Validation of antimicrobial activity of selected herbs against multidrug-resistant *Salmonella* Typhi isolates: development of antityphoid herbal formulation

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By

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CERTIFICATE FROM THE SUPERVISOR(S)

This is to certify that the thesis entitled "**Validation of antimicrobial activity of selected herbs against multidrug-resistant Salmonella Typhi isolates: development of antityphoid herbal formulation**", Submitted by Smt. **Sunayana Saren** who got her name registered on **02.11.2020** for the award of Ph. D. (Science) Degree of Jadavpur University, is absolutely based upon her own work under the supervision of **Dr. Shanta Dutta, Former Director and Scientist G, ICMR - National Institute for Research in Bacterial Infections** and that neither this thesis nor any part of it has been submitted for either any degree/diploma or any other academic award anywhere before.

Shanta Dutta 25/4/2025

(Signature of the Supervisor(s) date with official seal)

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-:Abstract:-

Index no.: **50/20/Life Sc./27**

Thesis Title: **Validation of antimicrobial activity of selected herbs against multidrug-resistant *Salmonella* Typhi isolates: development of antityphoid herbal formulation**

Salmonella enterica subsp. enterica serotype Typhi, or *Salmonella* Typhi, is a common food-contaminating bacterium that causes human-restricted typhoid. In 2021, over 9 million cases of typhoid and 100,000 deaths were reported. Over time, typhoid has disappeared from many developed countries, but the emergence of drug-resistant strains has become a growing concern in low- to middle-income countries. The rise of resistant strains of *S.* Typhi demands the development of new antibacterial agents against this pathogen. Plants can serve as a valuable natural source of antibacterial agents due to their cost-effectiveness and sustainability. The methanolic leaf extract of *Senna occidentalis* and the methanolic root extract of Scoparia dulcis were tested against clinical isolates of *S.* Typhi. Both extracts demonstrated antibacterial activity against *S.* Typhi; however, the root extract of *Scoparia dulcis* exhibited greater efficacy, with MIC and MBC values of ≤ 7.5 (> 3 75) mg/ml and ≥ 15 (> 7.5) mg/ml, respectively, compared to the leaf extract of Senna occidentalis. Both crude extracts were fractionated by column chromatography, and each collected fraction was tested against *S.* Typhi to identify the most active fractions. A total of forty-three phytochemicals were identified through LC-MS from five selected fractions. All identified phytochemicals were virtually screened to assess their physicochemical properties and predict their druglikeness and toxicity class. Furthermore, five phytochemicals were selected for further evaluation: three flavonoids- acacetin, apigenin, and luteolin and two benzoxazinoids- 6-methoxybenzoxazolinone and benzoxazolinone, to delve deeper into their effectiveness against *S.* Typhi. Luteolin showed comparatively greater effectiveness than the other four, with the lowest MIC of ≤ 0.03 (> 0.01) mg/ml. The effect of flavonoids was bacteriostatic, while it was bactericidal for benzoxazinoids. All phytochemicals inhibited biofilm formation, inhibition of invasion of *S.* Typhi into macrophage cells and inhibition of the activity of *S.* Typhi DHFR protein. In *S.* Typhimurium challenged mice, phytochemical treatment resulted in 100% survivability with significantly less bacterial colonization in the intestine and liver of infected models. This study suggests that the root of *S. Dulcis* can serve as a herbal remedy for *S.* Typhi infection and as a source for new antibacterial agents. Additionally, the five studied phytochemicals- acacetin, apigenin, luteolin, 6-methoxybenzoxazolinone, and benzoxazolinone- show great potential to become anti-typhoid drugs and may inform future studies.

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-:Declaration:-

I, **Sunayana Saren**, do hereby declare that the work embodied in this thesis entitled "**Validation of antimicrobial activity of selected herbs against multidrug-resistant *Salmonella* Typhi isolates: development of antityphoid herbal formulation"** Submitted for the Degree of Doctor of Philosophy (Sciences) in Life Science and Biotechnology, under Jadavpur University is the completion of work carried out under the supervision of **Dr. Shanta Dutta**, former Director and Scientist G at Division of Bacteriology, ICMR-National Institute for Research in Bacterial Infections, Kolkata, India. Neither this thesis nor any part of it has been submitted for any equivalent degree/ diploma or any other academic award elsewhere.

Date: 25.4.25

Place: Kolkata

Sunayana Saren

Signature of the Candidate

SUNAYANA SAREN

Dedicated to

My grandfather

Late Ganesh Chandra Saren

He who planted the curiosity in me for plants and health

For

Maa and Baba

***

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Sunayana Saren

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-:Abbreviation:-

°C: Degree Celsius

°F: Degree Fahrenheit

µg/ml: microgram per mililitre

µl: Microliter

µm: Micrometer

3D: Three-dimensional

ADME: Absorption, Distribution, Metabolism, Excretion

ALA: Alanine

AMR: Antimicrobial Resistance

ARG: Arginine

ASN: Asparagine

ASP: Aspartic Acid

ATCC: American Type Culture Collection

BALB/c: Bagg Albino Laboratory-Bred Mouse, subline "c"

BBB: Blood-Brain-Barrier

BCE: Before Common Era

BOA: 2-Benzoxazolinone/ Benzoxazolinone

bp: Base Pair

BPI: Bactericidal Permeability-Increasing Protein

CFU: Colony Forming Unit

CLSI: Clinical and Laboratory Standards Institute

cm: Centimeter

CO_2: Carbon dioxide

CUH: Calcutta University Herbarium

CV: Crystal Violet

DHFR: Dihydrofolate Reductase

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic Acid

dNTPs: deoxynucleoside triphosphates

EPS: Extracellular Polymeric Substances

EU/EEA: European Union/European Economic Area

F primer: Forward primer

FASTA: Fast Alignment Search Tool for Amino Acid or Nucleotide Sequences

FBS: Fetal Bovine Serum

GI: Gastrointestinal

Glide SP: standard-precision Glide docking

GLU: Glutamic acid

gm: Gram

GMQE: Global Model Quality Estimate

H_2S: Hydrogen Sulfide

HCL: Hydrochloric acid

HICs: High-Income Countries

HIV: Human Immunodeficiency Virus

HPLC: High Pressure Liquid Chromatography

IC50: Inhibitory Concentration 50

ICMR: Indian Council of Medical Research

ID: Identification

IL6: Interleukin-6

IL8: Interleukin-8

kb: Kilobase pairs

kDa: Kilodalton

KOL: Kolkata

LB: Luria Bertani

LB_{Amp}: Luria Bertani media with ampicilin

LC-MS: Liquid Chromatography-Mass Spectrometry

LD_{50}: Lethal Dose 50

LMICs: low- and middle-income countries

LPS: Lipopolysaccharide

M cells: Microfold cells

mb: Mega basepair

MBC: Minimum bactericidal concentration

MBOA: 6-Methoxybenzoxazolinone

MDR: Multidrug resistant

mg/ml: Milligram per millilitre

MHB: Mueller-Hinton broth

MIC: Minimum inhibitory concentration

min: Minute

ml: Millilitre

MLEE: Multilocus Enzyme Electrophoresis

MLST: Multilocus Sequence Typing

mm: Millimeter

mM: Millimolar

MOI: Multiplicity of infection

MRP: Multidrug resistant-associated protein

MRSA: methicillin-resistant *Staphylococcus aureus*

MTCC: Microbial Type Culture Collection and Gene Bank

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

NA: Nutrient agar

NaCl: Sodium chloride

NADP: Nicotinamide Adenine Dinucleotide Phosphate

NADPH: Nicotinamide Adenine Dinucleotide Phosphate (reduced form)

NCBI: National Center for Biotechnology Information

NDGA: Nordihydroguaretic acid

NEB: New England Biolab

ng: Nanogram

Ni-NTA: Nickel-nitrilotriacetic acid

NIPER-K: National Institute of Pharmaceutical Education and Research, Kolkata

NIRBI: National Institute for Research in Bacterial Infections

nm: Nanometre

NTS: Non-Typhoidal *Salmonella*

OD: Optical density

OMP: Outer membrane proteins

OSS: Outside *Salmonella* Sample

PBS: Phosphate-Buffered Saline

PCR: Polymerase Chain Reaction

PDB: Protein Data Bank

pH: Power of Hydrogen

PHE: Phenylalanine

PMDB: Protein Model DataBase

pmol: Picomole

PMSE: Phenylmethylsulfonyl fluoride

R primer: Reverse primer

rpm: Rotation per minute

RPMI1640: Roswell Park Memorial Institute 1640

S. dulcis*: Scoparia dulcis*

S. occidentalis*: Senna occidentalis*

***S.* Paratyphi A:** *Salmonella enterica* subsp. enterica serotype Paratyphi A

***S.* Paratyphi B:** *Salmonella enterica* subsp. enterica serotype Paratyphi B

***S.* Paratyphi C:** *Salmonella enterica* subsp. enterica serotype Paratyphi C

***S.* Typhi**: *Salmonella enterica* subsp. enterica serotype Typhi

***S.* Typhimurium**: *Salmonella enterica* subsp. enterica serotype Typhimurium

SCV*: Salmonella*-containing vacuole

SD: Standard deviation

SDF: Structure Data File

SDFRC: *Scoparia dulcis* fraction

SDMe: Methanolic root extract of *Scoparia dulcis*

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sec: Second

SER: Serine

SMILES: Simplified Molecular Input Line Entry System

SOC: Super Optimal Broth with Catabolite repression

SOFRC: *Senna occidentalis* fraction

SOMe: Methanolic leaf extract of *Senna occidentalis*

SPI: Salmonella pathogenicity island

SS Agar: *Salmonella-Shigella* Agar

T3SS: Type III secretion system

TBE: Tris-borate-EDTA

TIC: Total Ion Chromatography

TLC: Thin-layer chromatography

TLR: Toll-like receptor

TNF-α: Tumor Necrosis Factor-alpha

Tris-HCl: Tris (hydroxymethyl) aminomethane hydrochloride

TSB: Tryptic Soy Broth

TSI: Triple Sugar Iron

U: Unit

UV: Ultraviolet

VAL: Valine

WHO: World Health Organization

XDR: Extensively Drug-Resistant

XLD: Xylose Lysine Deoxycholate

XP: Extra-precision

YLLs: Years of life lost

ΔOD: Change in optical density

A. Introduction:

Salmonella enterica subsp. enterica serotype Typhi, commonly referred as *Salmonella* Typhi, a gram-negative, rod-shaped, flagellated bacterium and a causal organism for human-restricted typhoid. Symptoms of typhoid may include a progressively rising fever that can reach up to 104.9 °F, accompanied by headache, fatigue, muscle pain, and general weakness. Other signs include sweating, abdominal discomfort, diarrhea or constipation, dry cough, loss of appetite, weight loss, rash, and significant abdominal swelling. *Salmonella* Typhi is primarily transmitted through consuming food or water that has been contaminated by the excrement of infected persons. In healthy people, the infectious dose of *S.* Typhi can range from 1,000 to 1 million organisms, though this may vary depending on the host's immune defenses (Parry et al., 2002).

Before the advent of antibiotics, approximately 8-12% of patients experienced relapses. However, this rate increased to 15-20% among those treated with chloramphenicol (Hornick et al., 1970). Over time, *S.* Typhi disappears from many high-income countries (HICs), while increasingly resistant variants continue to persist in numerous low- and middle-income countries (LMICs). Despite global warnings about antimicrobial resistance (AMR) and the World Health Organization's 1994 recommendation to use vaccines to combat resistant typhoid (Ivanoff et al., 1994), the excessive global use of third-generation cephalosporins and fourth-generation fluoroquinolones (such as gatifloxacin, introduced in 1999) has further driven the selection of resistant strains (Basnyat, 2007; Sp et al., 2003). The recent outbreak in Pakistan has shown resistance to all first-line medications, including quinolones and ceftriaxone. For numerous low-income patients, azithromycin remains the only effective and affordable treatment option, although strains resistant to azithromycin have already been reported (Hooda et al., 2019). Extensively drug-

resistant (XDR) H58 represents the latest and most concerning development in the biosocial history of typhoid (Kirchhelle et al., 2019). In 2021, over 9 million cases of typhoid and 100,000 deaths were reported.

The emergence of antibiotic-resistant strains of *Salmonella* Typhi demands the need to develop an alternative to traditional antibiotics, and Plants show great potential as a source of antibacterial agents. They possess a range of robust defence mechanisms, like producing secondary metabolites, to protect themselves from herbivores and pathogens. Plant-derived compounds offer an alternative that might circumvent current resistance mechanisms. Many tribal communities worldwide, particularly in biodiversity-rich areas, have developed extensive knowledge of medicinal plants through observation, trial and error, and traditional inheritance. In regions where access to modern healthcare is limited, these treatments offer a cost-effective and readily available alternative. Combining traditional knowledge with advancements in modern science, plant-based antibacterial agents hold great potential for enhancing global healthcare solutions. The accessibility and affordability of plant extracts make them a preferred choice. Extensive research has highlighted the effectiveness of various plant extracts against numerous bacterial strains, emphasizing the role of phytochemicals in contemporary medicine. Nonetheless, more studies are necessary to develop standardized formulations and optimize their therapeutic applications.

Two well-known plant species with medicinal properties are used by tribal communities in West Bengal, India, named *Senna occidentalis* and *Scoparia dulcis.* The leaves of *S. occidentalis* and the root of *S. dulcis* are used by the community to treat typhoid symptoms, but the effectiveness has not been well validated. This thesis discusses the efficacy of these two plant extracts against drug-resistant clinical isolates of *Salmonella* Typhi to validate their traditional use through experimental evaluation, along with the identification of phytochemicals that may be involved in

the activity. Then, dive deeper into the physicochemical and cytotoxicity of five selected phytochemicals with their activity against *Salmonella* Typhi and in vivo therapeutic efficacy is discussed.

B. Review of Literature:

B.1. The Earliest Descriptions of Typhoid:

Hippocrates Heraclide, a 5th century BCE physician, is thought to have been the first to document an illness resembling typhoid in his books I and III of Epidemic. Additionally, the Greek historian Herodotus mentioned an outbreak during the Battle of Salamis in his Histories (Adler and Mara, 2016). It has also been suggested that the Roman Emperor Augustus Caesar may have suffered from typhoid fever in 23 BCE. The Neapolitan epidemic of 1495-1496 may have been the one possible typhoid outbreak. Samuel Cooper, a British surgeon and professor in his analysis of the outbreak, characterized the disease as a febrile illness which does not transmit through intimate contact and was a different disease from syphilis spread by poor sanitation (Adler and Mara, 2016). In 1635, a febrile illness also appeared among the French-Dutch army soldiers during the midst of the 30-year war, which possibly was a typhoid outbreak (Adler and Mara, 2016). In the early 1900s, typhoid had a fatality rate of 10% and primarily impacted impoverished individuals living in urban areas. George Sober, in 1907, published the results of an investigation describing Mary Mallon, who was infamously termed “Typhoid Mary”, as a “healthy carrier” of Salmonella typhi in the United States. He stated that her actions were spreading illness and causing fatalities. That year, 3000 people in New York had typhoid, and it was assumed that Mary Mallon was the main reason for the outbreak (Marineli et al., 2013).

B.2. Casual Organism of Typhoid:

B.2.i. Discovery:

n 1829, C. A. Louis in Paris was the first to identify it as distinct from other fevers, linking its clinical characteristics to lesions found in the intestines, mesenteric lymph nodes, and spleen. Before the identification of the causal organism for typhoid was done, Karl Liebermeister stated that the disease was transmitted through drinking water contaminated by the excrement of individuals with typhoid (Liebermeister, 1896). In 1873, William Budd proposed that typhoid might spread via a specific toxin found in the excrement of infected patients, which contaminates water and is responsible for its propagation. Karl Joseph Eberth in 1879 isolated a bacillus from the spleen and abdominal lymph nodes, and later published his findings in 1880 and 1881 (Moorhead, 2002). Georg Theodor Gaffky, in 1884, named the rod-like bacillus, the agent of typhoid fever, as *Eberthella typhi* (Gryglewski and Chlipała, 2020). Later, it was identified under the genus *"Salmonella*", named in honour of Daniel Elmer Salmon, an American veterinary pathologist.

B.2.ii. Classification and Nomenclature:

Salmonella Typhi (*S.* Typhi) is now classified as one of the serovars of *Salmonella enterica* subspecies enterica. The genus *Salmonella,* a member of the family Enterobacteriaceae, comprises two species: *S. bongori* and *S. enterica*. The latter is further divided into six subspecies, with *Salmonella enterica* subspecies enterica being the most clinically significant. Based on disease syndromes, this subspecies is classified into Typhoidal *Salmonella* and Non-Typhoidal *Salmonella* (NTS). Typhoidal *Salmonella* cause enteric fever (typhoid and paratyphoid) exclusively in humans (host-restricted). Typhoid is caused by *S.* Typhi, while paratyphoid is caused by *S.* Paratyphi A, *S.*

Paratyphi B, and *S.* Paratyphi C. In contrast, Non-Typhoidal *Salmonella* are generally host-generalists and encompass a wide variety of serovars (Achtman et al., 2012; Sanderson et al., 2015). The complete nomenclature of *Salmonella* Typhi is *Salmonella enterica* subspecies enterica serovar Typhi (Brenner et al., 2000).

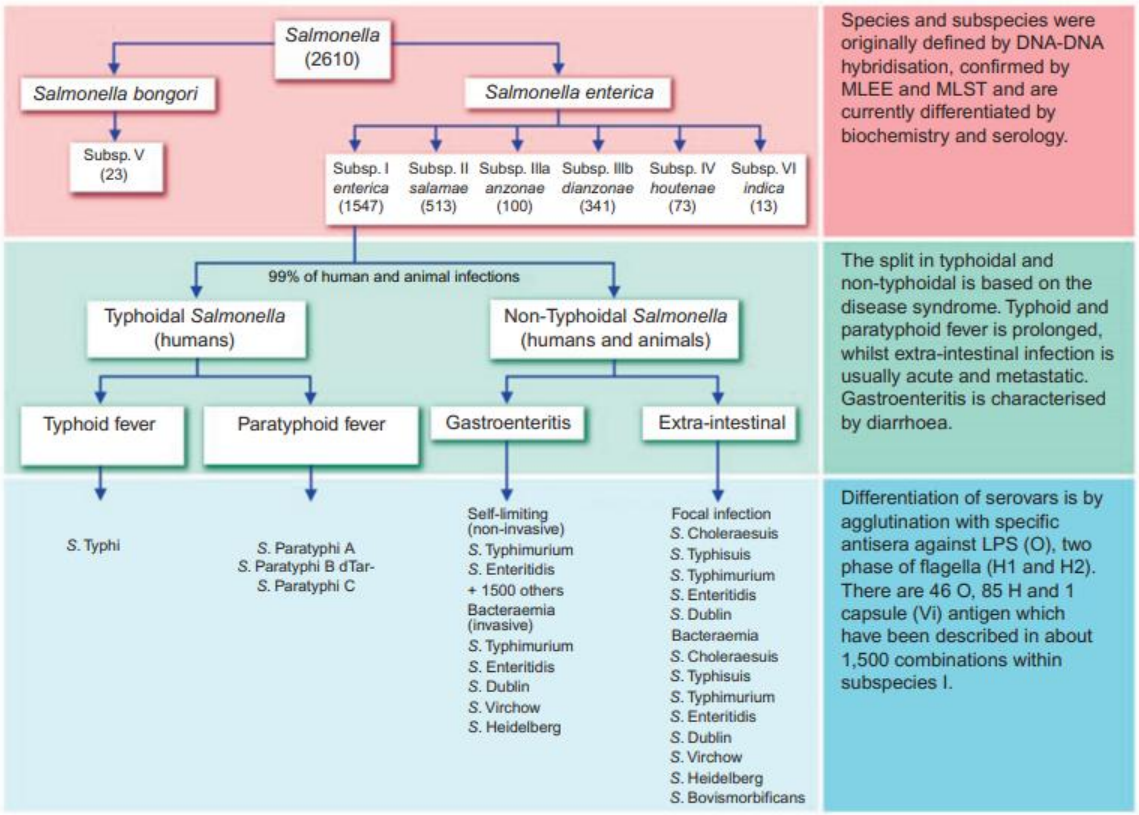


Figure B.2.ii: classification of bacteria in the genus *Salmonella* (Achtman et al., 2012;)

The most common method for the classification and nomenclature of the genus *Salmonella* is Kauffmann-White serological typing, which is based on the antigenic properties of serovars, especially the somatic (O) and flagellar (H) antigens. The O antigens are determined by the lipopolysaccharides of the outer membrane, whereas the H antigens are determined by the flagella

(Brenner et al., 2000; Sanderson et al., 2015). *S.* Typhi and a few other serovars may also contain Vi or capsular antigen. There are 48 O antigens, 85 H antigens switch between two phases except *S.* Typhi strains, which are monophasic as they lack genes that code for phase 2 (Baker et al., 2007), and 1 Vi antigen (Achtman et al., 2012). *Salmonella* serovars are recognized by their unique combination of antigens, which is their antigenic formula (Kauffman, 1966). The antigenic formula for the *S.* Typhi is 9,12,Vi:d.

B.2.iii. Structure:

Salmonella Typhi is a gram-negative, rod-shaped bacterium of about 1-3 µm × 0.5-0.6 µm size with a peritrichous 2-5 µm long flagella arrangement. It is a motile bacterium arranged singly or in pairs and is non-sporing and non-capsulated. The inner membrane is covered by a thin, rigid murein peptidoglycan cell wall, which provides rigidity for cell shape and is intimately attached to the inner side of the outer membrane. The lipoprotein bilayers present in both the inner and outer membranes serve the selective permeability of the cell and contain specific pores for the transport of molecules into and from the cytoplasm. The outer membrane is saturated externally with polymerized O antigen linked to core oligosaccharide, with its terminus attached to lipid A embedded in the outer membrane. The virulence (Vi) capsular antigen is an N-acetylglucosamine uronic acid polymer and is expressed on the outer cell surface. *S.* Typhi bears proteinaceous hair-like 10 nm in diameter fimbriae or pili, which help in adherence and biofilm formation and are composed of fimbrillin or pilin subunits of about 21 kDa (Jaroni, 2014).

