (n = 6) consisted of pathogenic strain-induced mice treated with TC extract at a dose of 320 mg/kg body weight/day, administered twice daily for 7 days.

Group C1: This group (n = 6) consisted of pathogenic strain-induced mice treated with the standard antibiotic levofloxacin at a dose of 10 mg/kg body weight, administered twice daily for 7 days.

Group C2: This group (n = 6) consisted of pathogenic strain-induced mice treated with the standard antibiotic levofloxacin at a dose of 20 mg/kg body weight, administered twice daily for 7 days.

Group U: This group (n = 6) consisted of pathogenic strain-induced mice that received no treatment after induction and served as the positive control.

***In vivo* study:** For the *in vivo* study, the abdominal hair of the mice was shaved using a sterile razor. A 1 mL single-use sterile insulin syringe was used to inoculate the bacteria. From a young culture of *Staphylococcus* broth, approximately 10 μ L (0.01 mL) containing $\sim 10^{14}$ CFU was injected subcutaneously into the mice model.

After 3 days of inoculation, skin and soft tissue infections (SSTIs), such as abscesses near the injection site and below the ear, developed. Different oral doses—TC20, TC40, TC80, TC160, TC320, Levo 10, and Levo 20—were then administered twice daily to the respective groups: Group M1 (n = 6; TC-20), Group M2 (n = 6; TC-40), Group M3 (n = 6; TC-80), Group M4 (n = 6; TC-160), Group M5 (n = 6; TC-320), Group C1 (n = 6; Levo 10), Group C2 (n = 6; Levo 20), and Group U (n = 6; infected but not treated with any drug).

3.20. Confirmation after treatment: After 7 days of treatment, 1 mL of blood was drawn from the tail of each animal into culture vials and incubated at 37°C for 24 hours. The samples were then inoculated onto Petri dishes containing Mueller-Hinton Agar (MHA) and incubated at 37°C for another 24 hours. The number of emerging colonies was counted to evaluate the efficacy of the treatment.

3.21. Data collection: The study was conducted in the Microbiology Laboratory, Institute of Nutrition and Food Science (INFS), University of Dhaka, and data were collected from laboratory observations.

3.21. Data analysed and interpreted by: Direct Observation and results were compared with different groups.

3.22. Results:

The antibacterial activities of these extracts were evaluated against different human pathogenic strains, and the preclinical trial results were documented following standard guidelines.

Results

In the *in vitro* study, it was observed that the negative controls with demineralized water, pure ethanol, and a blank did not show any zone of inhibition. On the other hand, the positive control with Levofloxacin showed a significant zone of inhibition (average 12–16 mm) against the pathogenic bacterial strains.

4.1. Table 1: Antibacterial activity (zone of inhibition) of different *Tinospora cordifolia* (TC) concentration against different pathogenic strains after 24 hours

	Zone of Inhibition (mm) by Well diffusion method after 24 hours		
Pathogenic strain	TC20	TC40	TC80
<i>Vibrio cholerae</i>	10	12	14
<i>Escherichia coli</i>	8	13	16
<i>Streptococcus pyogenes</i>	12	16	16
<i>Staphylococcus aureus</i>	12	14	14
<i>Salmonella typhi</i>	8	10	12
<i>Klebsiella pneumoniae</i>	9	12	13

Table 1 shows that the maximum zones of inhibition (mm) 16, 16, 14, 14, 13, and 12 were measured for TC 80 against *Escherichia coli*, *Streptococcus pyogenes*, *Vibrio cholerae*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Salmonella typhi*, respectively. The minimum zones of inhibition (8 mm) were measured for TC 20 against *Salmonella typhi* and *Escherichia coli*



Fig 13: Zone of Inhibition of different *Tinospora cordifolia* (TC) concentration against different pathogenic strains after 24 hours

4.2. Table 2: Antibacterial activity (zone of inhibition) of different *Tinospora cordifolia* (TC) concentration against different pathogenic strains after 48 hours.

	Zone of Inhibition (mm) by Well diffusion method after 48 hours		
Pathogenic strain	TC20	TC40	TC80
<i>Vibrio cholerae</i>	10	12	14
<i>Escherichia coli</i>	8	13	16
<i>Streptococcus pyogenes</i>	12	16	16
<i>Staphylococcus aureus</i>	12	14	16
<i>Salmonella typhi</i>	8	10	12
<i>Klebsiella pneumoniae</i>	9	12	13

Table 2 demonstrates that the maximum zones of inhibition (mm) 16, 16, 16, 14, 13, and 12 were measured for TC 80 against *Escherichia coli*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Vibrio cholerae*, *Klebsiella pneumoniae*, and *Salmonella typhi*, respectively. The minimum zones of inhibition (8 mm) were measured for TC 20 against *Salmonella typhi* and *Escherichia coli*.



Fig 14: Zone of Inhibition of different *Tinospora cordifolia* (TC) concentration against different pathogenic strains after 48 hours

4.3. Table 3 Antibacterial activity (zone of inhibition) of different *Tinospora cordifolia* (TC) concentration against different pathogenic strains after 72 hours

	Zone of Inhibition (mm) by Well diffusion method after 72 hours		
Pathogenic strain	TC20	TC40	TC80
<i>Vibrio cholerae</i>	10	12	14
<i>Escherichia coli</i>	8	13	16
<i>Streptococcus pyogenes</i>	12	16	16
<i>Staphylococcus aureus</i>	12	14	16
<i>Salmonella typhi</i>	8	10	12
<i>Klebsiella pneumoniae</i>	9	12	13

Table 3 shows that the maximum zones of inhibition (mm) 16, 16, 16, 14, 13, and 12 were measured for TC 80 against *Escherichia coli*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Vibrio cholerae*, *Klebsiella pneumoniae*, and *Salmonella typhi*, respectively. The minimum zones of inhibition (8 mm) were measured for TC 20 against *Salmonella typhi* and *Escherichia coli*.



Fig 15: Zone of Inhibition of different *Tinospora cordifolia* (TC) concentration against different pathogenic strains after 72 hours

4.4. Table 4: Antibacterial activity (zone of inhibition in mm) of different *Nigella sativa* concentration against different pathogenic strains after 24 hours

	Zone of Inhibition (mm) by Well diffusion method after 24 hours		
Pathogenic strain	NS20	NS40	NS80
<i>Vibrio cholerae</i>	8	8	9
<i>Escherichia coli</i>	0	0	0
<i>Streptococcus pyogenes</i>	0	8	10
<i>Staphylococcus aureus</i>	7	8	9
<i>Salmonella typhi</i>	0	0	0
<i>Klebsiella pneumoniae</i>	0	0	0

Table 4 represents that the maximum zones of inhibition (mm) 10 and 9 were measured for NS 80 against *Streptococcus pyogenes*, *Staphylococcus aureus*, and *Vibrio cholerae*, respectively. The minimum zones of inhibition (7 and 8 mm) were measured for NS 20 against *Staphylococcus aureus* and *Vibrio cholerae*. No zones of inhibition were observed at NS 40 and NS 80 concentrations against *Salmonella typhi*, *Klebsiella pneumoniae*, and *Escherichia coli*.



Fig 16: Zone of Inhibition of different NS concentration against different pathogenic strains after 24 hours

4.5. Table 5: Antibacterial activity (zone of inhibition in mm) of different *Nigella sativa* concentration against different pathogenic strains after 48 hours

	Zone of Inhibition (mm) by Well diffusion method after 48 hours		
Pathogenic strain	NS20	NS40	NS80
<i>Vibrio cholerae</i>	8	8	9
<i>Escherichia coli</i>	0	0	0
<i>Streptococcus pyogenes</i>	0	10	8
<i>Staphylococcus aureus</i>	7	8	9
<i>Salmonella typhi</i>	0	0	0
<i>Klebsiella pneumoniae</i>	0	0	0

Table 5 shows that the maximum zones of inhibition (mm) 10 and 9 were measured for NS 80 against *Streptococcus pyogenes*, *Staphylococcus aureus*, and *Vibrio cholerae*, respectively. The minimum zones of inhibition (7 and 8 mm) were measured for NS 20 against *Staphylococcus aureus* and *Vibrio cholerae*. No zones of inhibition were observed at NS 40 and NS 80 concentrations against *Salmonella typhi*, *Klebsiella pneumoniae*, and *Escherichia coli*



Fig 17: Zone of Inhibition of different NS concentration against different pathogenic strains after 48 hours.