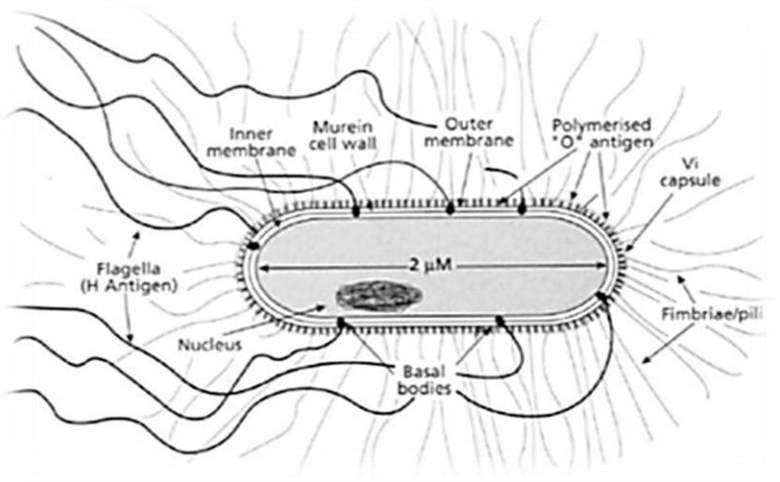


Figure B.2.iii: Schematic diagram of *Salmonella* Typhi structure

B.2.iv. Culture Condition:

S. Typhi is an aerobic and facultative anaerobic bacterium, can grow in the presence or absence of oxygen. In the laboratory, 37 °C is the optimum temperature, and at 6.5-7.5 pH, maximum growth can be observed, but it can also survive at 6-46 °C and 4.1-9.0 pH. *S.* Typhi can grow without the need for specialized nutrition and grows well on standard nutrient agar media. In laboratory settings, the most frequently used media include Nutrient agar and MacConkey agar. For isolating the organism from fecal samples, Selenite F broth and Xylose Lysine Deoxycholate (XLD) medium are commonly employed (Tankeshwar, 2015).

B.2.v. Colony Morphology:

Colonies of *S. Typhi* on agar media are generally Broad, circular, measuring 2-3 mm across, with a gently curved surface and a sleek texture. On Nutrient Agar, it is off-white, moist, circular with a slightly raised appearance. On MacConkey agar, colonies are pale or colorless due to non-lactose fermentation, whereas on XLD Agar, colonies are red with a black center due to hydrogen sulfide production, and on Bismuth Sulfite Agar, they are black colonies with a metallic sheen. On Blood

Agar, colonies are white and non-hemolytic and on Salmonella-Shigella (SS) Agar, colonies appear colorless with black centers due to hydrogen sulfide production (Aryal, 2016; BATRA, 2018; Tankeshwar, 2015).

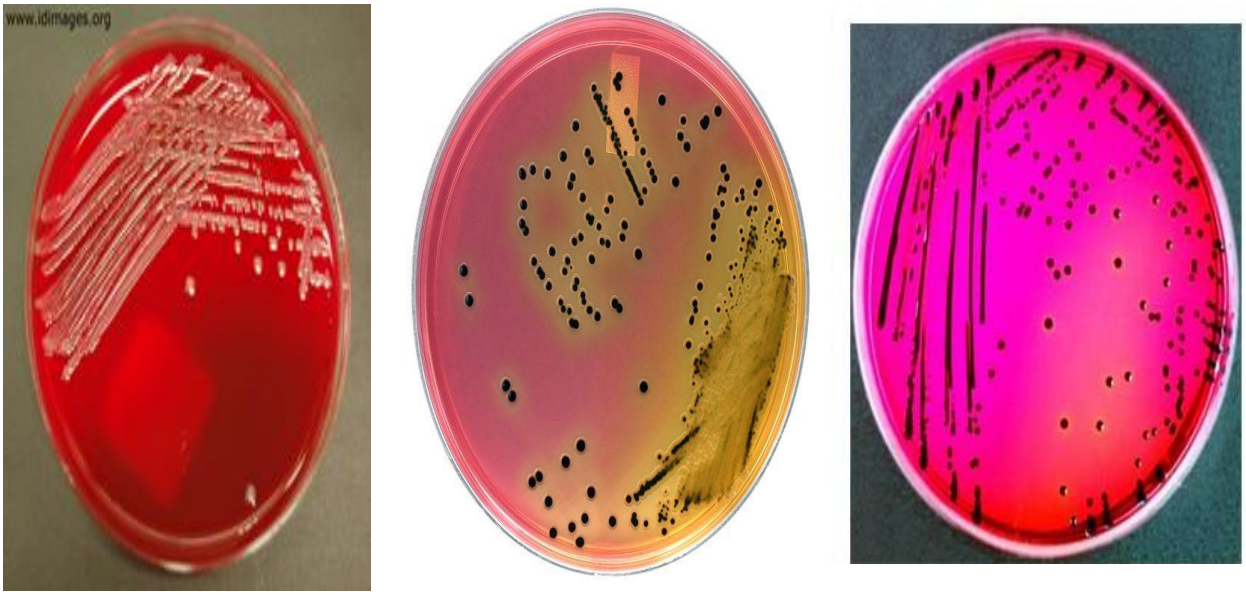


Figure B.2.v: Colonies of *Salmonella* Typhi on Xylose Lysine Deoxycholate, *Salmonella-Shigella*, Blood Agar plates (lift to right) [Source: web image (Aryal, 2015); https://hardydiagnostics.com/g327; https://www.idimages.org/.]

B.2.vi. Biochemical Reaction:

Salmonella Typhi, the causative agent of typhoid fever, exhibits distinct biochemical reactions that aid in its identification. The common biochemical tests for *S.* Typhi and their outcome are presented in **Table B.2.vi**.

Table B.2.vi: Biochemical tests and their outcome for Salmonella Typhi (Aryal, 2022, 2015; BATRA, 2018b)

Test		Result
Catalase		+
Citrate Utilization		-
Indole		-
Hydrogen Sulfide (H_2S) Production		+
Methyl Red		+
Voges-Proskauer		-
Urease		-
Oxidase		-
Triple Sugar Iron (TSI) Agar		Alkali/Acid
Fermentation	Glucose	+
	Maltose	+
	Mannitol	+
	Mannose	+
	Melibiose	+
	Sorbitol	+
	Trehalose	+
	Xylose	+
	Lactose	-
	Sucrose	-

B.2.vii. Genetic Makeup:

The genome of S. Typhi spans approximately 5 mb and encodes around 4,000 genes, over 200 of which are categorized as pseudogenes and lack functionality. (Baker and Dougan, 2007; Zhang et al., 2008). In 2001 first complete genome sequence of S. Typhi was studied from the strain CT18 (Parkhill et al., 2001). The chromosome structure of *Salmonella* strains contains a core genome that comprises 90% of the genome, conserved across all strains and is responsible for essential functions, and an accessory genome responsible for host specificity, pathogenicity, and is unique to specific strains (Sanderson et al., 2015). The core genes are about 90% similar among E. coli and S. enterica and about 99% similar among S. enterica serovars (Baker and Dougan, 2007).

There are 13.1% genes which were unique to *S.* Typhi compared to S. Typhimurium, which may be acquired by horizontal gene transfer (Hacker et al., 1997; Parkhill et al., 2001).

There are three major types of accessory genome, *i.e*. Salmonella pathogenicity island (SPI), Prophages and remnants of prophages that are inserted into the genome and operons (Sanderson et al., 2015). There are 22 different types of SPIs in serovars of *Salmonella,* with 16 found in *S.* Typhi (Fookes et al., 2011). The SPI-1 and SPI-2 codes for the type III secretion system (T3SS), involved in transferring virulence factors into host cells (Zhou and Galán, 2001). The inactivation of SPI-1, SPI-2, SPI-3, SPI-4, and SPI-5 leads to inability to express several virulence-associated traits (Zhang et al., 2008). The SPI-7, SPI-8, SPI-15 and SPI-18 are specific to *S.* Typhi. The SPI-7 is the largest SPI, 134 kb long, that produces the Vi polysaccharide capsule and also encodes for the Vi locus (Baker and Dougan, 2007). The prophages and their remnant had a great role in the evolution of bacterial virulence (Cooke et al., 2007). The prophages may carry "cargo genes" that may influence the pathogenic potential of strains by lysogenic conversion (Figueroa-Bossi et al., 2001; Sanderson et al., 2015). Some of the known prophages in *S.* Typhi are Gifsy-1, Gifsy-2, and ST64 B (Trofeit et al., 2023). The operons determine fimbriae on the surface of bacteria and vary between and within serovars (Sanderson et al., 2015). Some of the fimbrial operons identified in *S.* Typhi are *sef, fim, saf, tcf, bcf, sta, stb, ste, std, stc, stg,* and *sth*. These operons play roles in motility, host invasion, and persistence (Dufresne et al., 2018; Townsend et al., 2001).

Some strains of *S.* Typhi contain plasmids carrying virulence or antibiotic-resistance genes (Baker and Dougan, 2007). *S.* Typhi CT18 contains two 218 kb and 106 kb long plasmids, namely pHCM1 with multiple-drug-resistance genes, and pHCM2, respectively, but is absent in virulent wild-type *S.* Typhi strains (Parkhill et al., 2001; Sanderson et al., 2015). In 2007, a linear plasmid named

pBSSB1 was identified in a strain of *S.* Typhi isolated from Indonesia that can code for an additional z66 flagellar antigen (Baker et al., 2007).

The large number, *i.e*. over 200 pseudogenes of *S.* Typhi, which may occurred due to the accumulation of mutations, are the cause of its host restriction (Sanderson et al., 2015).

B.2.viii. Pathogenesis:

S. Typhi has a prolonged incubation period of 7 to 14 days, leading to a restricted intestinal infection that typically does not involve diarrhoea. However, it causes a more severe systemic enteric fever. During the infection process, the bacteria invade the liver and spleen, frequently establish a carrier state in the gall bladder, and may reinfect the intestines via bile from the gall bladder. This infection has the potential to be fatal. *S.* Typhi primarily transmitted through ingestion food or water that has been contaminated by the excrement of infected persons (Sanderson et al., 2015). *S.* Typhi, ingested by contaminated food or water, from the gastrointestinal tract enters the small intestine where it invades specialized epithelium cells called M cells that overlie the Peyer's patches by using flagella and T3SS-1 and colonizes the cells by adhering to the mucosal surfaces. Bacteria within the submucosal layer interact with dendritic cells, enterocytes, and macrophages, migrate to intestinal lymphoid follicles and mesenteric lymph nodes, move into the reticuloendothelial cells of the liver and spleen, enter the bloodstream, and spread throughout the body. Bacteremia can lead to secondary infections in the liver, spleen, bone marrow, gallbladder, and Peyer's patches. *S.* Typhi can go into a chronic 'carrier' state where it remains reserved in the gallbladder and may reinvade the intestine or be excreted in the faeces. The chronic infection persists for many decades, and carriers are very contagious but asymptomatic (Everest et al., 2001; Hk et al., 2012; Parry et al., 2002; Tartera and Metcalf, 1993).

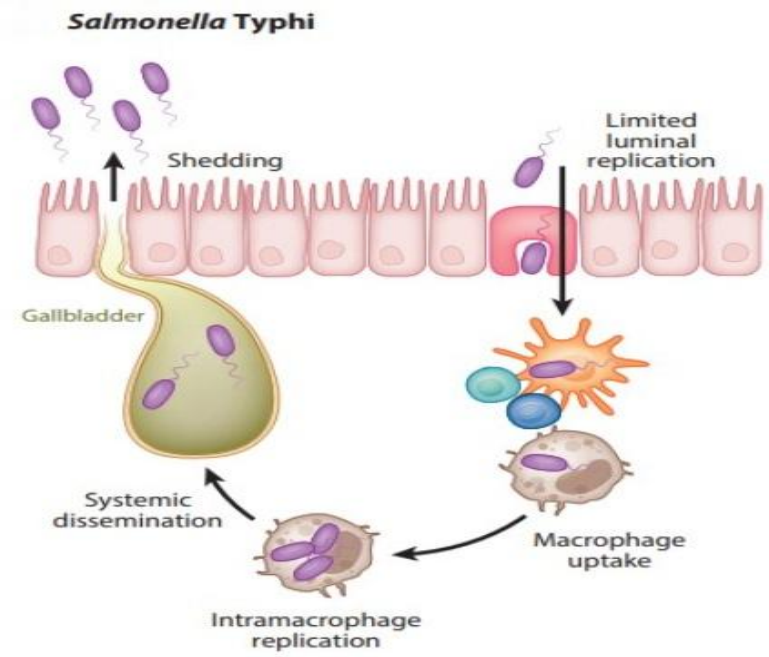


Figure B.2.viii.a: Schematic diagram S. Typhi pathogenesis (Dougan and Baker, 2014).

The *S.* Typhi during the infection faces hostile environments, in the stomach, most of the bacteria are killed by gastric acid, and those that survive the acid may act as a stimulus to replicate (Ahirwar et al., 2014). The mucus layer that consists of highly glycosylated proteins, mucins, and is enriched with antimicrobial peptides, upon infection, changes dramatically. Beneath the mucus layer lies the glycocalyx, made up of glycolipids and glycoproteins that extend from the plasma membrane of intestinal epithelial cells, giving extra defense against infection (J. Barton et al., 2021).

The fimbriae of *S.* Typhi play a key role in attachment to epithelial cells and may also have a role in host specificity (Berrocal et al., 2015; J. Barton et al., 2021). Within the high osmolarity environment of the intestinal lumen, EnvZ, a sensor kinase, activates the downstream regulator OmpR by phosphorylation, which suppresses the TviA, resulting in expression of T3SS-1 and flagellin, allowing the invasion of the intestinal epithelium (Winter et al., 2009). The T3SS-1 inject

a variety of effector proteins into the host cells that includes SipA promotes actin rearrangements to facilitate bacterial invasion, SptP reverses actin rearrangements after invasion, SopE and *Sop*E2 activate host cell signaling pathways to promote bacterial entry, SopB modulates host cell membrane trafficking and ion flux, AvrA suppresses host inflammatory responses and SteA and SteC are involved in intracellular survival and replication (dos Santos et al., 2020; Winnen et al., 2008; Zhou et al., 2023).

S. Typhi invade macrophages to disseminate through the body. To invade the cells, Vi antigen masks lipopolysaccharide (LPS) to evade recognition by toll-like receptor-4 (TLR-4), preventing release of TNF-α, IL6 and IL8 and phagocytosis by BPI, a LPS binding antimicrobial protein (Balakrishnan et al., 2016; Wilson et al., 2008). *S.* Typhi forms a vacuole within the cell called the *Salmonella*-containing vacuole (SCV). The acidic environment of SCV induces the expression of SPI-2, which prevents the assembly of NADPH oxidase on the membrane of phagosome and evades killing (Gallois et al., 2001; Liew et al., 2019).

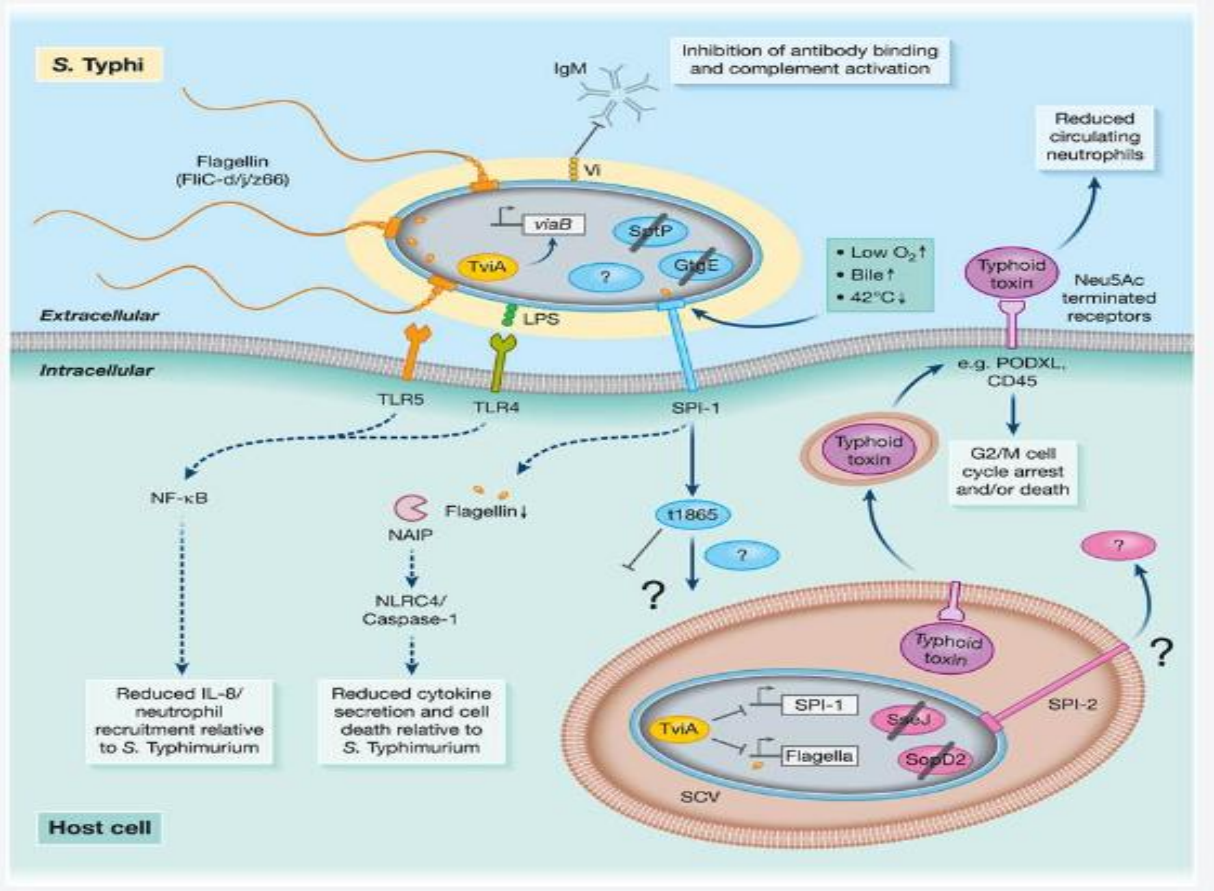


Figure B.2.viii.b: Molecular pathogenesis of S. Typhi (Johnson et al., 2018)

B.2.ix. Virulence Factors:

S. Typhi has several virulence factors that enable it to infect humans effectively and are engaged in different infection phases, such as toxin production, colonization, adhesion, invasion, and survival within host cells (Madigan and Brock, 2007).

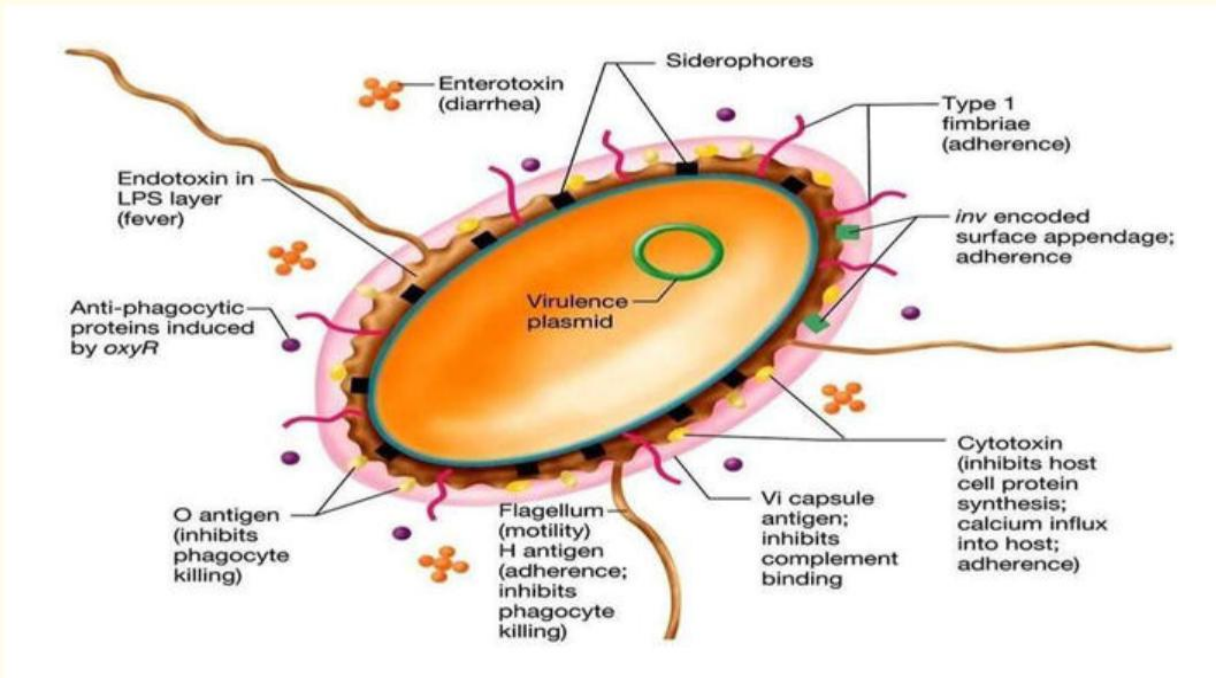


Figure B.2.ix: Virulence factors of *S.* Typhi (Madigan and Brock, 2007).

B.2.ix.a. Vi Antigen:

A key distinguishing feature of S. Typhi compared to NTS is its ability to produce the Vi polysaccharide capsule, also known as the Vi antigen. This antigen plays a role in inhibiting phagocytosis and enhancing serum resistance, potentially by concealing the O-antigen from antibody recognition (Hart et al., 2016; Wilson et al., 2008). The genes responsible for coding the Vi antigens are located within the *via*B locus of SPI-7, which also contains genes for the T3SS effector SopE and a type IVB pilus (Pickard et al., 2003).

B.2.ix.b. SPI-1 and SPI-2:

Two of the main factors for *Salmonella* virulence are the SPI-1 and SPI-2, which are encoded by the T3SS. The SPI-1 facilitates the invasion of nonphagocytic cells (Bishop et al., 2008), while the function of the SPI-2 T3SS is not well defined. While the interruption of SPI-2 did not affect the

survival of *S.* Typhi in macrophages (Forest et al., 2010), but strains that have inserted transposons in the SPI-2 components *ssa*Q, *ssa*P, or *ssa*N showed adverse selection during competitive growth in human macrophages (Sabbagh et al., 2012).

B.2.ix.c. O Antigen and OMPs:

The outer L-layer beneath the capsular material contains O antigen. The O antigens are composed of the side chains of repeating sugar units that extend outward from the lipopolysaccharide layer and the bacterial cell wall surface. These antigens are hydrophilic, thermophilic, and utilized in serological diagnosis (Al-Khafaji et al., 2021). The L-layer also contains specific outer membrane proteins (OMPs), that exhibit antigenic properties. These OMPs consist of both porin and non-porin substances. Porins act as channel-forming pores that facilitate solute uptake, while non-porin proteins contribute to the structural framework (Benz, 1988). These antigens are highly immunogenic, eliciting a strong antibody response in patients with typhoid fever (Calderón et al., 1986).

B.2.ix.d. H Antigen:

Flagella or H antigen play a significant role in the virulence of *S*. Typhi. They are not only essential for motility but also help host cells to invade and evade immune responses. The flagellar protein, flagellin, is recognized by the host's immune system through receptors like TLR-5, triggering an innate immune response. However, S. Typhi has evolved mechanisms to modulate this response, aiding in immune evasion (Hayashi et al., 2001; Kortmann et al., 2015). Additionally, flagella facilitate the bacteria's interaction with host epithelial cells and macrophages, which is crucial for establishing infection (Schreiber et al., 2015). Generally, *S.* Typhi strains are monophasic, characterized by the expression of the H:d antigen through FliC. But, some *S.* Typhi strains isolated

from Indonesia express the H:j antigen attributed to an in-frame deletion in fliC, which is a variant of H:d (Frankel et al., 1989). Few *S.* Typhi strains are reported to be biphasic, producing a plasmid-encoded FljB analogue of the H:z66 antigen. The emergence of H:j and H:z66 antigenic variants is believed to be a recent development in S. Typhi evolution, influenced by immune selection in areas with a high prevalence of the disease (Baker et al., 2008, 2007).