4.6. Table 6: Antibacterial activity (zone of inhibition in mm) of different *Nigella sativa* concentration against different pathogenic strains after 72 hours

	Zone of Inhibition (mm) by Well diffusion method after 72 hours		
Pathogenic strain	NS20	NS40	NS80
<i>Vibrio cholerae</i>	8	8	8
<i>Escherichia coli</i>	0	0	0
<i>Streptococcus pyogenes</i>	0	10	0
<i>Staphylococcus aureus</i>	7	8	7
<i>Salmonella typhi</i>	0	0	0
<i>Klebsiella pneumoniae</i>	0	0	0

Table 6 shows that the maximum zones of inhibition (mm) 10 and 9 were measured for NS 80 against *Streptococcus pyogenes*, *Staphylococcus aureus*, and *Vibrio cholerae*, respectively. The minimum zones of inhibition (7 and 8 mm) were measured for NS 20 against *Staphylococcus aureus* and *Vibrio cholerae*. No zones of inhibition were observed at NS 40 and NS 80 concentrations against *Salmonella typhi*, *Klebsiella pneumoniae*, and *Escherichia coli*



Fig 18: Zone of Inhibition of different NS concentration against different pathogenic strains after 72 hours.

4.7. Table 7: Antibacterial activity (zone of inhibition in mm) of different *Ocimum tenuiflorum* concentration against different pathogenic strains after 24 hours

	Zone of Inhibition (mm) by Well diffusion method after 24 hours		
Pathogenic strain	OT20	OT40	OT80
<i>Vibrio cholerae</i>	8	9	13
<i>Escherichia coli</i>	0	0	0
<i>Streptococcus pyogenes</i>	0	11	13
<i>Staphylococcus aureus</i>	0	12	13
<i>Salmonella typhi</i>	0	0	0
<i>Klebsiella pneumonia</i>	0	0	0

Table 7 shows that the maximum zones of inhibition (mm)—13, 13, and 13—were measured for OT 80 against *Streptococcus pyogenes*, *Vibrio cholerae*, and *Staphylococcus aureus*. No zones of inhibition were observed against *Klebsiella pneumoniae*, *Salmonella typhi*, and *Escherichia coli* at OT 20, OT 40, and OT 80 concentrations



Fig 19: Zone of Inhibition of different OT concentration against different pathogenic strains after 24 hours

4.8. Table 8: Antibacterial activity (zone of inhibition in mm) of different *Ocimum tenuiflorum* concentration against different pathogenic strains after 48 hours

	Zone of Inhibition (mm) by Well diffusion method after 48 hours		
Pathogenic strain	OT20	OT40	OT80
<i>Vibrio cholerae</i>	8	9	13
<i>Escherichia coli</i>	0	0	9
<i>Streptococcus pyogenes</i>	0	11	13
<i>Staphylococcus aureus</i>	0	12	13
<i>Salmonella typhi</i>	0	0	0
<i>Klebsiella pneumoniae</i>	0	0	0

Table 8 shows that the maximum zones of inhibition (mm)—13, 13, and 13—were measured for OT 80 against *Streptococcus pyogenes*, *Vibrio cholerae*, and *Staphylococcus aureus*. No zones of inhibition were observed against *Klebsiella pneumoniae*, *Salmonella typhi*, and *Escherichia coli* at OT 20, OT 40, and OT 80 concentrations.



Fig 20: Zone of Inhibition of different OT concentration against different pathogenic strains after 48 hours

4.9. Table 9: Antibacterial activity (zone of inhibition) of *Ocimum tenuiflorum* against different pathogenic strains after 72 hours

	Zone of Inhibition (mm) by Well diffusion method after 72 hours		
Pathogenic strain	OT20	OT40	OT80
<i>Vibrio cholerae</i>	8	13	9
<i>Escherichia coli</i>	9	0	9
<i>Streptococcus pyogenes</i>	0	11	0
<i>Staphylococcus aureus</i>	0	14	10
<i>Salmonella typhi</i>	11	12	12
<i>Klebsiella pneumonia</i>	0	8	0

Table 9 shows that the maximum zones of inhibition (mm)—13, 13, and 13—were measured for OT 80 against *Streptococcus pyogenes*, *Vibrio cholerae*, and *Staphylococcus aureus*. No zones of inhibition were observed against *Klebsiella pneumoniae*, *Salmonella typhi*, and *Escherichia coli* at OT 20, OT 40, and OT 80 concentrations.



Fig 21 : Zone of Inhibition of different OT concentration against different pathogenic strains after 72 hours.