B.2.ix.e. Fimbriae and Pili:

Fimbriae and pili are major adhesion factors in *S.* Typhi, crucial for infection and host colonization through various cellular interactions. Among the six fimbriae operons specific to *S.* Typhi, the Stg operon has been recently identified as playing a role in cellular invasion and *in vitro* degradation of epithelial cells. Furthermore, it enables *S.* Typhi to target enterocytes more effectively than M cells, allowing it to bypass Peyer's patches and evade the innate immune response (Berrocal et al., 2015; Gonzales et al., 2017).

B.2.ix.f. Virulence Plasmid:

The virulence plasmid of *S.* Typhi is integral to its ability to cause disease. It contains the *spv* operon, which encodes proteins that enable the bacteria to bypass the host's immune defenses and enhance their ability to survive and replicate within host cells. These proteins include SpvB, SpvC, and SpvD, which interfere with host cell processes like actin polymerization and immune signaling. The plasmid also carries other virulence factors, such as fimbrial operons, which aid in adhesion and invasion (Al-Khafaji et al., 2021; Kang et al., 2024; Silva et al., 2017).

B.2.ix.f. Invasiveness:

S. Typhi employs a complex process called bacterial-mediated endocytosis to invade host cells. During this process, *S.* Typhi injects its proteins into the host cell, manipulating the signaling pathways to control cytoskeletal membrane trafficking and gene expression. This process ultimately leads to the endocytosis of *S.* Typhi into the host cell. *S.* Typhi primarily targets macrophages, and its survival within these phagocytic cells is made possible by bacterial proteins that enable it to resist both oxygen-dependent and oxygen-independent defense mechanisms of the macrophages (Al-Khafaji et al., 2021; Ohl and Miller, 2001; Sultana, 2012).

B.2.ix.g. Biofilm:

The mechanisms and signaling pathways involved in *Salmonella* biofilm development are highly intricate. The structure of a *Salmonella* biofilm is composed of four primary components: curli, cellulose, capsular polysaccharides, and lipopolysaccharides. Curli, also known as thin aggregative fibers (Tafi), are a significant part of the extracellular polymeric substances (EPS) matrix (Corcoran, 2013). Research by Di Domenico et al., 2017 suggests that biofilm production by *S.* Typhi could be a critical factor in promoting persistent infections within the gallbladder, triggering chronic localized inflammation and repeated epithelial damage due to carcinogenic toxins.

B.2.ix.h. Endotoxin of Salmonella Typhi:

Endotoxins, primarily made up of LPS, are an integral part of the outer membrane of Gram-negative bacteria and are important for pathogenicity. When cells divide or die, they release endotoxins, which stimulate immune responses. This process may result in inflammation and, in severe situations, septic shock. Endotoxins interact with TLR4 receptors on immune cells, triggering a cascade of signaling processes that result in the secretion of pro-inflammatory

cytokines, like TNF-α and IL-6. While these cytokines are vital in fighting infections, excessive production can lead to severe inflammation and systemic issues ("*Salmonella* Toxins," 2025).

B.3. Typhoid Outbreaks and Its Treatments Throughout History:

Over the years, improvements in sanitation, the introduction of new vaccines, gall bladder removal procedures (cholecystectomy), and the identification of carriers have played a crucial role in significantly lowering disease incidence in High-Income Countries (HICs) (Kirchhelle et al., 2019). From 1940 onward, researchers concentrated on systematic typhoid eradication, for which they introduced two eradication plans: epidemiologically driven bacteriophage typing and effective chemotherapy using antibiotics (Hardy, 2015). With bacteriophage typing, declining typhoid cases and centralized bacteriological resources enabled scientists to detect and manage outbreaks, as well as identify and document asymptomatic (healthy) carriers. Their goal was to eliminate endemic reservoirs through education and, when necessary, carrier isolation. After 1945, international phage typing surveys enhanced outbreak surveillance and helped identify unknown carriers among civilians, food handlers, travellers, and returning military personnel (Felix, 1955). Simultaneously, clinicians tested newly mass-produced antibiotics to combat outbreaks and prevent carriers from spreading the disease. Early trials with sulphonamides, penicillin, and tetracyclines yielded disappointing results (Buttle et al., 1937; Fairbrother and Taylor, 2014; Watson, 1955). Though in 1948, investigators in Malaya found that chloramphenicol successfully treated typhoid patients during outbreaks (Kirchhelle et al., 2019). By 1961, ampicillin became a second-line treatment, proving partially effective in curing carriers. Shortly thereafter, reports surfaced of a rising number of *S.* Typhi strains worldwide developing resistance to multiple antibiotics (MDR) from the 1960s onward.

In the following decades, *S.* Typhi effectively vanished from many HICs. However, increasingly resistant typhoid variants continued to persist in many low- and middle-income countries (LMICs). Alarmingly, the shifting perceptions of typhoid in HICs coincided with the realization of 1960s predictions about transferable multidrug resistance becoming a pressing issue in LMICs (Kirchhelle et al., 2019). The 1970s witnessed severe outbreaks of *S.* Typhi resistant to multiple antibiotics due to plasmid-mediated resistance, including chloramphenicol, in regions such as Central America, India, and Vietnam. For instance, during the Mexican typhoid outbreak between 1972 and 1974, more than 10,000 cases were reported (Datta and Olarte, 1974; Olarte and Galindo, 1973). In response, over five million doses of inactivated vaccines were administered with ampicillin treatment and hygiene interventions. Around 1980, a live attenuated *S.* Typhi (Ty21a) strain's oral vaccine was introduced as a tool for typhoid control. This vaccine, commercially developed, underwent trials in various countries, including Egypt (1978), Chile (1982, 1983, 1984 & 1986), and Indonesia (1986). Alongside vaccine development, new effective antibiotics (trimethoprim-sulfamethoxazole, 1974) were also in use to tackle MDR typhoid variants in endemic areas. In 1984, an international workshop on typhoid, "engendered a sense of optimism among participants for improved, worldwide control of typhoid fever", highlighted advancements in diagnostics, new vaccines, and the apparent instability of *S.* Typhi R-factors. (Edelman and Levine, 1986).

Four years after that workshop, an outbreak of typhoid in Kashmir involving 230 individuals revealed *S.* Typhi strains resistant to all three first-line antibiotics. The MDR was plasmid-mediated and transferable to *E. coli*. Subsequently, strains with transferable resistance were identified in Shanghai (Kirchhelle et al., 2019). The early 1990s saw additional MDR outbreaks in Pakistan and the Mekong Delta (Cooke and Wain, 2004). By 1993, sporadic quinolone resistance had emerged,

and during the 1996-1998 typhoid outbreak in war-stricken Tajikistan, which impacted 24,000 people, over 90% of strains exhibited MDR, with 82% showing ciprofloxacin resistance. Despite escalating international warnings about AMR and the WHO's 1994 recommendation to use vaccines against resistant typhoid (Ivanoff et al., 1994), excessive global use of third-generation cephalosporins and fourth-generation fluoroquinolones (e.g., gatifloxacin introduced in 1999) contributed to further AMR evolution (Basnyat, 2007; Sp et al., 2003). In the case of the cephalosporins, sporadic ceftriaxone resistance was observed in the early 2000s, and Pakistan's current typhoid outbreak has revealed strains resistant to all first-line drugs, quinolones, and ceftriaxone. Notably, extensively drug-resistant (XDR) *S.* Typhi strains from this outbreak were H58 isolates containing the plasmid responsible for multidrug resistance, commonly found in *E. coli* and other enteric pathogens, confirming Anderson's 1960s prediction about environmental AMR selection.

In 2000, 21.7 million cases of typhoid were estimated, and 216,000 global deaths. By 2010, the International Vaccine Institute reported 11.9 million cases of typhoid and 129,000 deaths in LMICs (Crump et al., 2004; Mogasale et al., 2014). For many low-income victims, azithromycin remains the final effective and affordable antibiotic option. However, azithromycin-resistant strains have already been reported (Hooda et al., 2019). The emergence of XDR H58 represents the latest and most alarming development in typhoid's biosocial history (Kirchhelle et al., 2019).

B.4. Current Global Burden of Typhoid:

Enteric fever (typhoid and paratyphoid) continues to be a major global health challenge, with over 9 million reported cases and 100,000 deaths in 2021. South Asia, especially India, accounts for the majority of global cases and deaths, contributing 62% of cases worldwide. India alone reported

5.4 million cases, equivalent to 58% of global cases. The case-fatality rate globally has improved, standing at approximately 1.2%. Children under the age of five are the most affected, representing nearly half of the global years of life lost (YLLs) and accounting for about 40% of all fatalities. The highest mortality rates for this age group are observed in South Asia. Antimicrobial resistance poses an increasing threat, particularly in South Asia and Sub-Saharan Africa, impacting treatment success (Piovani et al., 2024).

Between 2016 and 2018 in the United States, an estimated 400 culture-confirmed cases of typhoid fever were reported annually. Around 85% of these cases involved international travelers, predominantly those returning from South Asia, especially Bangladesh, India, and Pakistan (Hughes et al., 2024).

In the European Union/European Economic Area (EU/EEA), typhoid and paratyphoid fevers are rare, with most cases linked to travel outside the region, especially to southern Asia. In 2021, a total of 304 laboratory-confirmed cases were reported across 19 EU/EEA countries ("Typhoid and paratyphoid fever - Annual Epidemiological Report for 2021," 2024).

In certain areas of Latin America, typhoid is widely recognized as endemic, with the region estimated to have a moderate incidence rate of 53 cases per 100,000 people, totaling over 273,000 cases annually (Crump et al., 2004; Crump and Mintz, 2010). Despite these figures, the disease burden in individual Latin American countries, along with the epidemiology and population dynamics of the pathogens involved, remains poorly understood. Among the countries in the region, Colombia is noted for having a particularly low prevalence of typhoid fever (Diaz-Guevara et al., 2020).

In Oceania, Samoa experienced a significantly high typhoid incidence, with 342 cases per 100,000 person-years, surpassing the rates observed in the majority of South Asian nations. This raises concerns about potential under-reporting of local cases in Samoa (Sikorski et al., 2020). Moreover, annual surveillance reports from New Zealand, where typhoid is also a notifiable disease, showed that Samoa ranked as either the first or second most common source of travel-associated infections (alongside India) between 2010 and 2017, with 5-21 cases reported annually (Forster and Leder, 2021). A 2021 study from Australia reported a total of 923 typhoid cases, with 96% acquired overseas and only 4% were locally acquired during a 7.5-year retrospective review. The majority of cases were linked to travel in South Asia (668 cases, 72%), followed by Southeast Asia (94 cases, 10%) and Oceania (75 cases, 8%), with India being the most frequently reported country of acquisition (496 cases, 54%). Individuals aged 15 years and older accounted for 669 cases (73%) (Forster and Leder, 2021).

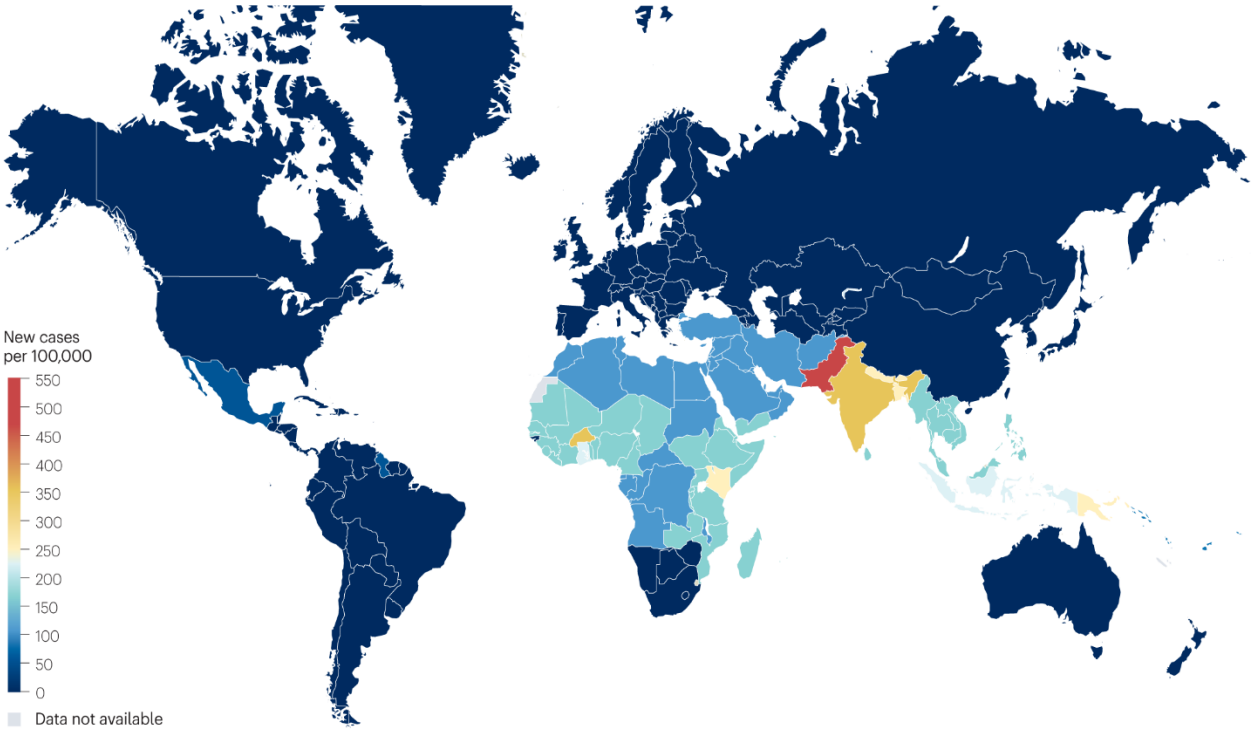


Figure B.4: Number of new cases of typhoid per 100,000 person-years worldwide (Meiring et al., 2023)

Typhoid a significant public health concern in sub-Saharan Africa, with 7.2 million estimated cases occurring each year and an incidence rate of 762 per 100,000 person-years (Kim et al., 2022). The Democratic Republic of the Congo experiences the greatest burden, with one study documenting 315 cases per 100,000 people, while Madagascar reports incidence rates higher than 100 cases per 100,000 (Dall, 2024; Marks et al., 2024).

Typhoid remains a significant public health concern across numerous Asian countries, particularly in South and Southeast Asia, driven by issues such as inadequate sanitation, contaminated water, and restricted healthcare access. Among these, India faces the highest burden worldwide (Antillón et al., 2017; Hancuh, 2023; Ochiai et al., 2008). Although the incidence of typhoid fever is typically lower in East Asia than in Southeast Asia, it continues to present a public health concern in certain

regions (Crump and Mintz, 2010). Typhoid fever poses a serious public health concern across Southeast Asia, with varying incidence rates among countries. Vietnam has an annual rate of 24.2 cases per 100,000 people (Ochiai et al., 2008), while Indonesia reports a broader range of 71.44 to 371.65 cases per 100,000 ("Indonesia," 2018). In Thailand, 2019 data show 120 cases per 100,000 people, with children under 15 years making up 32% of the cases. The disease disproportionately affects younger populations in the region ("Potential of typhoid conjugate vaccines in Thailand," 2024). During the study period of 2017 to 2021, South Asia had the highest number of incident cases, 6.7 million, and the highest age-standardized incidence rate, 379.6/100,000 person-years, in 2021 (Piovani et al., 2024). A study conducted in Dhaka, Bangladesh, reported an incidence rate of 913 cases per 100,000 persons annually. It also identified incidence rates of 330 and 268 cases per 100,000 person-years in Kathmandu and Kavrepalanchok, Nepal, respectively, while Karachi, Pakistan, showed an incidence range of 103–176 cases per 100,000 person-years (Garrett et al., 2022).

In 2021, India accounted for the largest share of incident cases globally, with a total of 5.4 million, representing about 58% of all cases worldwide, followed by Pakistan with 8%, and Bangladesh with 5%. Additionally, India recorded the highest age-standardized incidence rate, reaching 411.5 per 100,000 person-years (Piovani et al., 2024). A 2023 study reported the incidence rates of 574 cases in Delhi, 714 cases in Kolkata, 35 cases in Pune, and 1,173 cases in Vellore, India, per 100,000 person-years among children, during the period from 2017 to 2020 (John et al., 2023).

B.5. Emergence of AMR Strains of *Salmonella* Typhi:

The emergence of AMR in *S.* Typhi has become a worldwide health issue. In 1948, chloramphenicol was first successfully used to treat typhoid (Butler et al., 1977), but just two years

later, in 1950 from England first chloramphenicol-resistant strain of *S.* Typhi was isolated (Colquhoun and Weetch, 1950). During the early to mid-1970s, plasmid-mediated chloramphenicol-resistant *S.* Typhi rapidly spread across the globe, affecting regions like Mexico, India, Vietnam, and Korea (Chowdhury et al., 2024). MDR *S.* Typhi strain was first identified in the 1970s and quickly spread across the globe. Mexico experienced a severe MDR S. Typhi epidemic with nearly 10,000 cases reported, alongside smaller outbreaks globally over the subsequent two decades, which underscored the critical need for the development of new anti-Salmonella drugs (Olarte and Galindo, 1973; Wain et al., 1999). In 1992, fluoroquinolone-resistant MDR *S.* Typhi was first discovered in England (Threlfall et al., 1997). In the following years, major outbreaks of MDR *S.* Typhi also resistant to quinolone were recorded in South Asian, particularly India and Pakistan, with resistance levels increasing dramatically from 2001 to 2006, multidrug resistance rose from 34.2% to 48.5%, while quinolone resistance climbed from 1.6% to 64.1% (Britto et al., 2018; Hasan et al., 2008). In 2016, an XDR strain of *S.* Typhi was identified in Sindh, Pakistan. This strain is resistant to multiple first-line antibiotics, including chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole, along with fluoroquinolones and cephalosporins and the resistance was linked to a plasmid carrying resistance genes (Klemm et al., 2018). The World Health Organization in 2018 highlighted the first significant outbreak of XDR typhoid, with 5,274 cases out of 8,188 individuals affected by typhoid in Hyderabad, Sindh, Pakistan, between November 2016 and December 2018 ("Typhoid fever-Islamic Republic of Pakistan," 2018). From late 2018 to early 2019, XDR-related typhoid infections were transmitted internationally from Pakistan to the USA, the UK, and Canada (Akram et al., 2020; Chirico et al., 2020; Godbole et al., 2018; Wong et al., 2019). Resistance to azithromycin, one of the last remaining oral treatments for typhoid, has been reported in South Asia, first from Bangladesh in

2019 and then from Nepal, India, and Pakistan as well. This resistance is associated with specific genetic mutations, such as the AcrB-R717Q/L mutation (Sajib et al., 2021).

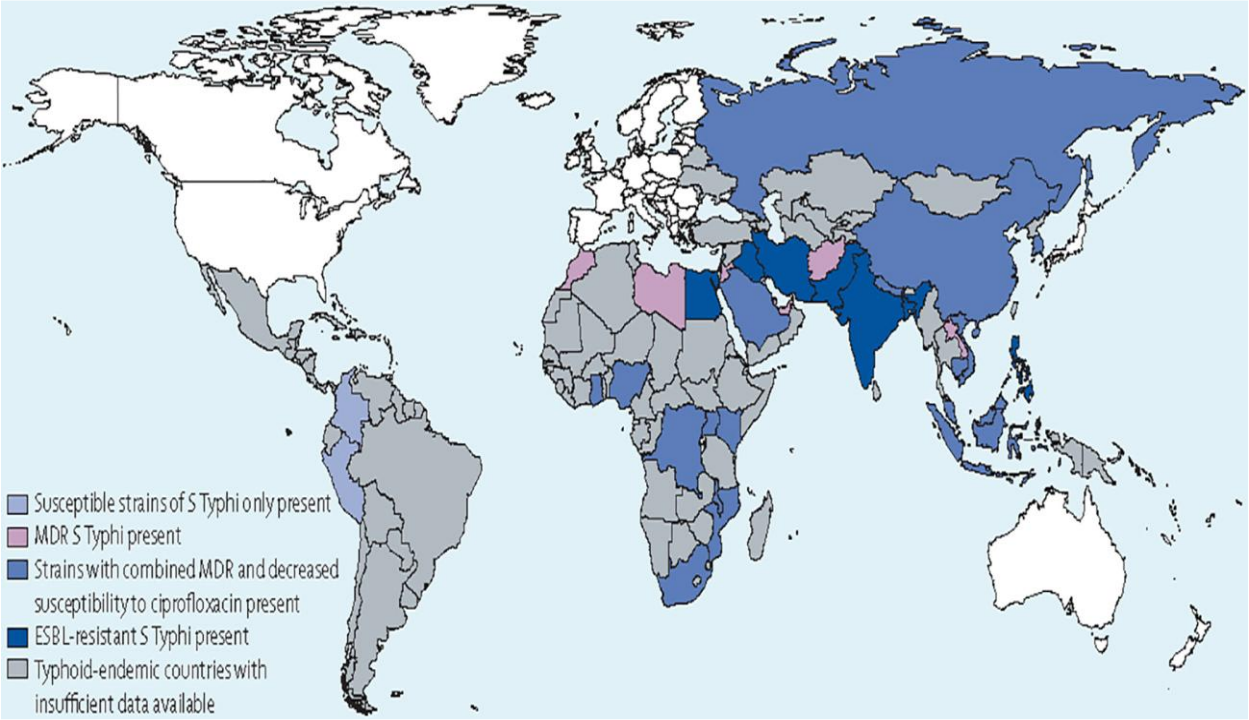


Figure B.5: Worldwide distribution of antimicrobial drug resistance in *S.* Typhi (Crump et al., 2015)

B.6. Plants as a Source of Antibacterial Agents against *Salmonella* Typhi:

As antibiotic-resistant strains of *Salmonella* Typhi continue to rise, plant-derived compounds offer an alternative that might circumvent current resistance mechanisms. Plants show great potential as a source of antibacterial agents. They possess a range of robust defense mechanisms, like producing secondary metabolites, to protect themselves from herbivores and pathogens. Over more than 350 million years of co-evolution, plants and other organisms have developed strategies to counteract each other's defense systems (Clarke et al., 2011; Magallón and Hilu, 2009). Secondary metabolites are essential for plants' environmental adaptation, acting as an internal regulatory

system. These compounds, derived from non-essential metabolic pathways, contribute to the distinct odors, flavors, and colors of plant tissues. Furthermore, secondary metabolites support plants in managing abiotic stresses such as UV radiation and facilitate communication with various organisms including herbivores, pathogens, neighboring plants, pollinators, and fruit dispersers). These functions make secondary metabolites essential for plant growth and development (Kessler and Kalske, 2018; Wink, 2020; Zaynab et al., 2018). Plant chemicals frequently act synergistically, boosting their antibacterial effectiveness and proving valuable for developing combination therapies. Unlike synthetic drugs that often depend on non-renewable resources, plant-based compounds are renewable, contributing to sustainability.

B.6.i.Traditional Use of Plants against Typhoid:

The relationship between humans and plants for medicinal purposes dates back thousands of years. Many tribal communities all over the world in biodiversity-rich areas have developed extensive knowledge of medicinal plants through observation, trial and error, and inheritance. In areas with limited access to modern healthcare, these treatments provide an affordable and accessible option. Many Indigenous communities have relied on their knowledge of medicinal plants and traditionally used medicinal plants to treat typhoid symptoms and prevent its spread, and this indigenous knowledge can assist modern scientific research in discovering effective antibacterial agents.

The use of plants in traditional medicine to combat typhoid fever has a rich history rooted in ethnomedicinal practices across various cultures. In Jharkhand, India, home to 32 different indigenous tribes, a biodiversity-rich area has rich knowledge of the ethnic use of medicinal plants to treat disease. Bhaunra et al., 2024 documented 17 plant species belonging to 15 families

traditionally used by the indigenous people from Jharkhand, India (**Table B.6.i.a**). Similarly Khadka et al., 2020 in their report cited 20 different plant species that were used against typhoid by ethnic peoples of Lwangghalel, Kaski district, central Nepal. The plant species include *Achyranthes bidentata, Abelmoschus manihot, Berberis aristata*, *Imperata cylindrical, Eurya acuminata*, *Rubus ellipticus* and *Oxalis corniculata* , The plant species like *Centella asiatica* and *Azadiracta indica* were also documented in article by Raj et al., 2018 for the use to treat typhoid by the forest-dependent communities of Northern Bengal, India.

Table B.6.i.a: Medicinal Plants to Treat Typhoid by The Indigenous People from Jharkhand, India (Bhaunra et al., 2024)

Sl.No.	Botanical Name	Family	Vernacular Name(s)	Habit	Parts used
1.	*Adhatoda vasica* Nees.	Acanthaceae	H.- Adusa, N.- Bakaspati, M.- Arandi ba, Here ba	Shrub	Leaf
2.	*Andrographis paniculata* (Brum.f.) Nees.	Acanthaceae	H.-Kiryat, Mahatita, N.-Kalmegh, M.-Bhuinim	Herb	Leaf
3.	*Achyranthes aspera* L.	Amaranthaceae	H.-Chirchira, Latjira, M.-Sitirkad, Chirchiti	Herb	Leaf
4.	*Centella asiatica* L.	Apiaceae	H.-Mandukparni, N.-Beng sag, M.-Choke ara, Huring Chatom ara	Herb	Leaf/ Whole plant
5.	*Rauwolfia serpentina* (L.) Benth. ex Kurz.	Apocynaceae	H.-Sarpagandha, N.-Nagbail, M.-Ara ba, Huring supururid ba,, Daru jikipota	Shrub	Leaf
6.	*Ocimum tenuiflorum Linn.*	Lamiaceae	H.-Tulsi,, Kala tulsi, M.-Tunrusi	Herb	Leaf
7.	*Azadirachta indica* A. Juss.	Meliaceae	H.-Nimb, Nim, N.-Nim, M.-Nim daru	Tree	Leaf
8.	*Ficus religiosa* L.	Moraceae	H.- Pipal, M.- Jitia hesa	Tree	Leaf
9.	*Moringa oleifera* Lamk.	Moringaceae	H.-Sahijan, Munga, N.-Munga sag, M.-Munga ara	Tree	Bark
10.	*Nyctanthes arbor-tristis* L.	Oleaceae	H.-Harsinghar, M.-Saparom, Kula marsal, Chamgar	Tree	Leaf
11.	*Oxalis corniculata* L.	Oxalidaceae	H.-Amrul, N.-Amboti, Chalmori, M.-Pusigunju, Pirikatam, Husuki,, Piri Chatom ara	Herb	Leaf/ Whole plant
12.	*Cynodon dactylon* Pers.	Poaceae	H.- Dub, N.- Dubla ghans, M.- Dubila tasad	Creeper	Leaf
13.	*Aegle marmelos* Correa.	Rutaceae	H. & N.- Bel, M.- Lora daru, Sinju daru	Tree	Leaf
14.	*Scoparia dulcis* L.	Scrophulariacece	H.- Meetha ghas, M.-Madukam Koara, Chini buta, Chini sakam, Ote kantara, Merom med, Guru ara	Herb	Whole plant
15.	*Pterospermum acerifolium* Willd.	Sterculiaceae	H.-Kaniar, Machkunda, N.- Machkunda, M.- Makchund	Tree	Flower
16.	*Vitex negundo* L.	Verbenaceae	H.- Nirgundi, Sambhalu, N.- Sinduar, M.- Bigana, Huhuri, Sursing, Huri, Bigna	Shrub	Leaf
17.	*Vitex peduncularis* Wall.	Verbenaceae	H.-Nagbail, Nagpheni, N.-Charaigorwa,, Minjurgorwa, M.- Simkata, Marakata, Simjanga	Tree	Bark, Leaf

Abbreviations: H= Hindi, N= Nagpuri, M= Munda

Oyedeji-Amusa et al., 2024 also in their systematic review listed a total of 227 plant species that have traditional use to treat typhoid in various parts of Nigeria (**Table B.6.i.b**). Akwa and Nguimbous, 2021 in their review article documented the most commonly used plants, namely *Azadirachta indica, Harungana madagascariensis, Glycyrrhiza, glabra, Paullinia pinnata*, *Aleo barbadensis, Cassia siamea, Carica papaya, Moringa oleifera,* and *Allium sativum* in treatment of typhoid in Kenya. The Nupes of Bida in Niger State, Nigeria, uses plants like *Euphobia hirta*, *Citrus aurantifolia*, *Cassia occidentalis*, and Cassia eucalyptus in treating typhoid fever (Evans et al., 2002).

Table B.6.i.b: List of Locally Used Medicinal Plants in Nigeria to Treat Typhoid, Adopted from Oyedeji-Amusa et al., 2024.

	Plant name	Plant family	Common Name	Part Used	Plant Extraction Method
1	*Acacia albida*	Fabaceae	Ana Tree	Leaves	Decoction
2	*Acacia nilotica*	Mimosaceae	Gum Arabic Tree	Fruit, Leaves	Powder
3	*Acanthospermum hispidum*	Asteraceae	Bristly Starbur	Leaves, Whole Plant	Decoction, Infusion, Maceration
4	*Acanthus montanus*	Acanthaceae	Mountain Thistle	-	-
5	*Achillea nobilis*	Asteraceae	Noble Yarrow Plant	-	-
6	*Adansonia digitata*	Malvaceae	Baobab	-	-
7	*Aframomum melegueta*	Zingerberaceae	Alligator Pepper	Leaves	-
8	*Ageratum conyzoides*	Asteraceae	Billy-Goat Weed	Leaves	-
9	*Albizia ferruginea*	Fabaceae	West African Albizia	Whole Plant	-
10	*Alchornea cordifolia*	Euphorbiaceae	Christmas Bush	Leaves, Stem Bark	-
11	*Alchornea laxiflora*	Euphorbiaceae	Lax-Flowered Alchornea	Leaves, Root	-
12	*Allium ascalonicum*	Amaryllidaceae	Wild Onion	Bulb	Decoction
13	*Allium cepa*	Amaryllidaceae	Onions	Bulb	Infusion
14	*Allium sativum*	Amaryllidaceae	Garlic	Bulb	Decoction
15	*Aloe barbadensis*	Asphodelaceae	Aloe Vera	Leaves	Infusion
16	*Aloe ferox*	Asphodelaceae	Cape Aloe	Whole Plant	Decoction, Infusion, Maceration
17	*Aloe vera*	Asphodelaceae	Aloe Vera	Leaves	Maceration

18	*Alstonia boonei*	Apocynaceae	Alstonia	Leaves, Stem Bark	-
19	*Alstonia capensis*	Apocynaceae	Cheese Wood	Leaves, Stem Bark	-
20	*Alstonia congensis*	Apocynaceae	Cheese Wood	Stem Bark	-
21	*Amaranthus spinosus*	Amaranthaceae	Spiny Amaranth	Leaves, Whole Plant	Decoction, Infusion, Moistening, Maceration
22	*Anacardium occidentale*	Anacardiaceae	Cashew Tree	Stem Bark, Leaves, Fruit	-
23	*Ananas comosus*	Bromeliaceae	Pineapple	Fruit, Leaves, Root	-
24	*Annickia chlorantha*	Annonaceae	Africa Yellow Wood	Stem Bark, Leaves, Root	-
25	*Annona muricata*	Annonaceae	Soursop	Fruit Sap, Leaves	-
26	*Annona senegalensis*	Annonaceae	African Custard Apple	Stem Bark, Leaves	-
27	*Annona squamosa*	Annonaceae	Custard Apple	-	-
28	*Anogeissus leiocarpus*	Loranthaceae	Axle Wood Tree	Stem Bark	-
29	*Anthocleista vogelii*	Gentianaceae	Cabbage Tree	Leaves	Decoction
30	*Artemisia absinthium*	Asteraceae	Wormwood	Leaves	-
31	*Artemisia dracunculus*	Asteraceae	Tarragon	Leaves	Decoction, Infusion
32	*Asparagus africanus*	Asparagaceae	Bush Asparagus	Whole Plant	Powder
33	*Azadirachta indica*	Meliaceae	Neem Tree	Leaves, Stem Bark, Fruit, Flower	-
34	*Balanites aegyptiaca*	Zygophyllaceae	Egyptian Balsam	Leaves, Stem Bark, Fruit, Root	Decoction, Infusion, Moistening, Maceration
35	*Bambusa vulgaris*	Poaceae	Bamboo	Leaves	-
36	*Blighia sapida*	Sapindaceae	Akee	Leaves	-
37	*Boswellia dalziellii*	Burseraceae	Indian Frankincense	Stem Bark, Flowers, Leaves	Decoction, Infusion
38	*Bridelia ferruginea*	Phyllantaceae	Bridelia	Stem Bark	-
39	*Bridelia micrantha*	Phyllanthaceae	Imitseeri	Leaves	-
40	*Byrsocarpus coccineus*	Conniraceae	Crimson Thyme	Leaves, Stem Bark	-
41	*Cadaba farinosa*	Capparaceae	Kadhab	Leaves	Decoction
42	*Caesalpinia bonduc*	Fabaceae	Grey Nicker	Leaves, Root, Seed	Decoction
43	*Caesalpinia pulcherrima*	Fabaceae	Pride Of Barbados	Leaves, Fruit	Decoction
44	*Cajanus cajan*	Fabaceae	Pigeon Bean	Leaves	-
45	*Calotropis procera*	Asclepiadaceae	Apple Of Sodom	Leaves, Root	Decoction
46	*Canna indica*	Cannaceae	Indian Shot	Leaves	-
47	*Capsicum frutescens*	Solanaceae	Chilli Pepper	Fruit	Decoction
48	*Carica papaya*	Caricaceae	Pawpaw	Latex, Root, Leaves, Fruit	Maceration
49	*Carissa carandas*	Apocynaceae	Bengal Currant	Leaves, Stem Bark	Decoction, Infusion
50	*Cassia alata*	Fabaceae	Ringworm Bush Plant	Leaves	-
51	*Cassia eucalyptus*	Fabaceae	Gum Tree	Stem Bark, Inflorescence	-
52	*Cassia occidentalis*	Fabaceae	Negro Coffee	Leaves, Whole Plant	Decoction

53	*Cassia sieberiana*	Fabaceae	Drumstick Tree	Leaves, Stem Bark, Root	Decoction
54	*Cassia tora*	Fabaceae	Sickle Senna	Leaves	Infusion
55	*Cassytha filiformis*	Lauraceae	Laurel Dodder	-	-
56	*Ceiba pentandra*	Bombaceae	Silk Cotton Tree	Leaves, Stem Bark	Decoction
57	*Celastrus paniculatus*	Celastraceae	Bittersweet	Leaves	Decoction
58	*Celtis integrifolia*	Ulmaceae	Nettle Tree	Leaves	Decoction
59	*Chassalia kolly*	Rubiaceae	Na	Whole Plant	Maceration
60	*Chromolaena odorata*	Asteraceae	Armstrong's Weed Plant	Leaves	Infusion
61	*Chrysophyllum albidum*	Sapotaceae	White Star Apple	-	Maceration
62	*Cinnamomum verum*	Lauraceae	True Cinnamon Tree	Stem Bark, Leaves, Oil	-
63	*Citrullus colocynthis*	Cucurbitaceae	Bitter Gourd	Fruit	-
64	*Citrullus vulgaris*	Cucurbitaceae	Watermelon	Fruit	Powder
65	*Citrus aurantiifolia*	Rutaceae	Lime	Fruit, Leaves, Seed	-
66	*Citrus aurantium*	Rutaceae	Bitter Orange	Leaves, Root	Infusion
67	*Citrus limon*	Rutaceae	Lemon	Leaves, Fruit	Decoction
68	*Citrus medica*	Rutaceae	Fingered Citron	Leaves, Fruit	Decoction
69	*Citrus paradisi*	Rutaceae	Grape	Fruit, Leaves	-
70	*Citrus sinensis*	Rutaceae	Sweet Orange	Fruit, Leaves	-
71	*Cleistopholis patens*	Annonaceae	Na	-	Infusion
72	*Cnestis ferruginea*	Connaraceae	Na	Leaves, Stem Bark	-
73	*Cochlospermum planchonii*	Bixaceae	Buttercup Tree	Leaves, Root	Decoction
74	*Cochlospermum religiosum*	Bixaceae	Buttercup Tree	Leaves, Flowers, Stem Bark	Decoction
75	*Cochlospermum tinctorium*	Bixaceae	Na	Root	Decoction
76	*Cocos nucifera*	Arecaceae	Coconut Palm	Stem Bark, Leaves, Fruit Husk	-
77	*Cola hispida*	Malvaceae	Na	Fruit	-
78	*Cola millenii*	Malvaceae	Monkey Cola	Leaves	-
79	*Combretum glutinosum*	Combretaceae	Na	Leaves	Decoction
80	*Combretum micranthum*	Combretaceae	African Bush Willow	Leaves	Infusion
81	*Combretum molle*	Combretaceae	Velvet Bush Willow	Stem Bark	Maceration
82	*Combretum paniculatum*	Combretaceae	Rangoon Creeper	Twig	-
83	*Commiphora africana*	Burseraceae	African Myrrh	Stem Bark, Leaves	-
84	*Commiphora kerstingii*	Burseraceae	Myrrh	Stem Bark	-
85	*Conocarpus erectus*	Combretaceae	Buttonwood	Leaves, Stem Bark	-
86	*Conospermum capitatum*	Proteaceae	Drumsticks	-	-
87	*Cordia africana*	Boraginaceae	Boran	Stem Bark	Decoction
88	*Costus anomocalyx*	Costaceae	Twisted Ginger	Leaves	Decoction
89	*Curcuma longa*	Zingiberaceae	Turmeric	Leaves	-
90	*Cymbopogon citratus*	Poaceae	Lemongrass	Leaves, Whole Plant, Fruit	-
91	*Cymbopogon giganteus*	Poaceae	Na	-	-
92	*Cyperus tonkinensis*	Cyperaceae	Sedge	Leaves	Decoction

93	*Daniellia oliveri*	Fabaceae	African Copaiba Balsam Tree	Leaves, Stem Bark	-
94	*Daucus carota*	Apiaceae	Carrot	Fruit	Maceration
95	*Delonix regia*	Fabaceae	Flamboyant Tree	Leaves, Stem Bark	-
96	*Detarium microcarpum*	Fabaceae	Sweet Dattock	Root, Leaves	Powder
97	*Distemonanthus benthamianus*	Fabaceae	West African Ironwood	-	-
98	*Dodonaea viscosa*	Sapindaceae	Hopbush	Leaves	-
99	*Drypetes gossweileri*	Euphorbiaceae	Horseradish Tree	Stem Bark	-
100	*Enantia chlorantha*	Annonaceae	Africa Yellow Wood	Stem Bark	-
101	*Eriosema psoraleoides*	Fabaceae	Canary Pea	Leaves	-
102	*Erythrina senegalensis*	Fabaceae	Coral Tree	Stem Bark, Leaves	-
103	*Erythrina senegalensis*	Fabaceae	Coral Tree	Stem Bark, Leaves	Decoction
104	*Eucalyptus camaldulensis*	Myrtaceae	River Red Gum	Leave, Seed, Stem Bark	-
105	*Eucalyptus citriodora*	Myrtaceae	Lemon Scented Gum	Leaves, Root	Decoction
106	*Eucalyptus globulus*	Myrtaceae	Tasmanian Blue Gum	Leaves	-
107	*Eugenia caryophyllus*	Myrtaceae	Clove	Leaves, Fruit	Decoction, Infusion
108	*Euphorbia heterophylla*	Euphorbiaceae	Spurge Weed	Whole Plant	Decoction
109	*Euphorbia hirta*	Euphorbiaceae	Asthma Plant	Root, Leaves, Inflorescence	-
110	*Euphorbia lateriflora*	Euphorbiaceae	Spurge	Leaves	-
111	*Ficus carica*	Moraceae	Fig Tree	Leaves	-
112	*Ficus exasperata*	Moraceae	Sandpaper Tree	Leaves, Root	Decoction
113	*Ficus glumosa*	Moraceae	Mountain Fig	Leaves, Stem Bark	Decoction
114	*Ficus gnaphalocarpa*	Moraceae	Ficus Tree	-	Infusion
115	*Ficus platyphylla*	Moraceae	Flake Rubber Tree	Leaves	-
116	*Ficus sur*	Moraceae	Commercial Fig	Stem Bark, Root	-
117	*Ficus sycomorus*	Moraceae	Sycamore Fig	Fruit, Leaves	Powder, Decoction
118	*Ficus thonningii*	Moraceae	Strangler Fig	Leaves	Decoction
119	*Fimbristylis ferruginea*	Cyperaceae	Rusty Sedge	Leaves, Flower	-
120	*Floscopa africana*	Commelinaceae	African Floscopa	Leaves	Decoction
121	*Garcinia kola*	Clusiaceae	Bitter Kola	Leaves, Fruit, Stem Bark	Decoction
122	*Garcinia mangostan*	Clusiaceae	Purple Mangosteen	Leaves, Stem Bark	Decoction, Infusion
123	*Gardenia aqualla*	Rubiaceae	Bendigo	Root, Leaves	-
124	*Glyphaea brevis*	Malvaceae	False Coffee	Leaves	Powder
125	*Gmelina arborea*	Lamiaceae	Melina	Fruit, Leaves	-
126	*Gossypium arboreum*	Malvaceae	Cotton	Seed, Root, Leaves	-
127	*Gossypium herbaceum*	Malvaceae	Cotton	Leaves	Decoction
128	*Gossypium hirsutum*	Malvaceae	Cotton	Leaves, Seed	-
129	*Grewia mollis*	Malvaceae	Raisin	Stem Bark	-
130	*Guiera senegalensis*	Combretaceae	Sabara	Leaves	Powder
131	*Haematostaphis barteri*	Anacardiaceae	Na	Leaves	-
132	*Hibiscus sabdariffa*	Malvaceae	Roselle	Whole Plant	Decoction

133	*Hygrophila auriculata*	Acanthaceae	Talmak-Hana	Leaves	Decoction
134	*Hyphaene thebaica*	Arecaceae	Gingerbread Tree	-	-
135	*Ipomoea batatas*	Convolvulaceae	Sweet Potato	Leaves	Decoction
136	*Jatropha curcas*	Euphorbiaceae	Barbados Nut	Leaves, Fruit, Whole Plant	Decoction, Infusion, Moistening, Maceration
137	*Khaya senegalensis*	Meliaceae	Senegal Mahogany	Leaves, Stem Bark, Root	-
138	*Kigelia africana*	Bignoniaceae	Sausage Tree	Seed	Decoction
139	*Lawsonia inermis*	Lythraceae	Henna	Leaves	Infusion
140	*Lecaniodiscus cupanioides*	Sapindaceae	Lecaniodiscus	Leaves, Stem Bark, Seed, Root	Decoction
141	*Leea guineensis*	Vitaceae	West Indian Molly Plant	Leaves, Stem Bark	Decoction
142	*Leptadenia hastata*	Apocynaceae	Akamongot	Stem Bark	-
143	*Lophira alata*	Ochnaceae	Ironwood	Leaves, Root, Stem Bark	-
144	*Mangifera indica*	Anacardiaceae	Mango	Leaves, Stem Bark, Seed, Root, Fruit	Decoction
145	*Maytenus senegalensis*	Celastraceae	Spike Thorn	Leaves	Decoction
146	*Milicia excelsa*	Moraceae	African Teak	Leaves, Stem Bark	-
147	*Momordica balsamina*	Cucurbitaceae	Balsam Apple	Twig	-
148	*Momordica charantia*	Cucurbitaceae	Bitter Lemon	Leaves	-
149	*Monodora myristica*	Annonaceae	African Nutmeg	Leaves, Seed	-
150	*Morinda lucida*	Rubiaceae	Brimstone Tree	Stem Bark, Leaves, Root	-
151	*Moringa oleifera*	Moringaceae	Drumstick Tree	Seed, Stem Bark, Leaves, Root	Powder
152	*Musa acuminata*	Musaceae	Banana	Leaves, Fruit	-
153	*Musa angolensis*	Musaceae	Banana	Leaves, Fruit	Decoction
154	*Musa paradisiaca*	Musaceae	Plantain	Leaves, Fruit	-
155	*Musa sapientum*	Musaceae	Banana	Fruit, Leaves	Decoction
156	*Nauclea diderrichii*	Rubiaceae	Bilinga	Leaves	Decoction
157	*Nauclea latifolia*	Rubiaceae	African Peach	Stem Bark, Leaves, Fruit, Root	-
158	*Newbouldia laevis*	Bignoniaceae	Na	Stem Bark, Leaves	-
159	*Nicotiana tabacum*	Solanaceae	Tobacco	Leaves	Juice
160	*Ocimum gratissimum*	Lamiaceae	Scent Leaves	Whole Plant	-
161	*Olax subscorpioidea*	Olacaceae	Acagniba	Root	-
162	*Olea europaea*	Oleaceae	Common Olive	Leaves	-
163	*Parkia biglobosa*	Fabaceae	African Locust Bean	Fruit	Decoction
164	*Pennisetum purpureum*	Poaceae	Elephant Grass	Whole Plant	-
165	*Pergularia daemia*	Apocynaceae	Pergularia	Leaves	Decoction, Infusion, Maceration, Moistening
166	*Persea americana*	Lauraceae	Avocado	Leaves, Stem Bark, Seed, Fruit	Decoction, Juice Extraction
167	*Phaseolus lunatus*	Fabaceae	Lima Bean	Leaves	-
168	*Phyllanthus amarus*	Phyllanthaceae	Sleeping Plant	Leaves	-
169	*Piliostigma reticulatum*	Fabaceae	Piliostigma	Root, Leaves, Fruit	Decoction