4.10. Table 10: *In vivo* Plasma Bacterial clearance of *Staphylococcus aureus* in different dosages of TC-20, TC 40, TC 80 (n=6) with standard Levofloxacin 10 &20 (mg/day twice daily after 7 days of administration)

Group A (n=6) * ¹	Rx Group M1(n=6; TC-20)	Rx Group M2(n=6; TC-40)	Rx Group M3(n=6 TC-80)	Group C1 (n=6; Levo 10)	Rx Group M4(n= 6; TC- 160)	Rx Group M5(n= 6; TC- 320)	Group C 2*2 (n=6; Levo 20)	Group U*3 (n=6)
NB	NBCP	NBCP	NBCP	NBCP	NB	NB	NB	NBCP
NB	NBCP	NBCP	NBCP	NBCP	NB	NB	NB	NBCP
NB	NBCP	NBCP	NBCP	NBCP	NB	NB	NB	NBCP
NB	NBCP	NBCP	NBCP	NBCP	NB	NB	NB	NBCP
NB	NBCP	NBCP	NBCP	NBCP	NB	NB	NB	NBCP
NB	NBCP	NBCP	NBCP	NBCP	NB	NB	NB	NBCP

Note: *¹Group A: Negative Control (Water +Pellet); *² Group C1: Std Control; Levo-10mg bid; Group C2: Std Control; Levo-20mg bid; *³ Group U: Bacteria induced but No-Rx; NPBC-No Bacterial Clearance from Plasma; NB-No Bacteria; Rx-Treatment

Table 10 shows that Group A had no detectable bacteria in plasma after 7 days. Groups M1, M2, and M3 showed the presence of bacteria in plasma, and no bacterial clearance was observed from plasma. Groups C1 and U also showed no bacterial clearance after 7 days of drug administration. In addition, Group A showed no bacteria in plasma after 7 days, while Groups M4, M5, and Group C2 showed no detectable bacteria in plasma. Group U also showed no bacterial clearance from plasma after 7 days

Discussion

The study result showed that the maximum zones of inhibition (mm)—16, 16, 14, 14, 13, and 12 were measured for TC 80 against *Escherichia coli*, *Streptococcus pyogenes*, *Vibrio cholerae*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Salmonella typhi* respectively. The minimum zones of inhibition (mm) were observed for TC 20 against *Salmonella typhi* and *Escherichia coli*. One study conducted by Upadhyay et al. (2010) showed that at very low concentrations, *Tinospora cordifolia* exhibited notable antimicrobial activity against both gram-positive and gram-negative microorganisms such as *Klebsiella pneumoniae* (ATCC 15380), *Escherichia coli* (ATCC 25922), *Micrococcus luteus* (ATCC 9341), *Streptococcus pneumoniae* (ATCC 12755), *Staphylococcus aureus* (ATCC 25923), and *Bacillus cereus* (ATCC 11778). Another study conducted by Jeyachandran et al. (2013) found that the stem of *Tinospora cordifolia* was significantly effective against *Escherichia coli*, *Proteus vulgaris*, *Enterococcus faecalis*, *Salmonella typhi* (Gram-negative), and *Staphylococcus aureus*. These findings were coincided with the study results against *Klebsiella pneumoniae*, *Escherichia coli*, and *Staphylococcus aureus*.

The study also showed that the maximum zones of inhibition (mm) 16, 16, 16, 14, 13, and 12—were measured for TC 80 against *Escherichia coli*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Vibrio cholerae*, *Klebsiella pneumoniae*, and *Salmonella typhi*, respectively. The minimum zones of inhibition (mm) of 8 mm were observed for TC 20 against *Salmonella typhi* and *Escherichia coli*. Another study conducted by Sandhu et al. (2013) reported that *Tinospora cordifolia* is highly effective against *Serratia marcescens*, *Escherichia coli*, *Salmonella typhi*, *Streptococcus thermophilus*, *Fusarium oxysporum*, and *Aspergillus niger*. The findings regarding *E. coli* and *S. typhi* were similar with the study results; however, differences were observed for other pathogens, as those were not included in the study.

It was found that the alcoholic extract of *Tinospora cordifolia* produced a zone of inhibition of 10 mm against *Salmonella typhi*, while the standard drug Levofloxacin showed a zone of 12 mm. One study conducted by Nageswari (2016) reported that aqueous extracts of *T. cordifolia* exhibited effective antimicrobial activity against *S.*

typhi (26 mm at 50 mg/mL). The differences between the findings may be due to variations in extraction techniques and solvents used. Another study conducted by Tambekar et al. (2009) reported that *Tinospora cordifolia* showed antibacterial activity against *Salmonella typhi* (ZI: 19 mm). Both studies showed some variation compared to the study findings.