170	*Piper guineense*	Piperaceae	West African Black Pepper	Leaves	-
171	*Plumeria rubra*	Apocynaceae	Temple Tree	Leaves	-
172	*Prosopis africana*	Fabaceae	African Mesquite	Leaves	Infusion
173	*Pseudocedrela kotschyi*	Meliaceae	Dry-Zone Cedar	Root	-
174	*Psidium guajava*	Myrtaceae	Guava	Whole Plant, Fruit, Stem Bark, Leaves	Decoction
175	*Pterocarpus erinaceus*	Fabaceae	African Kino Tree	Leaves, Stem Bark	Decoction
176	*Pterocarpus santalinoides*	Fabaceae	Na	Stem Bark	-
177	*Rauwolfia vomitoria*	Apocynaceae	Indian Snack Roots Plant	Root	-
178	*Saccharum officinarum*	Poaceae	Sugarcane	Stem	Juice
179	*Schwenkia americana*	Solanaceae	Tabaco Cimarron	Whole Plant	Decoction, Infusion, Moistening, Maceration
180	*Sclerocarya birrea*	Anacardiaceae	Marula	-	-
181	*Securidaca longipedunculata*	Polygalaceae	Violet Tree	Leaves, Stem Bark, Root	-
182	*Senna alata*	Fabaceae	Emperor's Candlesticks Plant	Whole Plant	Decoction
183	*Senna hirsuta*	Fabaceae	Woolly Senna	Leaves	Powder
184	*Senna occidentalis*	Fabaceae	Coffee Senna	Whole Plant, Leaves	-
185	*Senna siamea*	Fabaceae	Golden Shower	Stem Bark, Leaves, Fruit	Decoction
186	*Senna singueana*	Fabaceae	Winter Cassia	Leaves	-
187	*Senna spectabilis*	Fabaceae	Spectacular Cassia	Stem Bark, Leaves	-
188	*Senna tora*	Fabaceae	Sickle Senna	-	-
189	*Sesamum indicum*	Pedaliaceae	Benne	Leaves, Whole Plant	Decoction, Infusion, Moistening, Maceration
190	*Sida acuta*	Malvaceae	Broom Weed	Leaves	Decoction
191	*Sida rhomboidea*	Malvaceae	Arrow-Leaves Sida	Leaves	-
192	*Solanum erianthum*	Solanaceae	Potato Tree	Stem Bark	Decoction
193	*Solanum lycopersicon*	Solanaceae	Tomato	Fruit	-
194	*Sorghum bicolour*	Poaceae	Millet	Leaves	-
195	*Spermacoce villosus*	Rubiaceae	Tropical Girdle Pod	Whole Plant	Decoction, Infusion, Moistening, Maceration
196	*Sphenocentrum jollyanum*	Menispermaceae	Red Medicine Plant	Root	Powder
197	*Stachytarpheta jamaicensis*	Verbenaceae	Blue Porter Weed	Leaves	-
198	*Stereospermum kunthianum*	Bignoniaceae	Pink Jacaranda	Stem Bark	Decoction

199	*Swietenia macrophylla*	Meliaceae	Honduran Mahogany	Leaves	-
200	*Syzygium guineense*	Myrtaceae	Woodland Water Berry	-	-
201	*Talinum triangulare*	Talinaceae	Water Leaves	Leaves	-
202	*Tamarindus indica*	Fabaceae	Tamarind	Fruit, Stem Bark, Root, Leaves	Maceration
203	*Terminalia avicennioides*	Combretaceae	Tropical Almond	Root, Leaves, Stem Bark	-
204	*Terminalia catappa*	Combretaceae	Tropical Almond	Leaves	Decoction
205	*Theobroma cacao*	Malvaceae	Cocoa	Stem Bark	-
206	*Thesium viride*	Santalaceae	Na	Whole Plant	Decoction, Potash, Infusion
207	*Tithonia diversifolia*	Asteraceae	Tree Marigold	Leaves, Root	Infusion, Decoction
208	*Trema guineensis*	Ulmnaceae	Charcoal Tree	Stem Bark	-
209	*Trema orientale*	Cannabaceae	Pigeon Wood	Leaves	-
210	*Triplochiton scleroxylon*	Malvaceae	African Whitewood	Leaves	Decoction
211	*Uvaria chamae*	Annonaceae	Bush Banana	Root, Stem Bark, Leaves	-
212	*Vernonia amygdalina*	Asteraceae	Bitter Leaves	Leaves, Stem	Decoction
213	*Vigna subterranea*	Fabaceae	Bambara	Leaves	-
214	*Viscum album*	Santalaceae	Mistletoe	-	-
215	*Vitex doniana*	Lamiaceae	Black Plum	Stem Bark, Root, Leaves	-
216	*Waltheria americana*	Sterculiaceae	Sleepy Morning	Root	Decoction
217	*Ximenia americana*	Olacaceae	Hog Plum	Stem Bark, Leaves	-
218	*Xylocarpus granatum*	Meliaceae	Mangrove Cannonball Tree	Leaves, Stem Bark	-
219	*Xylopia aethiopica*	Annonaceae	Ethiopian Pepper	Fruit, Leaves, Seed, Root	Decoction
220	*Zanthoxylum zanthoxyloides*	Rutaceae	Artar Roots	-	Infusion
221	*Zingiber officinale*	Zingiberaceae	Ginger	Rhizome, Corm, Stem Bark, Root	Powder
222	*Ziziphus abyssinica*	Rhamnaceae	Fruit Pulp	Root, Stem Bark, Leaves	-
223	*Ziziphus mauritiana*	Rhamnaceae	Indian Jujube	Root	-

The utilization of plants in disease treatment dates back to ancient times and remains relevant today. By integrating traditional knowledge with modern scientific advancements, plant-based antibacterial agents could contribute significantly to global healthcare solutions. Plant extracts are favored for their accessibility and cost-effectiveness. While some of these extracts are sold in local

markets, others are prepared and used domestically. However, many producers fail to provide evidence of their products' safety and efficacy before commercialization. As a result, the potential adverse effects and risks associated with consuming these products are often uncertain. Furthermore, plant extracts are not subject to regulation for purity and potency. Contamination and variations in the potency of these plant-based products may significantly contribute to negative outcomes after consumption (Nguimbous, 2021).

B.6.ii. Plants with Reported Antibacterial Activity:

There are numerous plants reported to have antibacterial activity. Chassagne et al., 2021 in their systematic review documented 958 plant species with antibacterial activity and stated that the most extensively researched families were Lamiaceae, Fabaceae and Asteraceae, while the most studied species were *Cinnamomum verum*, *Rosmarinus vulgaris,* and *Thymus vulgaris*. They also stated that plants belonging to monocotyledons were less promising in terms of antibacterial activity, whereas the members of the Zingiberaceae family had the lowest minimum inhibitory concentrations (MICs), ranging from 92 -185 µg/ml and *Origanum vulgaris,* a member of Lamiaceae, showed the lowest MIC range of 0.03-100 µg/ml against *E. coli*, *Acinetobacter baumannii, Listeria monocytogenes, Staphylococcus aureus, Pseudomonas aeruginosa, Acinetobacter baumannii* and *Streptococcus pyogenes*. Some of their documented plants with antibacterial activity are presented in **Table B.6.ii**. Aqueous extract of ten well-known spices namely *Amomum subulatum, Anethum graveolens, Cinnamomum zeylanicum, Elettaria cardamomum, Foeniculum vulgare, Glycyrrhiza glabra, Gnetum gnemon, Syzygium aromaticum, Trachyspermum ammi,* and *Viola odorata,* were reported to have antibacterial activity against some of the human pathogens live *E. coli* (MTCC 119), *P. aeruginosa* (MTCC 647 and MTCC 741), *Klebsiella pneumoniae* (MTCC 109 and MTCC 530), *S.* Typhi (MTCC 531), *S.* Typhimurium

(MTCC 98 and MTCC 1251), *S. aureus* (MTCC 96), and *Shigella flexneri* (MTCC 1457) (Arora and Kaur, 2007). Nimri et al. 1999 tested 15 plant species, which were traditionally used in Jordan and other Middle East countries for their medicinal property and stated that three of them, namely *Quercus infectoria, Punica granatum* and *Rhus coriaria* were most active with MIC of 0.98-31.25, 3.9-31.25 and 1.95-31.25 mg/ml, respectively against all the tested bacteria i.e., *E. coli*, *Bacillus cereus, Staphylococcus epidermidis, S. aureus*, *S. pyogenes*, *Shigella dysentriae*, *Enterococcus faecalis*, *K. pneumoniae*, *Yersinia enterocolitica*, *Proteus vulgaris*, and *P. aeruginosa*. Erdogrul, 2002 evaluated plant extracts of four plant species against *E. coli*, *Bacillus brevis*, *Bacillus megaterium*, *Bacillus subtilis*, *B. subtilis var. niger*, *Micrococcus luteus*, *Pseudomonas fluorescens, Listeria monocytogenes*, *S.* aureus, *Streptococcus thermophilus*, *Mycobacterium smegmatus*, and *Y. enterocolitica* and stated that the ethyl acetate, methanol, chloroform, and acetone extract of *Urtica dioica* and *Fumaria officinalis* whole plant did no showed antibacterial activity against any of the test bacterial strains but the ethyl acetate, methanol, and chloroform extract of *Rosmarinus officinalis* leaves showed inhibitory effects against all the test bacterial strains with inhibition zone ranged from 7 to 16 mm/20 µl whereas the Chloroform and ethyl acetate extracts of *Artemisia absinthium* whole plant exhibited inhibition to some of the test strains with zone of inhibition ranged from 8 to 16 mm/20 µl. *Cynodon dactylon* (Doob grass), which traditionally used to treat cuts, wounds, indigestion, and genitourinary disorders (Manandhar, 2002; Rajbhandari, 2001; Singh et al., 2012) showed moderate inhibition against methicillin-resistant *Staphylococcus aureus* (MRSA), MRD *S. typhi,* imipenem-resistant *P. aeruginosa*, and *S.* Typhimurium but promising inhibition was observed against *S. aureus* with a MIC of 31 *µ*g/ml (Marasini et al., 2015). *Curcuma longa* (Haldi), *Cinnamomum camphora* (Kapoor) and *Curculigo orchioides* (Kalo Musali) are well-known medicinal plants that possess a variety of ethnomedicinal uses (Manandhar, 2002;

Rajbhandari, 2001; Singh et al., 2012) showed promising inhibition to *S. pyogenes* with a MIC of 195, 49, 49 µg/ml, respectively (Marasini et al., 2015). The aqueous, ethanolic, and methanolic extracts of *Moringa oleifera* L. (Sojone) leaves and *Matricaria recutita* L.(Babuna) flowers are reported to exhibit inhibitory activity against MDR, XDR and pan drug-resistant isolates of *E. coli, P. aeruginosa, Klebsiella spp. Staphylococcus spp.,* and *P. mirabilis* with MICs ranging from 7.8-62.5 mg/ml (Atef et al., 2019). *Hibiscus sabdariffa* (roselle) a very common medicinal and economically utilized plant (Da-Costa-Rocha et al., 2014; "NParks | Hibiscus sabdariffa," n.d.) demonstrated to possess antibacterial activity against drug resistant *S. aureus* and *E. coli* with inhibition zones of 8.11 mm (± 1.5 SD) and 15.0 mm (± 4.2 SD) respectively (Ibn Awadh and Ahmed, 2025).

Table B.6.ii: List of plants with reported antibacterial activity (Chassagne et al., 2021)

	Plant Species	Common Name	Extract Types	Used Part	Family	Targeted Bacteria	MICs (µg/ml)	Reference
1	***Origanum vulgare***	Oregano	Essential Oil	Whole Plant	Lamiaceae	*P. aeruginosa*, *Listeria monocytogenes*, *S. aureus*, *E. coli*, *S. pyogenes*, and *Acinetobacter baumannii*	0.03–100	(Becerril et al., 2012; Helal et al., 2019; Santos et al., 2017; Thielmann et al., 2019)
2	***Origanum majorana***	Sweet Marjoram	Essential Oil	Flowering Aerial Parts		*S. aureus* and *E. coli*	0.19-6.25	(Lagha et al., 2019; Vaillancourt et al., 2018)

3	***Thymus vulgaris***	Thyme	Essential Oil	Aerial Parts		*S.* Typhimurium, *B. cereus, L. monocytogenes, E. coli, Legionella pneumophila*, and *S. aureus*	0.3–30	(Aliakbarlu and Shameli, 2013; Chaftar et al., 2015; Ghrairi and Hani, 2015; Vaillancourt et al., 2018)
4	***Thymus zygis***	Red Thyme	Essential Oil	Flowering Herb		*E. coli* and *S. aureus*	0.19–400	(Lagha et al., 2019; Thielmann et al., 2019)
5	***Rosmarinus officinalis***	Rosemary	Essential Oil	Aerial Parts		*E. coli, S. epidermidis*, *S. aureus, P. aeruginosa L. monocytogenes,* and *Legionella pneumophila*	0.3–70	(Chaftar et al., 2015; Jardak et al., 2017; Lagha et al., 2019; Santos et al., 2017)
			Ethanol Extracts			*P. aeruginosa, S. saprophyticus, S. epidermidis,* and *E. faecalis*	70–350 µg/mL	(Petrolini et al., 2013)
6	***Mentha x piperita***	Peppermint	Essential Oil	Whole Plant		*E. coli, S. aureus, S. pneumoniae, S.* Typhi, *P. aeruginosa,* and *K. pneumoniae*	0.5–8	(Abolfazl et al., 2014)
7	***Salvia officinalis L***	Sage	Essential Oil	Whole Plant		*S. aureus, E. coli, L. monocytogenes,* and *P. aeruginosa*	12.5–225	(Golestani et al., 2015; Santos et al., 2017; Vaillancourt et al., 2018)

			Ethanol Extracts			*S. aureus* and *S. pyogenes*	62.5 and 300	(Silva et al., 2019; Wijesundara and Rupasinghe, 2019)
8	***Ocimum basilicum***	Basil	Essential Oil	Aerial Parts		*S.* Typhimurium, *S. pyogenes* and *Vibrio cholerae*	0.6–50	(Gemechu et al., 2013; Helal et al., 2019; Ozdikmenli and Demirel Zorba, 2016; Snoussi et al., 2016)
			Methanol Extract	Seeds		*M. tuberculosis*	25	
9	***Dichrostachys cinerea***	-	Dichloromethane And Methanol (1:1)	Twigs	Fabaceae	*S. epidermidis*	0.19	(Nciki et al., 2016)
10	***Albizia myriophylla***	-	Ethanol Extract	Wood		*S. mutans*	3.9	(Limsuwan et al., 2018)
11	***Glycyrrhiza glabra***	Licorice	Methanol Extract	Stem		*P. aeruginosa*	10	(Chakotiya et al., 2016)
			Ethanol Extract	Root		*S. pyogenes*	62.5	(Wijesundara and Rupasinghe, 2019)
12	***Glycyrrhiza triphylla***	-	Essential Oil	Aerial Part		*S.* Typhi, *Micrococcus luteus, P. aeruginosa, Listeria monocytogenes, B. cereus* and *S. aureus*	2.7–87	(Shakeri et al., 2017)
13	***Copaifera reticulata***	Copaiba Trees	-Essential Oil	Oleoresins		*S. aureus, Porphyromonas gingivalis, S.*	6-100	(Bardají et al., 2016;

						salivarius, S. mitis, S. sanguinis, L. monocytogenes, and *E. faecalis*		Fernández et al., 2018; Vieira et al., 2018)
14	***C. paupera***	Copaiba Trees	Essential Oil	Oleoresins		*B. cereus, L. monocytogenes* and *S. aureus*	12.5–100	
15	***Acacia karroo***	-	Methanol Extract	Aerial Parts		*M. luteus, S. aureus,* and *P. aeruginosa,*	7.5–125	(Madureira et al., 2012; Nielsen et al., 2012)
				Stems		*MRSA, a*mpicillin-resistant *K. pneumoniae,* and beta-lactamase producing *E. coli*	78–156	
16	***Tanacetum polycephalum***	-	Essential Oil	Aerial Part	Asteraceae	*S. aureus, E.coli, B. subtilis,* and *S.* Typhi	0.36–10	(Rezazadeh et al., 2014)
17	***Xanthium strumarium***	Cocklebur	Essential Oil	Leaf		*S. aureus, K. pneumoniae, B. subtilis,* and *P.aeruginosa*	0.5–20.5	(Sharifi-Rad et al., 2015)
18	***Echinops kebericho***	-	Ethanol And Methanol Extracts	Root		*E. faecalis, S. aureus,* and *E. coli.*	3–25	(Lee et al., 2014)
19	***Cota palaestina***	-	Essential Oil	Flowers		*S. epidermidis, E. coli, S. aureus, B. subtilis,* and *P. aeruginosa*	6–75	(Bardawel et al., 2014)
20	***Mikania glomerata***	-	Methanol Extract	Whole Plant		*Cutibacterium acnes, A. naeslundii, E. faecalis, Prevotella nigrescens* and *P orphyromonas gingivalis*	6.25-18	(Moreti et al., 2017)
21	***Artemisia abyssinica***	-	Methanol Extract	Leaf		*M. tuberculosis* and	6.25 and 12.5	(Gemechu et al., 2013)

						M. bovis, respectively		
22	***A. indica***	-	Essential Oil	Whole Plant		*K. pneumoniae, S. Typhi, P. aeruginosa* and *S. dysenteriae*	32–128	(Rashid et al., 2013)
23	***Matricaria chamomilla***	Chamomile	Essential Oil	Whole Plant		*E. coli, MRSA, K. pneumoniae, P. vulgaris, P. mirabilis,* and *B. subtilis*	10–156	(Cvetanović et al., 2019)
24	***Psidium guajava***	Guava	Methanol Extracts	Leaves	Myrtaceae	*P. aeruginosa, S. aureus,* and *E. aerogenes*	31–128	(Gutiérrez et al., 2008)
			Acetone Extracts			*E. faecalis, S. Typhi,* and *S. flexneri*	78	(Bai et al., 2015; Bisi-Johnson et al., 2017; Dzotam and Kuete, 2017)
25	***Rhodomyrtus tomentosa***	-	Ethanol Extract	Leaf		*B. cereus, S. mutans, S. aureus* (including MRSA), *L. monocytogenes* and *S. agalactiae*	7.8–32	(Limsuwan and Voravuthikunchai, 2013; Na-Phatthalung et al., 2017; Odedina et al., 2015; Phoem and Voravuthikunchai, 2012; Zhao et al., 2019)
26		-	Hexane Extract	Stem Bark		*M. tuberculosis*	4	(Lawal et al., 2012)

	Eucalyptus camaldulensis			Leaf			6.25	
			Essential Oil			*Shigella spp, E.coli* .and *Bacillus spp.*	5	(Nasir et al., 2015)
27	***Syzygium aromaticum***	Clove	Essential Oil	Bud		*S. aureus, H. ducreyi,* and *E. coli*	50, 100 and 400	(Bersan et al., 2014; Lindeman et al., 2014; Thielmann et al., 2019)
				Leaf		*Porphyromonas gingivalis, Fusobacterium nucleatum,* and *S. mitis*	250–500	
28	***Anacardium occidentale***	Cashew	Aqueous	Bark	Anacardiaceae	*S. mutans, S. mitis,* and *S. salivarius*	3–6	(de Araújo et al., 2018)
			Methanol Extract	Leaf		*S. aureus, E. faecalis,* and *M. luteus*	7.5–15	(Madureira et al., 2012)
29	***Schinus terebinthifolia***	Brazilian Peppertree	Acetone	Fruits		*S. aureus, A. baumannii,* and *E . coli.*	8–16	(Salem et al., 2018)
			Essential Oil			*P. aeruginosa* and *M . flavus*	32 and 128	
30	***Pistacia terebinthus***		Essential Oil	Fruit, Galls and Leaves		MRSA	0.32, 0.64 and 1.28	(Pulaj et al., 2016)
31	***Pistacia lentiscus***	Mastic	Essential Oil	Aerial Parts		MRSA	120	(Lahmar et al., 2017)
32	***Mangifera indica***	Mango	Methanol Extract	Bark		*P. aeruginosa*	32	(Bai et al., 2015; Dzotam and Kuete, 2017; Tsouh Fokou et al., 2016)
				Leaf			125	
			Hexane Extract			*S. aureus*	250	
						M. smegmatis	250	

33	***Pavetta lanceolata***	Weeping Bride's Bush	Acetone Extract	Leaf	Rubiaceae	*M. aurum, M. smegmatis* and *M. tuberculosis*	12–120	(Aro et al., 2016)
34	***Cephalanthus natalensis***	Bush Berry	Acetone Extract	Leaves		*M. tuberculosis, M. flavus, M. aurum* and *M. smegmatis*	17–170	(Aro et al., 2016, 2015)
35	***Sarcocephalus latifolius***	-	Methanol Extract	Stem Bark		*S. T*yphi, *E. coli,* and *S. flexneri*	32	(Tekwu et al., 2012)
						B. cereu and *S. aureus*	64–128	
36	***Trachyspermum ammi***	Ajowan	Essential Oil	Fruit	Apiaceae	*B. cereus, E. aerogenes, S. aureus, P. putida, L. monocytogenes* and *E. coli*	0.4	(Moosavi-Nasab et al., 2016)
37	***Coriandrum sativum***	Coriander	Essential Oil	Whole Plant		*E. coli, P. mirabilis, F. nucleatum, S. hyicus* and *S. mitis*	0.4–62	(Bersan et al., 2014; Bogavac et al., 2015; Vaillancourt et al., 2018)
38	***Eryngium methystinum***		Essential Oil	Aerial Parts		*S. aureus*	2	(Matejić et al., 2018)
39	***E. campestre***		Acetone Extract	Whole Plant		*P. mirabilis, E. coli,* and *S. aureus*	4–10	

The exploration of plants with reported antibacterial activity highlights their potential as a valuable source of natural antimicrobial agents. Given the rise of antibiotic-resistant pathogens, plant-derived compounds offer promising alternatives for therapeutic applications. Numerous studies

have demonstrated the efficacy of various plant extracts against a wide range of bacterial strains, underscoring the importance of phytochemicals in modern medicine. However, further research, including clinical trials and toxicity assessments, is essential to establish standardized formulations and optimize their medicinal use.

B.6.ii.a. Against *Salmonella* Typhi:

The emergence of AMR strains of *S.* Typhi, as discussed earlier, has intensified the search for alternative treatments, including plant-derived antibacterial agents. Various medicinal plants have demonstrated promising inhibitory effects against *S.* Typhi, offering potential natural remedies for combating this pathogen. Studies have shown that the aqueous extracts of *Aloe secundiflora, Vernonia brachycalyx,* and *Terminalia brownii* exhibit inhibitory effects against *S.* Typhi, with zone of inhibition ranging from 4.4 to 18.5 mm (Ek et al., 2016). The aqueous extract of *Ficus sycomorus, Mangifera indica, Citrus aurantifolia* and *Carica papaya* was reported to inhibit the growth of *S.* Typhi clinical isolate with an inhibition zone of 16 mm, 15 mm, 14 mm and 13 mm, respectively and MIC and minimum bactericidal concentration (MBC) range between 12.5 - 50 mg/ml and 25 - 100 mg/ml, respectively (Bello et al., 2025). The *Cassia eucalyptus,* a medicinal plant claimed to be effective in treating typhoid by the local people of Nigeria, is reported to show inhibition against *S.* Typhi growth with MIC and MBC of 1 mg/ml and 2 mg/ml, respectively (Evans et al., 2002). Owolabi et al., 2024, in their report, indicated that the fractions derived from the acetone extract of *Hippocratea indica* exhibit an inhibitory effect on *S.* Typhi growth, with a MIC of 0.375 mg/mL and a MBC of 3 mg/ml. Pérez and Anesini, 1994 evaluated 132 aqueous plant samples of Argentine folk medicinal plants against *S.* Typhi and found that 24 of them exhibit inhibition which includes *Cassia occidentalis* roots, *Heimia salicifolia* aerial parts, *Punica granatum* fruit pericarp and *Rosa horhoniana* flowers. Akinyemi et al., 2005 screened ten

medicinal plants from South-west Nigeria commonly used in traditional medicine and found that the aqueous and ethanolic extracts of *Terminalia avicennioides*, *Momordica balsamina*, *Combretum paniculatum*, and *Trema guineensis* demonstrated effectiveness against MDR S. typhi strains. The MIC values ranged from 9.60 to 14 mg/ml, while the MBC values were between 24 and 33 mg/ml. In contrast, only the aqueous extracts of *Morinda lucida* and *Ocimum gratissimum* exhibited activity, with MIC values of 9.60 mg/ml and 40 mg/ml, respectively, and MBC values of 24 mg/ml and 55 mg/ml, respectively. Fifty-four methanolic and aqueous plant extracts that have importance in the Ayurvedic system of traditional medicine were screened, and the methanolic extract of eight showed strong antibacterial activity of *S.* Typhi that include *Terminalia arjuna* , *Aegle marmelos*, *Punica granatum, Salmalia malabarica*, *Myristica fragrans*, *Holarrhena antidysenterica*, and Triphal (mixture of *Emblica officinalis*, *Terminalia chebula* and *Terminalia belerica*) (Rani and Khullar, 2004).

In the previous report, medicinal plants have shown promising antibacterial activity against *S.* typhi, offering potential alternatives or complementary treatments for typhoid fever. These findings support traditional herbal medicine practices and emphasize the importance of harnessing plant-derived compounds in modern pharmaceutical development. The growing concern over antibiotic resistance further underscores the urgency of exploring natural antibacterial agents. The antibacterial properties of medicinal plants against *S.* typhi present an opportunity to develop more sustainable and effective approaches to combating bacterial infections. Continued research and investment in phytochemical studies could lead to innovative solutions that benefit global healthcare and address the growing challenge of antibiotic resistance.

B.6.iii. Phytochemicals with Reported Antibacterial Activity:

Phytochemicals, the bioactive compounds derived from plants, have garnered significant attention for their potential antibacterial properties. These naturally occurring substances, including alkaloids, flavonoids, tannins, terpenoids, and phenolic compounds, exhibit diverse mechanisms of action against bacterial pathogens. With the rise of antibiotic resistance, phytochemicals offer promising alternatives or complementary solutions to conventional antibiotics. Research has demonstrated that phytochemicals can inhibit bacterial growth by disrupting cell membranes, interfering with quorum sensing, and inhibiting essential enzymatic functions (Nag et al., 2022; Pammi S. S. and Giri, 2021). Their ability to target multidrug-resistant bacteria makes them valuable candidates for novel antimicrobial therapies. Additionally, some phytochemicals enhance the efficacy of existing antibiotics, reducing the likelihood of resistance development (Khameneh et al., 2019).

The exploration of plant-derived antibacterial agents continues to expand, with studies highlighting their effectiveness against various bacterial strains. Tsuchiya et al., 1996 evaluated thirteen types of flavanones against methicillin-resistant *Staphylococcus aureus* (MRSA) and demonstrated that tetrahydroxyflavanones, which was isolated from *Sophora exigua* and *Echinosophora koreensis,* exhibit growth inhibition to all tested MRSA strains at 3.13-6.25 µg/ml. The phytochemicals plumbagin found in *Plumbago indica* and nordihydroguaretic acid (NDGA) from creosote bush have been demonstrated to be antibacterial, which act on the efflux pump and increase the drug sensitivity of bacterial strains (Ohene-Agyei et al., 2014). Two isoflavonoids, erybraedin A and eryzerin C isolated from the roots of *Erythrina zeyheri,* exhibit a growth inhibitory effect against vancomycin-resistant enterococci with MICs of 1.56-3.13 µg/ml and 6.25 µg/ml, respectively and against MRSA at MICs 3.13-6.25 µg/ml (Sato et al., 2004). A

phytochemical named (6*E*,12*E*)-tetradecadiene-8,10-diyne-1,3-diol isolated from the roots of *Atractylodes japonica* was also reported to exhibit antibacterial activity against MRSA strains with a MIC of 4-32 μg/ml (Jeong et al., 2010). Kot et al., 2019 evaluated the antibacterial activity of commercial phytochemicals against *Aeromonas spp.* and stated that *trans*-cinnamaldehyde exhibits a significant inhibition of *Aeromonas salmonicida* subsp. salmonicida and *Aeromonas sobria* strains at a concentration of 0.01 mg/ml, whereas ferulic acid, *p*-coumaric acid, and caffeic acid showed inhibition to those strains at MICs ranging from 0.39 to 0.78 mg/ml. Khameneh et al., 2021 documented 81 phytochemicals that have been reported to be effective antibacterial agents, which are listed in **Table B.6.iii**.

Table B.6.iii: List of phytochemicals with reported antibacterial activity (Khameneh et al., 2021)

	Phytochemicals	**Class**	**Targeted organism**	**Mechanism of Action**
1	**Piperine**	Alkaloids	*B. subtilis* and *S. aureus*	Inhibition of the efflux pump
2	**Reserpine**		-	
3	**Sanguinarine**		Carbapenem-resistant *S.marcescens*	Inhibiting replication and transcription
4	**Chanoclavine**		-	Inhibition of the efflux pump
5	**Conessine**		*M. luteus* ATCC 9341	
6	**Chelerythrine**		MRSA and extended-spectrum β-lactamases *E. coli*	Damaging the bacterial cells
7	**Matrine**		*B. subtilis* and *E. coli*	Inhibiting the synthesis of proteins
8	**Camptothecin**		-	Cleaving the intermediate complex of DNA topoisomerase I

9	**Caffeine**		*P. aeruginosa*	Interaction with the quorum-sensing proteins and inhibiting biofilm formation
10	**Isothiocyanates**		-	Attacking the sulfhydryl groups of enzymes, damaging the cell wall integrity, and leakage of cellular metabolites
11	**Diallyl trisulfide (Allitridin)**		-	Destructing the bacterial cell membrane. Decreasing the activity of the bacterial membrane transporter system.
12	**Resveratrol**	Polyphenolic compounds	MDR Gram-negative bacteria	Inhibition of the efflux pump
13	**Baicalein**		*S.* Typhimurium	
14	**Biochanin A**		*S. aureus*	
15	**Chrysosplenol-D**		-	
16	**Chrysoplenetin**		-	
17	**Silybin**		-	
18	**Kaempferol**		-	
19	**Guttiferone-A**		-	β-lactamase inhibition
20	**4-Butanylanisole**		-	
21	**Gallic acid**		-	Cell membrane disruption, and Mg^{2+} Chelation
22	**Epigallocatechin gallate**		*S. aureus*	Inhibiting the B subunit of DNA gyrase, penicillinase, and β-lactamase

23	**3-p-trans-Coumaroyl-2-hydroxyquinic acid**		-	Damaging the cytoplasmic membrane
24	**Hydroxycinnamic acids (p-Coumaric, Caffeic, and Ferulic acids)**		-	Interfering with membrane integrity
25	**Naringenin**		-	Interacting with some crucial enzymes
26	**Eriodictyol**		*P. aeruginosa* and *S. mutans*	
27	**Taxifolin**		*H. pylori*	
28	**Curcumin**		*S. dysenteriae* and *C. jejuni*	Damaging the cell membranes
29	**Apigenin**		-	Interacting with some crucial enzymes
30	**Sophoraflavanone G**		MRSA	Interacting with peptidoglycan and inhibiting cell wall biosynthesis
31	**Acetosyringone**		*S. cerevisiae*	Depolarization of the bacterial cell membrane
32	**Chlorogenic acid**		*E. coli*, *P. alcalifaciens*, *S. aureus*, and *M. catarrhalis*	Interacting with some crucial enzymes
33	**Galangin**		*S. aureus*	Damaging of the cytoplasmic membrane and inhibition of β-lactamase
34	**Genistein**		-	Inhibition of the efflux pump
35	**Ononin**		-	
36	**Tangeritin**		-	Cell membrane disruption, DNA gyrase inhibition, Reduced protein

				synthesis, Interacting with some crucial enzymes
37	**5,6,7,4'-Tetramethoxyflavone**		-	Cell membrane disruption, DNA gyrase inhibition
38	**Chrysin**		*H. pylori*	
39	**Luteolin**		*S. aureus* and *Listeria monocytogenes*	Cell membrane disruption, DNA gyrase inhibition, Type III secretion inactivation, Interacting with some crucial enzymes
40	**Myricetin**		*S. aureus*	DNA gyrase inhibition, Type III secretion inactivation, Interacting with some crucial enzymes
41	**Nobiletin**		-	Cell membrane disruption, DNA gyrase inhibition, Reduced protein synthesis, Interacting with some crucial enzymes
42	**Totaral**		-	Reduced expression of enterotoxins, multi-drug efflux pump inhibitor
43	**Tannic acid**		*S. aureus*	Ion binding
44	**(+)-Catechin**		MRSA	Inhibition of bacterial gene expression
45	**Aegelinol**	Coumarins	*S. aureus*, *S. thypii*, *E. cloacae* and *E. earogenes*	Cell membrane Disruption

46	**Agasyllin**		*S. aureus*, *S. thypii*, *E. cloacae* and *E. earogenes*	
47	**Osthole**		-	DNA gyrase inhibitor
48	**Clorobiocin**		-	Inhibition of DNA topoisomerase type II (DNA gyrase)
49	**Novobiocin**		*S. aureus* and *S. gallinarum*	
50	**Coumermycin A1**		-	
51	**Bergamottin**		-	Inhibition of the efflux pump
52	**6-Geranyl coumarin**		-	
53	**Gallbanic acid**		-	
54	**Daphnetin**		*S. putrefaciens* and *P. fluorescens*	Cell membrane Disruption, Type III secretion inactivation
55	**Esculetin**		*R. pseudosolanacearum*	
56	**Umbelliferone**		*R. pseudosolanacearum*	
57	**Carvacrol**	Terpenes	*S. pyogenes*	Disrupting cell membrane integrity, Inhibition of efflux pump
58	**Thymol**		*B. cereus*	
59	**Soyasaponin V**		-	Inhibition of the New Delhi Metallo-β-lactamase 1
60	**Eugenol**		*E. coli*	Disrupting cell membrane integrity
61	**α-Pinene**		*H. pylori*	
62	**Limonene**		*S. aureus* and resistant *P. aeruginosa*	
63	**Menthol**		*C. albicans*	
64	**Farnesol**		Lactobacillus spp.	
65	**Nerolidol**		*S. aureus*, *S. mutans*, *P. aeruginosa* and *K. pneumoniae*	

66	**Ursolic acid**		Carbapenem-resistant *E. cloacae*	Disrupting cell membrane integrity and inhibition of β-lactamase
67	**α-Amyrin**		-	
68	**Cinnamaldehyde**		*E. coli*	Disrupting cell membrane integrity, decreasing membrane potential, and metabolic activity
69	**Artemisinin**		-	Free radicals' formation
70	**Linalool**		*P. aeruginosa*	Disrupting cell membrane integrity, changes in the nucleoid morphology, and interfering with cellular respiration
71	**Sabinene**		MDR strains	Disrupting cell membrane integrity and inhibiting DNA synthesis
72	**α-Terpineol**		*E. coli*	Lossing membrane-bound autolytic enzymes, the cytoplasm leakage and inability to osmoregulate
73	**α-Bisabolol**		*S. epidermidis* and *P. acnes*	Disrupting cell membrane integrity

Phytochemicals have emerged as promising antibacterial agents due to their diverse bioactive properties and ability to combat multidrug-resistant bacteria. Continued research into phytochemicals is essential to isolate specific bioactive compounds, understand their mechanisms of action, and develop innovative treatments for bacterial infections. This approach not only

addresses the growing challenge of antibiotic resistance but also contributes to the advancement of modern medicine.

B.6.iii.a. Against *Salmonella* Typhi:

The anti-*Salmonella* Typhi activity of the phytochemical was not a well-researched area. There few reports of phytochemicals evaluated against *S.* Typhi. The phytochemicals Thymol and Piperine synergistically act with amikacin and kanamycin to inhibit *S.* Typhi ATCC 6539 (Almuzaini, 2023). Tannic acid, a polyphenolic compound, is reported to have anti-quorum-sensing activity against S. Typhi at a concentration of 400 µg/ml and also synergistically acts with amikacin, ampicillin, ciprofloxacin, azithromycin, chloramphenicol, and gentamycin against the pathogen (Sivasankar et al., 2020).

The exploration of phytochemicals not only provides an alternative to conventional antibiotics but also addresses the growing concern of antibiotic resistance. Phytochemicals represent a valuable resource in the quest for novel antibacterial agents. Their potential to combat S. typhi and other resistant pathogens offers hope for developing sustainable and effective treatments. Continued interdisciplinary research, combining ethnobotany, microbiology, and pharmacology, will be pivotal in unlocking the full potential of these natural compounds.

B. Aim and Objectives:

The growing emergence of drug-resistant *Salmonella* Typhi is becoming a serious health concern that demands the finding of new alternatives to traditional antibiotics. Antimicrobial agents of plant source can be a path to quench that demand. Plants have an ancient history of being used for their medicinal properties by humankind to improve health. This ancient knowledge of plants' medicinal value can be a source of information to tackle modern health problems. There were herbs which were in use by the Indian tribal community to treat symptoms of typhoid. But the use of these herbs was for the symptoms, not for the disease, and was not well documented nor validated through scientific evaluation. This thesis aims to validate those uses through scientific discussion and understanding.

- To validate the antimicrobial activity of crude extract of selected herbs against MDR *Salmonella* Typhi clinical isolates.
- Identification of active phytochemicals.
- Determination of antimicrobial activity of selected phytochemicals against MDR clinical *Salmonella* Typhi isolates.
- Identification of potential mechanism of action of selected phytochemicals.
- Toxicity assay of the selected phytochemicals.
- To determine the therapeutic potential of selected phytochemicals in *in vivo* animal model.

D. ***Materials and Methods:***

D.1. Collection of Bacterial Samples:

The Bacterial samples used in this study included the reference strain MTCC734, 15 clinical isolates of *Salmonella* Typhi and one clinical isolate of *Salmonella* Typhimurium collected from the Bacterial repository of ICMR- National Institute for Research in Bacterial Infections (ICMR-NIRBI), Kolkata.

D.2. Collection of Plant Samples:

The two plant samples selected for the study were *Senna occidentalis* leaves and *Scoparia dulcis* roots and collected from a tribal village of East Burdwan, West Bengal, India (23°09'03.6"N 88°17'43.4"E) where the plants were grown naturally. The *S. occidentalis* leaves and *S. dulcis* root were collected from their natural habitat during the May-June season. The young, healthy leaves of *S. occidentalis* were selected and plucked. The *S. dulcis* plants were uprooted, and the roots were cut from the aerial part and collected.

D.3. Preparation of Crude Extract:

The crude extracts of *S. dulcis* roots and *S. occidentalis* leaves were prepared by the maceration technique (Pandey and Tripathi, 2014). First, the freshly collected plant samples were thoroughly washed in running water and were shade-dried for 2 to 3 weeks. The dried samples were then ground using a mechanical grinder. 200 gm of dried leaf samples of *S. occidentalis* were soaked in ethyl acetate for 24 hours with mild stirring to eliminate the chlorophyll contents. After soaking, the supernatant was removed, and the residual was collected. The collected residual of *S. occidentalis* and the dried root samples of *S. dulcis* (200 gm) were soaked in methanol (separately)

with continuous stirring at room temperature for 24 hours, and the supernatant was collected. This step was repeated until the supernatant became colourless. The methanol from the collected supernatant was completely evaporated at room temperature. After the removal of methanol, the collected crude extract was stored at 4 °C (for long-term storage, the extract was stored at - 20 °C).

D.4. Detection of Secondary Metabolites:

The presence of secondary metabolites like flavonoids, tannins, phenolic compounds, alkaloids, terpenoids, and saponins in the crude extracts was tested by the methods described below.

D.4.i. Detection of Flavonoid:

100 mg of extracts were dissolved in 10 ml ethyl acetate and placed in a boiling water bath for 3 min. The mixture was then filtered through a 0.22 µm filter unit. In 4 ml filtered solution, 1 ml of diluted ammonia was added and shaken well. If the solution turned yellow, it indicated the presence of flavonoids (Adeogun et al., 2016).

D.4.ii. Detection of Tannins and Phenolic Compounds:

500 mg of extracts were dissolved in water and boiled for 10 min. Then the boiled extract was filtered by Whatman filter paper (grade 1). In the filtered extract few drops of 0.1% ferric chloride were added. The solution turned blue-green, green, blue or blue-black, indicating the presence of tannins, whereas the appearance of precipitation indicated the presence of phenolic compounds (Wetungu Martin et al., 2014)

D.4.iii. Detection of Alkaloids (Mayer's test):

[Preparation of Mayer's reagent: 1.36 g mercuric chloride was dissolved in 60 ml distilled water, and 5 g potassium iodide in 30 ml distilled water. Both solutions were mixed, and 10 ml of distilled water was added to make up the volume to 100 ml.]

2 ml concentrated HCL was added to the 2 ml aqueous extract solutions, and then to this mixture, a few drops of Mayer's reagent were added. The appearance of precipitation indicated the presence of alkaloids (Ali et al., 2018).

D.4.iv. Detection of Terpenoids (Salkowski test):

2 ml of chloroform was added to 0.5 g of extract and mixed well. Then to this mixture, 3 ml of concentrated sulfuric acid was added. If a reddish-brown colouration appeared at the junction, it indicated the presence of terpenoids (Wetungu Martin et al., 2014).

D.4.v. Detection of Saponins:

0.5 g of extract was dissolved in 5 ml distilled water and shaken vigorously to form a stable froth. A few drops of olive oil were added to the froth and shaken vigorously. The formation of an emulsion indicated the presence of saponins (Mojab et al., 2010; Wetungu Martin et al., 2014).

D.5. Evaluation of the Antibacterial Activity of Crude Extract:

D.5.i. Determination of MICs and MBCs:

The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were determined by the microbroth dilution method (Balouiri et al., 2016; Wiegand et al., 2008). The bacterial isolates were sub-cultured on nutrient agar (NA) plates and incubated overnight at

37 °C. After incubation, two to three colonies were inoculated in sterile 3 ml Mueller-Hinton broth (MHB) and incubated at 37 °C in a shaker incubator at 250 rpm for 2 to 3 hours. The crude extracts were dissolved in sterile MHB with 10% Dimethyl sulfoxide (DMSO) to make working solutions of 60 mg/ml concentration. The standard anti-typhoid drug chloramphenicol was used as the control, 10% DMSO was used as the growth-positive control and sterile MHB was used as the growth-negative control. Sterile 96-well microtiter plates were used for the assay. The wells of the 12^{th} column and the 11^{th} column were used for growth-negative and growth-positive control, respectively and poured with 100 µl sterile MHB and 50 µl sterile MHB with 10% DMSO, respectively. The wells of columns 2 to 10 were poured with 50 µl sterile MHB. 100 µl of the working solution of crude extracts was poured into the wells of 1^{st} column. With the help of a multichannel pipette, 50 µl crude extract solutions from wells of the 1^{st} column were removed and serially diluted (two-fold serial dilution) up to the 10^{th} column. The Wells of the 1^{st} column had the highest concentration of extract or drug, whereas the Wells of the 10^{th} column had the lowest concentration (after the addition of 50 µl of culture, the highest concentration was 30 mg/ml, and the lowest concentration was 0.05 mg/ml). The incubated bacterial cultures were taken out, and optical density (OD) was adjusted to 0.08 – 0.135 OD at 600 nm, which is almost equivalent to 1×10^8 CFU/ml bacterial density. After obtaining the desired density, bacterial cultures were further diluted to 100-fold (20 µl culture was added to 1980 µl sterile MHB) to get a bacterial density of 1×10^6 CFU/ml. 50 µl diluted bacterial cultures were added to the wells of microtiter plate from the 1^{st} column to the 11^{th} column (the final density of bacterial culture was 5×10^5). Each row in microtiter plates represented a different test organism. The microtiter plates were covered with aluminum foil and incubated at 37 °C with mild shaking (80 rpm) overnight. After incubation appearances of visual growth were observed, and the lowest concentration with no visual growth

was recorded as MIC. Next, the whole content of wells with no visual growth was transferred to MHA plates and incubated overnight at 37 °C. After incubation appearances of bacterial growth were observed, and the lowest concentration with no growth was recorded as MBC.

D.5.ii. TLC-Bioautography:

The thin-layer chromatography (TLC)-bioautography is a technique which combines TLC with both biological and chemical detection methods (Balouiri et al., 2016). This method was applied to investigate the presence of antimicrobial compounds in the crude extract. First, 10 mg/ml working solution of crude extracts was prepared in methanol, and 10 µl were spotted on TLC plates (2×6 cm) and kept at room temperature for 5 min to dry. Meanwhile, in 150 ml beakers, a 2 ml mixture of chloroform and ethyl acetate (5:4) was poured as a solvent system (mobile phase) and covered with aluminium foil for 1-2 min for saturation. Then the dry spotted TLC plates were placed on the solvent systems, covered with aluminum foil and waited to let the solvent to run through the TLC plates. Then the plates were taken out and kept at room temperature for complete evaporation of the solvent. The TLC plates were visualized under UV light to check the development of bands of separated phytochemicals of the crude extract. The developed TLC plates were dipped into bacterial suspension (3 *Salmonella* Typhi isolates, i.e., MTCC734, KOL557 and KOL558, were selected for this experiment, and the density of the bacterial suspension was 1×10^8 CFU/ml). The plates were incubated for 24 hours at 37 °C in humid conditions. After incubation, plates were soaked with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and incubated at 37 °C for 5 to 8 hours. MTT underwent a conversion to the corresponding intensely coloured formazan by the dehydrogenases of living cells.

D.6. Fractionation of Crude Extracts by Column Chromatography:

The crude methanolic extracts were fractionated by column chromatography using different solvents like petroleum ether, ethyl acetate, and methanol as mobile phases. First, the methanolic crude extracts were mixed with silica gel (60-120 mesh) and dried well with the help of a drier. Absorbent cotton was placed inside the column (60×2.5 cm), and on top of that, 100 g pure silica gel was poured as a solid phase. Then 100 ml of petroleum ether was poured to saturate the column, and the stopcock was opened to collect the solvent. The extract and silica gel mixture were placed on top of the solid phase, and then absorbent cotton was placed on top of that. First, petroleum ether was added to the column as a mobile phase and allowed to pass through the column by the force of gravity and collected in a beaker. Continued this process until there was no colour in the collected petroleum ether. After petroleum ether, the process was continued with solvents of increasing polarity, viz., petroleum ether-ethyl acetate in a ratio of 9:1, 4:1, 7:3, 3:2, 1:1, 2:3, 3:7, 1:4, 1:9, 0:1 and lastly 100% methanol and water. All the solvents from the collected fractions were evaporated at room temperature.