The study also revealed that the maximum zones of inhibition (mm)—10 and 9 were measured for NS 80 against *Streptococcus pyogenes* and *Staphylococcus aureus* respectively. The minimum zones of inhibition (7 and 8 mm) were measured for NS 20 against *Staphylococcus aureus* and *Vibrio cholerae*. No zones of inhibition were observed at NS 40 and NS 80 concentrations against *Salmonella typhi*, *Klebsiella pneumoniae*, and *Escherichia coli*. A study conducted by Mohammad Tariq Salman et al. (2016) showed that *Nigella sativa* (black cumin) seed extract was effective against 38 multidrug-resistant strains of *Staphylococcus aureus* out of 40 isolates. Both studies were consistent regarding activity against *S. aureus*, although other pathogens were not evaluated in that study.

Another study conducted by M.S. Hanafy and M.E. Hatem (1991) and showed that diethyl ether extract of *Nigella sativa* seeds (25–400 µg/disc) showed concentration-dependent inhibition of Gram-positive bacteria such as *Staphylococcus aureus* and Gram-negative bacteria such as *Pseudomonas aeruginosa* and *Escherichia coli*, but not *Salmonella typhi*. These findings were almost similar with the study results for *S. aureus* and *E. coli*.

The study results showed that the maximum zones of inhibition (mm) 10 and 9 were measured for NS 80 against *Streptococcus pyogenes*, *Staphylococcus aureus*, and *Vibrio cholerae*, respectively, while the minimum zones (7 and 8 mm) were measured for NS 20 against *Staphylococcus aureus* and *Vibrio cholerae*. No zones of inhibition were observed at NS 40 and NS 80 concentrations against *Salmonella typhi*, *Klebsiella pneumoniae*, and *Escherichia coli*. A study by Zuridah et al. (2008) reported that *N. sativa* seed extracts at different concentrations (25, 50, and 100 mg/20 µL) showed increasing inhibition zones in *S. aureus* (14, 17, and 25 mm) and *P. aeruginosa* (8, 9, and 10 mm), respectively. Both studies were consistent regarding *Staphylococcus aureus*.

It was found that the maximum zones of inhibition (mm) 13, 13, and 13 were measured for OT 80 against *Streptococcus pyogenes*, *Vibrio cholerae*, and *Staphylococcus aureus*. No zones of inhibition were observed against *Klebsiella pneumoniae*, *Salmonella typhi*, and *Escherichia coli* at OT 20, OT 40, and OT 80 concentrations respectively. A study conducted by Jabeen et al. (2008) also reported antibacterial activity of OT against *E. coli*, *S. aureus*, *Bacillus subtilis*, and *Salmonella typhi*. Both studies were consistent regarding activity against *E. coli*, *S. aureus*, and *S. typhi*.

Tiwari et al. (2011) observed that OT aqueous and alcoholic extracts were effective against *E. coli*, *Salmonella typhi*, *Shigella dysenteriae*, *Vibrio cholerae*, and *S. aureus*. Another study by Rathnayaka (2013) reported significant antibacterial activity of *Ocimum tenuiflorum* extract against *Staphylococcus aureus*, which is consistent with the findings.

The *in vivo* study showed that Group A had no detectable bacteria in the plasma after 7 days of administration. Groups M1 (TC-20), M2 (TC-40), M3 (TC-80), and C1 (Levo 10) showed the presence of bacteria in plasma, and no bacterial clearance from plasma was observed. Groups M4 (TC-160), M5 (TC-320), and C2 (Levo 20) showed no detectable bacteria in plasma after 7 days of drug administration. Group U also showed no bacterial clearance after 7 days. A study conducted by Debnath et al. (2014) reported that *Tinospora cordifolia* extract enhances bacterial clearance and improves the phagocytic activity of PMN cells in mice models. The study concludes that alcoholic extracts of *Tinospora cordifolia* (Gulancha) exhibit significant antibacterial effects against *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus pyogenes*, *Vibrio cholerae*, *Klebsiella pneumoniae*, and *Salmonella typhi*.

The *in vivo* study showed that low concentrations (20, 40, and 80 mg/kg) of *Tinospora cordifolia* did not exhibit antibacterial activity against *Staphylococcus aureus* in plasma. In contrast, higher concentrations (160 and 320 mg/kg), administered twice daily, showed significant bacterial clearance from plasma after 7 days of oral administration. This effect may be attributed to differences in pathophysiological responses and host defence mechanisms in the mice model.