D.7. Selection of Most Active Fractions:

The collected fractions of the crude extracts from the above steps were tested against selected 3 *Salmonella* Typhi isolates, MTCC734, KOL557, and KOL558. Microbroth dilution was performed to detect the MIC and MBC following the standard steps described in section **D.5.i.**

The five fractions with the lowest MICs and MBCs were selected for phytochemical profiling.

D.8. Identification of Phytochemicals:

The phytochemical profiling of selected fractions was outsourced to the National Institute of Pharmaceutical Education and Research, Kolkata (NIPER-K), for Liquid Chromatography-Mass Spectrometry (LC-MS).

D.9. Computational Screening of Identified Phytochemicals:

The physicochemical properties of identified phytochemicals were virtually screened using the online tool SwissADME. This website computes the physicochemistry and estimates the lipophilicity, water solubility, pharmacokinetics, druglikeness, and medicinal chemistry of small molecules (Daina et al., 2017). First, the Simplified Molecular Input Line Entry System (SMILES) of each phytochemical was retrieved from the PubChem database. Then, the retrieved SMILES were submitted to SwissADME, and the program was run. The LD_{50} and toxicity class were also virtually predicted by ProTox-II (upgraded version ProTox-3.0), a virtual lab for the prediction of toxicities of small molecules (Banerjee et al., 2024, 2018). The SMILES were submitted to the search box, and the prediction started.

D.10. Procurement of Selected Phytochemicals:

Five, out of all the identified phytochemicals were selected to delve deeper and purchased from Sigma-Aldrich.

D.11. Cytotoxicity Test of Selected Phytochemicals:

The cytotoxicity of selected phytochemicals was determined by performing MTT assay (Van Meerloo et al., 2011) with the RAW264.7 macrophage cell line. The RAW264.7 cells were suspended in 96-well microtiter plates (flat-bottom) with RPMI1640 + 10% FBS media and

incubated overnight at 37 °C in 10% CO_2, achieving 2×10^4 cells/well. Different concentrations, viz. 0 mg/ml, 0.01 mg/ml, 0.02 mg/ml, 0.05 mg/ml,0.1 mg/ml, 0.2 mg/ml, 0.5 mg/ml and 1 mg/m of each phytochemical were prepared in RPMI1640 + 10% FBS media with 0.2% DMSO. After overnight incubation of RAW264.7 cells, the media was aspirated gentility. 100 µl of previously prepared phytochemical concentrations were poured in triplicate into wells containing RAW264.7 cells. 100 µl 0.2% DMSO in RPMI1640 + 10% FBS media was also added to cells in triplicate as a control. The cells were then incubated overnight at 37 °C in 10% CO_2. After the incubation period, 50 µl 0.5% MTT was added to the wells and again incubated for 4 hours at 37 °C in 10% CO_2. Then, DMSO was added as a solubilizer and kept for 15 min at room temperature. The cell viability was quantified at 595 nm.

D.12. Evaluation of Antibacterial Activity of Selected Phytochemicals:

D.12.i. Detection of MICs and MBCs:

The microbroth dilution was performed to detect the MIC and MBC of selected phytochemicals following the steps described in section **D.5.i.** The phytochemicals were dissolved in 10% DMSO to prepare working solutions of 2 mg/ml concentration.

D.12.ii. Time-kill Assay:

The time-kill assay was performed to gather information about the dynamic interaction between the antimicrobial agent and the microbial strain. It also revealed a time-dependent or concentration-dependent antimicrobial effect (Balouiri et al., 2016). It was performed against three of the selected *S.* Typhi strains, viz. MTCC734, KOL557 and KOL558 using MIC×0.5, MIC, and MIC×2 concentrations of phytochemicals. The bacterial isolates were sub-cultured on nutrient agar (NA) media plates and incubated overnight at 37 °C. After incubation, a single colony was

inoculated into sterile 3 ml MHB and incubated at 37 °C overnight. After the incubation period, the optical density at 600 nm (OD_{600}) was measured and adjusted to OD_{600} 0.08-0.135, which was equivalent to 1×10^8 CFU/ml inoculum. In 50 ml conical flasks, 5 ml of selected concentrations (**Table D.12.ii**) of phytochemicals were prepared in sterile MHB. From 5 ml phytochemical solutions, 25 µl was removed, and 25 µl OD_{600} adjusted bacterial culture was added, achieving the final bacterial density of 5×10^5 CFU/ml and incubated at 37 °C, 120 rpm. Then bacterial growth was checked every 2-hour interval (0 h, 2 h, 4 h, 6 h, 8 h, 10 h, and 12 h) and after 24 hours by removing 100 µl and serially diluting in sterile MHB up to 10^{-7}. From each serial dilution, 100 µl was taken out to spread in MHA and incubated overnight at 37°C. After incubation, colonies were counted, and CFU/ml was calculated.

Table D.12.ii: Selected concentrations of phytochemicals against each *S.* Typhi isolate.

Phytochemicals	**Concentration used (mg/ml)**								
	MTCC734			KOL557			KOL558		
	0.5×MIC	MIC	2×MIC	0.5×MIC	MIC	2×MIC	0.5×MIC	MIC	2×MIC
MBOA	0.25	0.5	1	0.5	1	2	0.5	1	2
BOA	0.5	1	2	0.5	1	2	0.5	1	2
Luteolin	0.125	0.25	0.5	0.25	0.5	1	0.125	0.25	0.5
Apigenin	0.125	0.25	0.5	0.125	0.25	0.5	0.125	0.25	0.5
Acacetin	0.125	0.25	0.5	0.125	0.25	0.5	0.125	0.25	0.5

D.12.iii. Antibiofilm Assay:

The effect of phytochemicals on biofilm formation and disruption was checked using MIC, MIC×0.5, and MIC×2 concentrations (**Table D.12.ii**) against three selected *Salmonella* Typhi strains MTCC734, KOL557, and KOL558. The assay was performed on biofilm grown on 96-well plates (Haney et al., 2021). Two to three colonies of test bacterial strains were inoculated in sterile 3 ml Mueller-Hinton broth (MHB) and incubated at 37 °C in a shaker incubator at 250 rpm for 2

to 3 hours. After incubation, the culture was adjusted to OD_{600} 0.08-0.135 (equivalent to 1×10^8 CFU/ml) and then diluted to 1:100 by sterile broth to achieve the culture density of 1×10^6 CFU/ml. The selected concentrations of phytochemicals (**Table D.12.ii**) were prepared in sterile MHB with 10% DMSO. For the biofilm formation study, 100 µl of diluted culture was poured into each well of 96-well plates. For biofilm disruption, 50 µl diluted culture and 50 µl sterile broth were poured into each well of 96-well. To check the effect on biofilm formation, 100 µl of phytochemical concentrations was added to the well immediately in triplicate. Whereas for biofilm disruption, the 96-well plate with culture was first incubated for 24 hours at 37 °C, and then 100 µl of phytochemical concentrations were added. The sterile MHB with 10% DMSO was used as a control. After adding phytochemicals, plates were incubated for 24 hours at 37 °C. After the incubation, the culture media was aspirated, and wells were washed with Phosphate Buffered Saline (PBS) and then incubated at 50 °C for 1 hour. The wells were then rinsed with 0.5% crystal violet (CV) and incubated for 15 min. CV was aspirated, wells were washed with PBS and then 95% ethanol was added. The OD was taken at 590 nm to quantify the biofilms.

D.12.iv. Invasion Assay:

The invasion assay or gentamicin protection assay (Sharma and Puhar, 2019) was performed with RAW264.7 macrophage cells, which were infected with MTCC734 [multiplicity of infection (MOI) 20], and three concentrations, i.e. 0.01 mg/ml, 0.02 mg/ml and 0.05 mg/ml of phytochemicals were used as treatment. The RAW264.7 cells were suspended in 24-well plates with RPMI1640 + 10% FBS media and incubated overnight at 37 °C in 10% CO_2, achieving 1×10^6 cells/well. A single colony of test bacterial strains was inoculated in sterile 3ml Mueller-Hinton broth (MHB) and incubated overnight at 37 °C. The selected test concentrations of phytochemicals were prepared in RPMI1640 + 10% FBS media. After the incubation period, media from wells

containing RAW264.7 cells were removed gently, and 200 µl of prepared phytochemical concentrations were added to wells in triplicate. The sterile RPMI1640 + 10% FBS media was used as a control. The overnight bacterial culture was quantified and added to each well, maintaining the MOI 20 (for 1×10^6 macrophage cells/well, 2×10^7 bacterial cells/well was added). The cells were incubated for 4 hours at 37 °C in 10% CO_2. After the incubation cell culture media was aspirated and the wells were washed thrice with PBS. Then, 200 µl of 100 µg/ml gentamicin was added to each well and incubated for 2 hours to kill the non-invaded bacterial cells. Then, after the incubation wells were washed with PBS thrice again. Then cells were lysed by adding 100 µl Triton X-100 (0.1%) to each well and pipetted in and out vigorously, then the whole content was transferred to *Salmonella-Shigella* (SS) agar plates. The plates were incubated overnight at 37 °C. After the incubation, colonies were counted, and then CFU/well was calculated.

D.13. Computational Docking:

The computational docking was performed between phytochemicals (ligands) and the Dihydrofolate Reductase (DHFR) protein of *S.* Typhi.

D.13.i. Retrieval of SMILES and SDF of Phytochemicals:

The SMILES structures and SDF of all the phytochemicals were retrieved from NCBI's PubChem database.

D.13.ii. Retrieval of Protein Sequence:

The FASTA file of *Salmonella* Typhi DHFR (Q8Z9J9) was retrieved from and aligned with human DHFR (P00374) to compute identity in the Uniport database.

D.13.iii. Generation of DHFR's 3D Structure:

The 3D structure of *Salmonella* Typhi DHFR was generated by homology modeling using SWISS-MODEL (Waterhouse et al., 2018). Here, the FASTA sequence of DHFR was submitted and aligned with a template (which had the highest sequence identity with the target protein) to build the 3D model of the protein. The PDB file of the generated model was retrieved. The quality of the built model was evaluated on UCLA-DOE LAB -SAVES v6.1. Then the generated PDB file was submitted to the Protein Model DataBase (PMDB id: PM0084249)

D.13.iv. Protein-Ligand Interaction:

The interaction between DHFR and phytochemicals (ligands) was checked on Schrödinger's Maestro platform. The SMILE of phytochemicals and PDB of protein were submitted in the Maestro workbench. The ligPrep and Protein Preparation Wizard were used to prepare the phytochemicals and the protein, respectively. Then receptor grid was generated for possible binding sites in the DHFR. The docking was run utilizing the standard-precision Glide docking (Glide SP) as well as the extra-precision Glide docking (Glide XP).

D.14. Inhibition of DHFR Protein:

D.14.i. Isolation of Genomic DNA of *Salmonella* Typhi:

The *Salmonella* Typhi genomic DNA was isolated using Qiagen's DNeasy Blood & Tissue Kit (Cat. No: 69504). First, an overnight culture of *Salmonella* Typhi (KOL557) was centrifuged for 5 min at 300×g, and the pellets were collected and resuspended in 200 µl PBS. Then, 20 µl Proteinase K was added, followed by adding 200 µl Buffer AL (provided), mixed thoroughly by vortexing and incubated at 56 °C for 10 min. After the incubation, 200 µl of absolute ethanol was

added and mixed thoroughly. Then the mixture was transferred into a DNeasy Mini spin column placed in a 2 ml collection tube (provided) and centrifuged for 1 min at ≥ 6000×g. The DNeasy Mini spin column was again placed in a new 2 ml collection tube, 500 µl Buffer AW1 was added, and centrifuged for 1 min at ≥6000×g. The DNeasy Mini spin column was placed in a new 2 ml collection tube one more time and centrifuged for 3 min at 20,000×g. Next, the DNeasy Mini spin column was placed in a clean 1.5 ml tube, 200 µl Buffer AE (provided) was added directly onto the DNeasy membrane, incubated at room temperature for 1 min and then centrifuged for 1 min at ≥6000×g to elute. Then the eluted DNA was quantified in a biophotometer.

D.14.ii. Amplification of dhfr Gene by PCR:

The dhfr gene was amplified by Polymerase Chain Reaction (PCR) where isolated genomic DNA was used as the template. The forward (F) and Reverse (R) primers were designed incorporating the restriction digestion site of NdeI and XhoI restriction enzymes, respectively (**Figure D.14.iia**). The reaction mixture was prepared according to **Table D.14.ii**. The cycle condition is described in **Figure D.14.iib**. After the completion of PCR, the amplified gene was visualized by gel electrophoresis. The 1x Tris-borate-EDTA (TBE) buffer was used as a running buffer, and DNA was run through 1.2% agarose gel at 70 volts for 40 min.

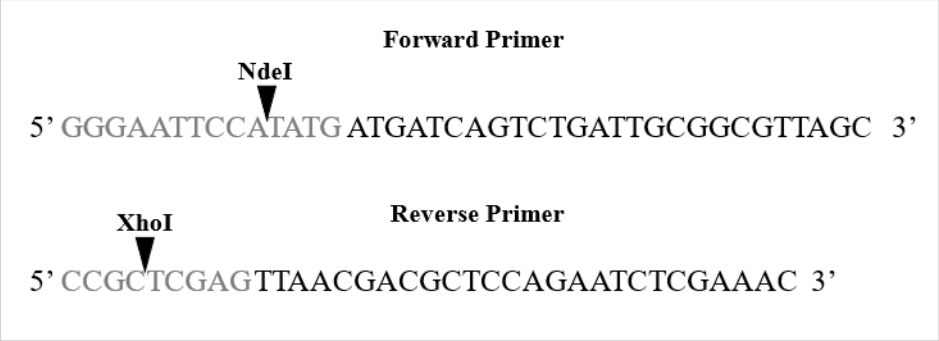


Figure D.14.iia: Sequences of primes with digestion site of NdeI and XhoI

Table D.14.ii: PCR Reaction mixture

	Reagent	Final Concentration	Volume (µl) for 25 µl Reaction
1	10× Assay Buffer	1×	2.5
2	2.5 mM dNTPs	0.25 mM	2.5
3	100 pmol/µl F primer	1 pmol/µl	0.25
4	100 pmol/µl R primer	1 pmol/µl	0.25
5	5U Taq polymerase/µl	1 U	0.2
6	Nuclease free water	-	14.3
Master mixture			20
7	Template	-	5

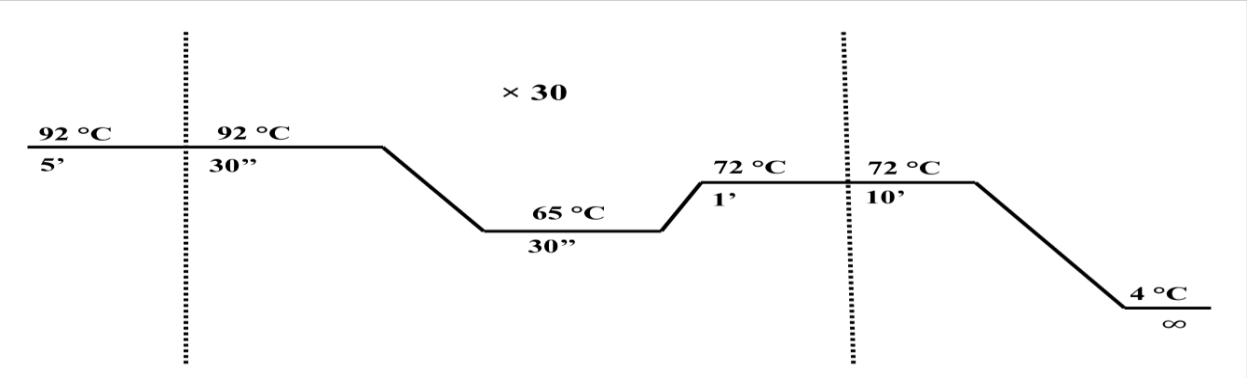


Figure D.14.iib: PCR condition

D.14.iii. Purification of PCR Product:

The amplified dhfr gene by PCR was purified by Qiagen's QIAquick PCR Purification Kit (cat. no. 28104). In a 25 µl PCR reaction, 125 µl Buffer PB (provided) was added and mixed to turn the mixture yellow. The mixture was transferred to the QIAquick column placed in a 2 ml collection tube and centrifuged for 1 min at 17,900×g. The column was then washed by adding 750 µl Buffer PE (provided) and centrifuged for 2 min at 17900×g. The QIAquick column was placed in a clean 1.5 ml microcentrifuge tube, 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) was added to the centre of the QIAquick membrane, and the column was centrifuged for 1 min at 17900×g. The eluted DNA was quantified in a biophotometer.

D.14.iv. Isolation of Vector Plasmid:

The pET-20b (+) plasmid was chosen as the vector plasmid for the insertion of *dhfr* gene. To isolate the plasmid, Qiagen's QIAprep Spin Miniprep Kit (cat. no. 27106) was used by following the prescribed protocol. The first 2 ml overnight culture of *E. coli* carrying pET-20b (+) plasmid was centrifuged at >8000 rpm for 3 min at room temperature, and the pellets were collected in a 1.5 ml microcentrifuge tube and resuspended in 250 µl Buffer P1 (provided). 250 µl Buffer P2 (provided) was added and mixed thoroughly by inverting the tube 4-6 times until the solution became clear. Then 350 µl Buffer N3 (provided) was added and mixed immediately and thoroughly by inverting the tube 4–6 times, followed by Centrifugation for 10 min at 13,000 rpm. The supernatant was collected, 800 µl was transferred to the QIAprep 2.0 spin column and centrifuged for 1 min. The QIAprep 2.0 spin column was washed by adding 750 µl Buffer PE and centrifuged for 2 min. The QIAprep 2.0 column was placed in a clean 1.5 ml microcentrifuge tube to elute DNA. 50 µl Buffer EB (10 mM TrisCl, pH 8.5) was added to the center of the QIAprep 2.0 spin column, let stand for 1 min, and centrifuged for 1 min. The eluted plasmid was quantified in a biophotometer and visualized by gel electrophoresis, where 1x TBE buffer was used as running buffer and run through a 0.8% agarose gel at 70 volts for 1h.

D.14.v. Enzymatic Restriction Digestion of *dhfr* and pET-20b (+) Plasmid:

The *dhfr* gene (containing the restriction digestion site of NdeI and XhoI restriction enzyme at 5' and 3' end, respectively) and pET-20b (+) plasmid were digested by NdeI and XhoI. Reaction mixtures were prepared following **Table D.14.v**. The reaction mixtures were incubated at 37 °C

for 4 hours. After the incubation, the reaction mixtures were run on a 1.2% agarose gel for 30 min at 80 volts using the 10X TBE buffer as running buffer.

Table D.14.v: Reaction mixture for restriction digestion

	Reagents	**Volume (µl)**	**Final concentration/reaction**
Digestion of pET-20b (+) plasmid (vector)			
1	pET-20b (+) plasmid	2.5	1 µg
2	NedI	1.5	15 U
3	XhoI	1.5	15 U
4	10X Buffer	2	1X
5	Nuclease-free water	12.5	-
Total		20	-
Digestion *dhfr* (insert)			
1	*dhfr*	7	1 µg
2	NedI	1.5	15 U
3	XhoI	1.5	15 U
4	10X Buffer	2	1X
5	Nuclease-free water	8	-
Total		20	-

D.14.vii. Purification of Digested dhfr Gene and pET-20b (+) Plasmid:

The double-digested dhfr gene and pET-20b (+) plasmid, after visualization by gel electrophoresis was excised from agarose gel with a clean, sharp scalpel and purified by Qiagen's QIAEX II Gel Extraction Kit (Cat. No.: 20021). Up to 250 mg of excised agarose gels with DNA bands were transferred to a 1.5 ml microfuge tube. 250 mg gel 750 µl Buffer QX1 (provided) was added, followed by 30 µl QIAEXII and mixed well, incubated at 50°C for 10 min with shaking to solubilize the agarose and bind the DNA. Centrifuged for 30 s, and the supernatant was removed.

The pellet was resuspended in 500 µl QX1, centrifuged for 30 s, and the supernatant was removed. The pellet was again resuspended in 500 µl Buffer PE, centrifuged for 30 s, and the supernatant was removed (this step was performed twice). The pellet was air-dried until the pellet became white. After the pellet becomes white, resuspend it in 20 µl of 10 mM Tris·Cl, pH 8.5. The *dhfr* samples were incubated at room temperature for 5 min, whereas the pET-20b (+) plasmid samples were incubated at 50 °C for 10 min. After incubation, centrifuge for 30 s, the supernatant was collected in a clean tube and quantified.

D.14.viii. Ligation and Transformation:

The *dhfr* gene was inserted into pET-20b (+) plasmid by performing a ligation reaction using New England Biolabs' (NEB) Quick Ligation Kit (M2200S). A reaction mixture was prepared by following **Table D.14.viii**. The reaction mixture was mixed by pipetting up and down and then microcentrifuged for 15 sec. The mixture was then incubated for 15 minutes at room temperature. After incubation, chilled on ice and then followed by the transformation procedure. For the transformation, the Invitrogen's One Shot TOP10 Chemically Competent *E. coli* cells (C404003) were used. The competent cells were thawed on ice, and then 50 µl of cells were added to 5 µl of ligation mixture, mixed gently by pipetting up and down. The mixture was then placed on ice for 30 min and followed by a heat shock at 42 °C for 45 sec. 950 µl of room temperature Super Optimal Broth with Catabolite repression (S.O.C) media was added to the mixture and incubated at 37 °C, 250 rpm for 1 hour. After the incubation, 100 µl was spread onto a warm Luria Bertani (LB) agar plate containing 50 µg/ml ampicillin (LB_{Amp}) and further incubated overnight at 37 °C. After incubation, only the transformed *E. coli* cells carrying the pET-20b (+) plasmid would grow on the selective LB_{Amp} agar plate as pET-20b (+) plasmid carries the ampicillin-resistant gene.

Table D.14.viii: Reaction mixture for ligation

	Reagent	Volume (µl)	Final concentration
1	2X Reaction buffer	10	1X
2	pET-20b (+) plasmid (vector)	0.8	100 ng/reaction
3	*dhfr* (insert)	5.4	135 ng/reaction
4	Nuclease-free water	2.8	-
5	Ligase	1	
Total		20	

D.14.ix. Confirmation by PCR and Plasmid DNA Mapping Using Restriction Enzymes:

The developed *E. coli* colonies from the previous step **(D.14.viii)** were collected, inoculated in Tryptic Soy Broth (TSB) and incubated overnight at 37 °C. After the incubation, the plasmid was isolated by following the steps discussed in **Section D.14.iv.** The successful insertion of the *dhfr* gene in vector pET-20b (+) plasmid is confirmed by PCR detection of the *dhfr* gene using the isolated plasmid as template and then digestion of the plasmid by NdeI and XhoI restriction enzymes. The PCR and digestion were performed by following the steps discussed in **Section D.14.ii** and **D.14.v,** respectively.