6. Conclusion

The *in vitro* study revealed that the ethanolic extract of *Tinospora cordifolia* has a significant antibacterial effect against *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus pyogenes*, *Vibrio cholerae*, *Klebsiella pneumoniae*, and *Salmonella typhi*. The *in vivo* study demonstrated that low concentrations (20, 40, and 80 mg/kg) of *Tinospora cordifolia* did not show bacterial clearance from plasma against *Staphylococcus aureus*. However, higher concentrations (160 and 320 mg/kg), administered twice daily, showed significant bacterial clearance from plasma after 7 days of oral administration. This may be due to differences in pathophysiological and host defence mechanisms in the mice model. Large-scale clinical trials are recommended to develop these extracts as potential natural antibiotics.

7. References (APA)

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Annexure I:

Certificate of Animal Ethical committee

ডিন অফিস
 জীববিজ্ঞান অনুষদ
 ঢাকা বিশ্ববিদ্যালয়, ঢাকা-১০০০, বাংলাদেশ

University of Dhaka
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Ref. No. 169/Biol. Scs. June 20, 2022

Ethical Review Committee

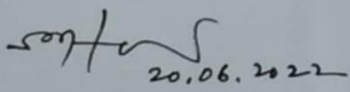
Professor Dr. Sharmin Rumi Alim
 Institute of Nutrition and Food Science
 University of Dhaka

Subject: Ethical Clearance

Dear Dr. Sharmin Rumi Alim,

With reference to your application on the above subject, this is to inform you that your research proposal entitled “In-vitro and In-vivo studies on antibacterial activity of *Ocinum transflorum*, *Nigella sativa* and *Tinosporn corditolia* extracts against some human pathogenic bacterial strain” has been reviewed and approved by the Ethical Review Committee of the Faculty of Biological Sciences, University of Dhaka.

I wish for the success of your research project.


 20.06.2022

Professor Dr. A K M Mahbub Hasan
 Dean, Faculty of Biological Sciences
 University of Dhaka

Image of *in vitro* findings



Image of *in vitro* study findings

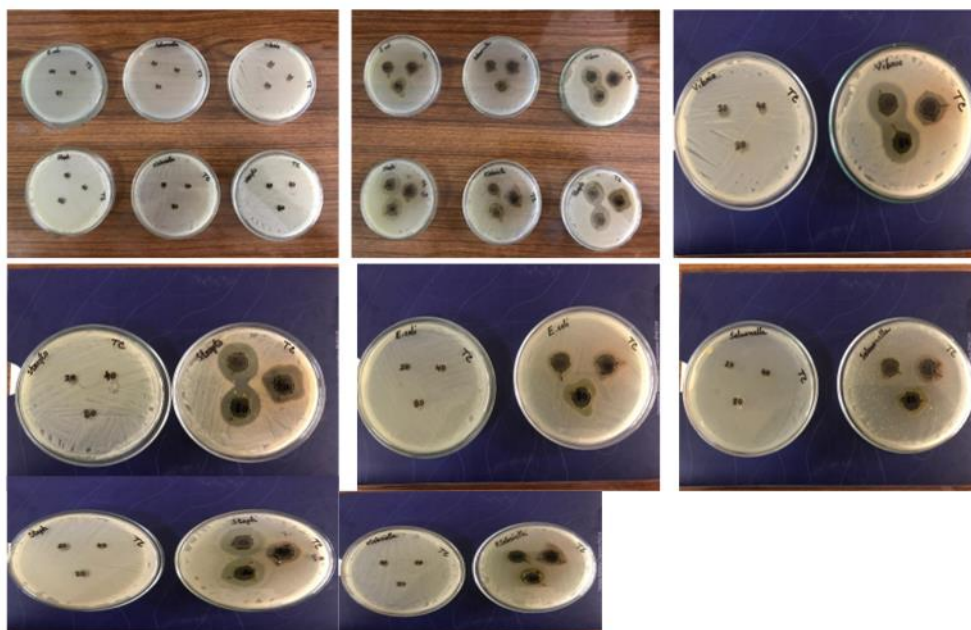


Image of *in vivo* (Animal study) during orally administered tested drug and collection of blood sample from tail vein

Published Title: *In-vitro* study showed that *Tinospora cordifolia* is having significant antibacterial effect against different human pathogenic strains in different dose dependent manner.

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