D.14.x. DHFR Protein Expression and Purification:

The isolated pET-20b (+) plasmid with inserted dhfr gene was introduced into competent *E. coli* BL21(DE3) by transformation following the steps discussed in **Section D.14.viii**. After the successful transformation, colonies were picked and inoculated in LB broth (with 50 µg/ml ampicillin) and incubated overnight at 37 °C, 180 rpm. 1 ml overnight culture was added to 250 ml LB broth (with 50 µg/ml ampicillin) and incubated at 37 °C, 180 rpm till the OD_{600} reached 0.6, after which 1M IPTG was added (final concentration 1 mM) and incubation was continued for another 5 hours. After incubation, cells were harvested by centrifugation at 6000 rpm, 4 °C for 10 min. The pellet was resuspended in 5 ml lysis buffer, and 1 mM Phenylmethylsulfonyl fluoride

(PMSF) was added. The cell suspension was then lysed by sonication. The lysed cell suspension was taken in an Oakridge tube and centrifuged at 13000 rpm, 4 °C for 40 min. After centrifugation, the supernatant was collected in a 5 ml tube, and 1 ml nickel-nitrilotriacetic acid (Ni-NTA) was added and incubated at 4 °C, 10 rpm for 3 hours. After incubation, the mixture was transferred to a PD-10 Column and washed with 40 ml of lysis buffer first. Then, the protein was eluted by adding 10 ml elution buffer to the column and collected in a clean 15 ml falcon. The eluted protein was dialyzed against PBS. The dialyzed protein was collected. The protein was quantified by Bradford assay and then visualized and analyzed by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Table D.14.xi: Preparation of Buffers

100 ml Lysis Buffer (pH 8)			
	Reagent	Concentration (mM)	Amount (g)
1	Tris-HCl	50	0.7
2	NaCl	500	2.922
3	Imidazole	20	0.136
100 ml Elution Buffer (pH 8)			
1	Tris-HCl	50	0.7
2	NaCl	500	2.922
3	Imidazole	300	2.642

D.14.xi. Enzyme Assay:

The inhibition of *Salmonella* Typhi DHFR as well as human DHFR by selected phytochemicals were tested by performing *in vitro* enzyme assay using the Dihydrofolate Reductase Assay Kit (SIGMA-ALDRICH, Cat no.: CS0340). This assay is based on the ability of DHFR to catalyze the NADPH-dependent reduction of dihydrofolic acid to tetrahydrofolic acid (**Figure D.14.xi**). The

reaction progress was monitored by the decrease in absorbance at 340nm. The 10 mM NADPH , 10mM Dihydrofolic acid and 1X assay buffer were prepared following the kit's instruction guidelines. 1mg/ml stock solutions of each phytochemical (inhibitor) were prepared. All the reagents were kept on ice except the 1X assay buffer. First, following the **Table D.14.xi** 1X assay buffer, DHFR and the concentration of inhibitors were mixed in a 1 ml tube. The mixture was transferred to a cuvette, 10 mM NADPH was added and mixed well. Then, 10mM dihydrofolic acid was added, mixed, and placed immediately in a biophotometer and absorbance at 340 nm was recorded at every 15 sec intervals for 2.5 min. The activity of DHFR was calculated by applying **Equation D.14.xi**.

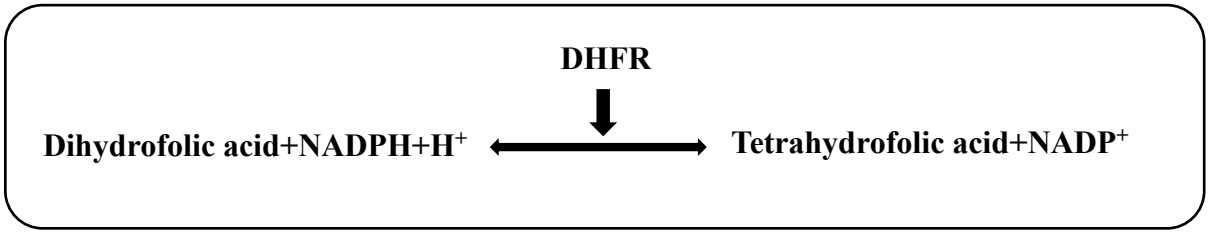


Figure D.14.xi: Reduction of Dihydrofolic acid to Tetrahydrofolic acid

Table D.14.xi: Reaction Scheme for DHFR activity test

	Reaction	1X Assay buffer (µl)	DHFR (µl)	Inhibitor (µl)	10mM NADPH (µl)	10mM Dihydrofolic Acid (µl)
1	Blank-Bacterial DHFR	900	100	-	6	-
2	Bacterial DHFR (Control)	900	100	-	6	5
3	Bacterial DHFR-0.01mg/ml MBOA	890	100	10	6	5
4	Bacterial DHFR-0.02 mg/ml MBOA	880	100	20	6	5
5	Bacterial DHFR-0.05 mg/ml MBOA	850	100	50	6	5
6	Bacterial DHFR-0.1mg/ml MBOA	800	100	100	6	5
7	Bacterial DHFR-0.01mg/ml BOA	890	100	10	6	5

8	Bacterial DHFR-0.02mg/ml BOA	880	100	20	6	5
9	Bacterial DHFR-0.05mg/ml BOA	850	100	50	6	5
10	Bacterial DHFR-0.1mg/ml BOA	800	100	100	6	5
11	Bacterial DHFR-0.01mg/ml Luteolin	890	100	10	6	5
12	Bacterial DHFR-0.02mg/ml Luteolin	880	100	20	6	5
13	Bacterial DHFR-0.05mg/ml Luteolin	850	100	50	6	5
14	Bacterial DHFR-0.1mg/ml Luteolin	800	100	100	6	5
15	Bacterial DHFR-0.01mg/ml Apigenin	890	100	10	6	5
16	Bacterial DHFR-0.02mg/ml Apigenin	880	100	20	6	5
17	Bacterial DHFR-0.05mg/ml Apigenin	850	100	50	6	5
18	Bacterial DHFR-0.1mg/ml Apigenin	800	100	100	6	5
19	Bacterial DHFR-0.01mg/ml Acacetin	890	100	10	6	5
20	Bacterial DHFR-0.02mg/ml Acacetin	880	100	20	6	5
21	Bacterial DHFR-0.05mg/ml Acacetin	850	100	50	6	5
22	Bacterial DHFR-0.1mg/ml Acacetin	800	100	100	6	5
23	Blank-Human DHFR	990	10	-	6	-
24	Human DHFR (Control)	990	10	-	6	5
25	Human DHFR-0.01mg/ml MBOA	980	10	10	6	5
26	Human DHFR-0.02 mg/ml MBOA	970	10	20	6	5
27	Human DHFR-0.05 mg/ml MBOA	940	10	50	6	5
28	Human DHFR-0.1mg/ml MBOA	890	10	100	6	5
29	Human DHFR-0.01mg/ml BOA	980	10	10	6	5
30	Human DHFR-0.02mg/ml BOA	970	10	20	6	5
31	Human DHFR-0.05mg/ml BOA	940	10	50	6	5
32	Human DHFR-0.1mg/ml BOA	890	10	100	6	5
33	Human DHFR-0.01mg/ml Luteolin	980	10	10	6	5
34	Human DHFR-0.02mg/ml Luteolin	970	10	20	6	5
35	Human DHFR-0.05mg/ml Luteolin	940	10	50	6	5
36	Human DHFR-0.1mg/ml Luteolin	890	10	100	6	5
37	Human DHFR-0.01mg/ml Apigenin	980	10	10	6	5

38	Human DHFR-0.02mg/ml Apigenin	970	10	20	6	5
39	Human DHFR-0.05mg/ml Apigenin	940	10	50	6	5
40	Human DHFR-0.1mg/ml Apigenin	890	10	100	6	5
41	Human DHFR-0.01mg/ml Acacetin	980	10	10	6	5
42	Human DHFR-0.02mg/ml Acacetin	970	10	20	6	5
43	Human DHFR-0.05mg/ml Acacetin	940	10	50	6	5
44	Human DHFR-0.1mg/ml Acacetin	890	10	100	6	5

Equation D.14.xi: DHFR activity calculation

$$\frac{Units}{mgP} = \frac{(\Delta OD/min_{sample} - \Delta OD/min_{blank}) \times d}{12.3 \times V \times mg\ P/ml}$$

where:

ΔOD/min blank = ΔOD/min. for the blank;

ΔOD/min sample = ΔOD/min. for the reaction;

12.3 = extinction coefficient (ε, mM–1 cm–1) for the DHFR reaction at 340 nm;

V = Enzyme volume in ml;

d = The dilution factor of the enzyme sample;

mg P/ml = enzyme concentration of the original sample before dilution;

Units/mg P = Specific activity in µmole/min/mg protein

D.15. Determination of *in vivo* Therapeutic Potential of Selected Phytochemicals:

The *in vivo* therapeutic activity of selected phytochemicals was checked by challenging 3 to 4-week-old BALB/c mice with *Salmonella* Typhimurium (OSS535) to develop a typhoid-like condition (non-typhoidal salmonellosis) as *Salmonella* Typhi is a human-restricted pathogen. The animal groups, except the control group (**Table D.15**), were orally administered with 200µl of 1×10^8 CFU/ml OSS535. After 24 hours, doses of phytochemicals were started and continued for 5 days. Based on virtually predicted LD_{50} value (**Table E.9**) three doses of each phytochemical were chosen *i.e.* $High\ dose = LD_{50}/10$; $Middle\ dose = High\ dose/2$; $Low\ dose = Middle\ dose/2$. 50 mg/kg chloramphenicol was used as a drug control. The control group (without infection) and the infection control group (without treatment) were administered with the vehicle (200µl DMSO). During the treatment, body weight, food and water consumption were recorded. On every alternative day stool samples were collected to check bacterial shedding. After completion of treatment, animals were sacrificed and the colonization and transmission of infections to different organs were recorded by aseptically collecting the liver, intestine, and blood. The excised organs were homogenized and plated on *Salmonella-Shigella* (SS) agar and incubated at 37 °C overnight. After the incubation colonies were counted.

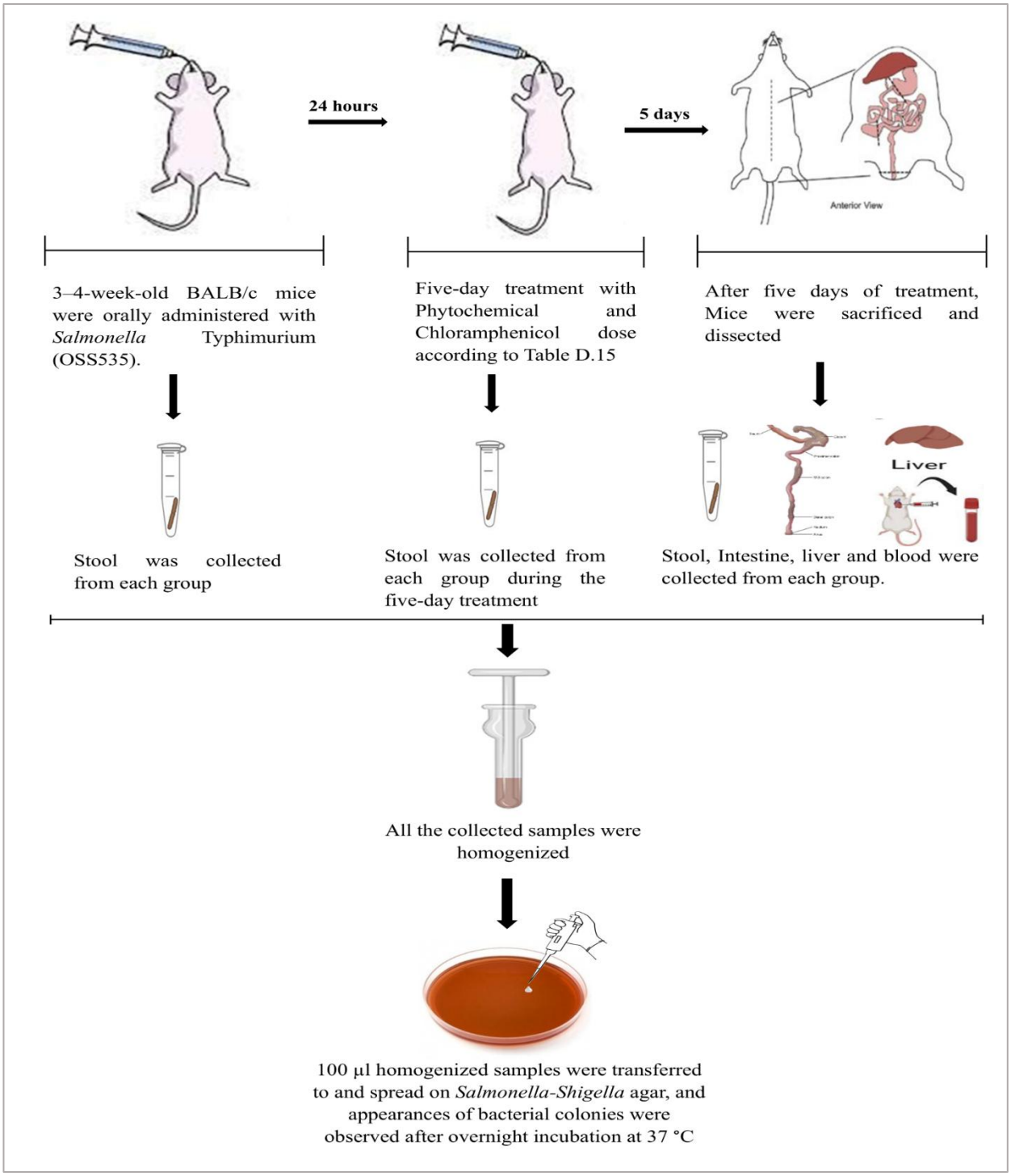


Figure D.15: Schematic diagram of the performed in vivo experiment

Table D.15: Animal groups and their treatment

Serial no.	Group (n=6)	Infection (200µl of 1×10^8 CFU/ml OSS535)	Treatment (200µl)
1	Control	-	Vehicle (DMSO)
2	Infection control	√	Vehicle (DMSO)
3	Drug control: Chloramphenicol	√	50 mg/kg Chloramphenicol
4	MBOA: High dose	√	131 mg/kg MBOA
5	MBOA: Middle dose	√	65.5 mg/kg MBOA
6	MBOA: Low dose	√	32.75 mg/kg MBOA
7	BOA: High dose	√	89 mg/kg BOA
8	BOA: Middle dose	√	44.5 mg/kg BOA
9	BOA: Low dose	√	22.25 mg/kg BOA
10	Luteolin: High dose	√	391.9 mg/kg Luteolin
11	Luteolin: Middle dose	√	195.95 mg/kg Luteolin
12	Luteolin: Low dose	√	97.97 mg/kg Luteolin
13	Apigenin: High dose	√	250 mg/kg Apigenin
14	Apigenin: Middle dose	√	125 mg/kg Apigenin
15	Apigenin: Low dose	√	62.5 mg/kg Apigenin
16	Acacetin: High dose	√	400 mg/kg Acacetin
17	Acacetin: Middle dose	√	200 mg/kg Acacetin
18	Acacetin: Low dose	√	100 mg/kg Acacetin

E. ***Result and Discussion:***

E.1. Collection of Bacterial Samples:

The antimicrobial-resistance profiles (CLSI, 2018) of collected clinical isolates of *S.* Typhi and *S.* Typhimurium, along with the isolation date and institute strain ID, were presented in **Table I.1**.

E.2. Collection of Plant Samples:

The East Burdwan, West Bengal, India (**Figure E.2.a**) from where the plant samples were collected is a tropical region – hot and humid with annual rainfall of 1400mm, and the soil is alluvial. *Senna occidentalis* (*Caccia occidentalis*) is a roadside plant about 1 M tall, locally known as Kalkasunda (**Figure E.2.b**) The stems are purplish with 12-20 cm long pinnately compound leaves. Leaflets are in the below 5 pairs, and they are ovate-oblong and acute. Flowers are yellow blooms in upper axils or terminal racemes. *Scoparia dulcis* is a 30-80 cm long weed of garden and wasteland, an erect herb commonly known as Chinidaare or Bandhonay with angular stems, and ovate-elliptic leaves (**Figure E.2.c[1]**). Flowers are regular, white blooms in axillary clusters. Roots are fibrous taproots, light brown to yellowish, with variable lengths (**Figure E.2.c[2]**). The plant samples were authenticated at the Department of Botany, the University of Calcutta, and voucher specimens (no. 20099 for *S. occidentalis* and 20100 for *S. dulcis*) were deposited in the Calcutta University Herbarium (CUH), Kolkata, and also in the host institute.

Traditionally, leaves of *S. occidentalis* were used to treat various skin diseases and gastric disorders (Singh et al., 2020). It was reported to be antibacterial against MRSA, *K. pneumoniae* (Zeeshan et al., 2022), *Staphylococcus spp*., *E. coli*, and *P. aeruginosa* (Imarenezor and Gaina, 2025) and as well as against *S.* Typhi (Sani, 2022). On the other hand, *S. dulcis* has been reported to treat

bronchitis, cough, cancer, diabetes, diarrhoea, fever, haemorrhoids, liver disorders, inflammation, rashes, abdominal pain, tuberculosis, ulcers, wounds, and more (De Farias Freire et al., 1993; Mishra et al., 2011; Mohandas et al., 2014; Reddy et al., 2012; Thirumurugan et al., 2018). Its antibacterial activity was reported against *Bacillus licheniformis, E. coli, K. pneumoniae, P. aeruginosa,* and *S. aureus* (Coulibaly et al., 2014; Md. Zulfiker et al., 2011; Niveditha and Prabavathy, 2015; Uma et al., 2014).

Figure E.2.a: map of West Bengal

Figure E.2.b: *Senna occidentalis*

Figure E.2.c[1]: *Scoparia dulcis*

Figure E.2.c[2]: *Scoparia dulcis root*

E.3. Preparation of Crude Extract:

The complete evaporation of methanol resulted in the final products, which were gooey in texture. The crude root extract of *Scoparia dulcis* (SDMe) was dark brown, and the final yield was ≈14%. Whereas the crude leaf extract of *Senna occidentalis* (SOMe) was greenish-black in colour with a total yield of ≈20%.

E.4. Detection of Secondary Metabolites:

Secondary metabolites are stress-induced organic compounds produced by organisms to tackle environmental stress. Plants also use secondary metabolites to defend against pathogens and herbivores. The chemical tests to detect secondary metabolites (**Table E.4**) showed that SDMe consisted of flavonoids, alkaloids, terpenoids, tannins and phenolic compounds, and there were no traces of saponins. Whereas SOMe was positive for flavonoids, alkaloids, terpenoids, tannins and saponins but no traces of phenolic compounds. Secondary metabolites were harvested by humans

in past to improve their health conditions (Thirumurugan et al., 2018). Thirumurugan et al., 2018 reported that tannins, terpenoids, alkaloids, and flavonoids exhibit antimicrobial properties. Additionally, alkaloids have been widely utilized in medicinal applications due to their antitumor, antipyretic, and antimalarial properties (Jordan and Wilson, 2004; Reyburn et al., 2009). Research on natural phenols has demonstrated their antimicrobial, antiviral, anti-inflammatory, and vasodilatory effects in vitro(Perez et al., 2003; Rauha et al., 2000; Santos et al., 2000).

Table B.1.iii.d: Secondary metabolites profile of crude extract

Secondary metabolites	**Methanolic root extract of *Scoparia dulcis* (SDMe)**	**Methanolic leaf extract of *Senna occidentalis* (SOMe)**
Flavonoids	+	+
Alkaloids	+	+
Terpenoids	+	+
Tannins	+	+
Phenolic compounds	+	-
Saponins	-	+

E.5. Evaluation of the Antibacterial Activity of Crude Extract:

E.5.i. Determination of MIC and MBC:

The MIC value of an antimicrobial agent represents the minimum concentration required to inhibit the visible growth of the tested organism, whereas MBC indicates the lowest concentration needed to kill 99.9% final inoculum after 24 hours of incubation (Balouiri et al., 2016). The MIC and MBC of crude extracts were determined against the *S.* Typhi MTCC734 strain and 15 drug-resistant clinical isolates (**Table E.5.i**). The SDMe had MIC of $\leq$ 7.5 (> 3.75) mg/ml against most of the drug resistent test isolates except KOL42 and KOL89 and MBC of $\leq$ 15 (> 7.5) mg/ml

against most of the test isolates except MTCC734 here it was ≤ 7.5 (> 3.75) mg/ml and for KOL42 and KOL89 it was > 15 mg/ml. On the other hand, the SOMe had MIC at a very high range, it was ≤ 60 (>50) mg/ml against MTCC734 and ≤ 80 (>70) mg/ml against other test isolates and MBC ranged from >80 mg/ml to >100 mg/ml. This result indicated that the SDMe is more effective against drug-resistant clinical isolates of *S.* Typhi in comparison to SOMe.

Table E.5.i: MIC and MBC of crude extract of *Scoparia dulcis* roots and crude extract of *Senna occidentalis* leaves against control MTCC734 and 15 clinical isolates of *S.* Typhi

***S. Typhi* Isolates**	**SDMe**		**SOMe**	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
MTCC734	≤ 7.5 (> 3.75)	≤ 7.5 (> 3.75)	≤ 60 (>50)	≤ 90 (>80)
KOL5	≤ 7.5 (> 3.75)	≤ 15 (> 7.5)	≤ 80 (>70)	≤ 90 (>80)
KOL18	≤ 7.5 (> 3.75)	≤ 15 (> 7.5)	≤ 80 (>70)	≤ 90 (>80)
KOL31	≤ 7.5 (> 3.75)	≤ 15 (> 7.5)	≤ 80 (>70)	≤ 90 (>80)
KOL42	≤ 15 (> 7.5)	> 15	≤ 80 (>70)	>100
KOL89	≤ 15 (> 7.5)	> 15	≤ 80 (>70)	>100
KOL112	≤ 7.5 (> 3.75)	≤ 15 (> 7.5)	≤ 80 (>70)	≤ 90 (>80)
KOL148	≤ 7.5 (> 3.75)	≤ 7.5 (> 3.75)	≤ 80 (>70)	≤ 90 (>80)
KOL162	≤ 7.5 (> 3.75)	≤ 15 (> 7.5)	≤ 80 (>70)	≤ 90 (>80)
KOL180	≤ 7.5 (> 3.75)	≤ 15 (> 7.5)	≤ 80 (>70)	≤ 90 (>80)
KOL244	≤ 7.5 (> 3.75)	≤ 15 (> 7.5)	≤ 80 (>70)	≤ 90 (>80)
KOL288	≤ 7.5 (> 3.75)	≤ 15 (> 7.5)	≤ 80 (>70)	≤ 90 (>80)
KOL301	≤ 7.5 (> 3.75)	≤ 15 (> 7.5)	≤ 80 (>70)	≤ 90 (>80)
KOL343	≤ 7.5 (> 3.75)	≤ 15 (> 7.5)	≤ 80 (>70)	≤ 90 (>80)
KOL557	≤ 7.5 (> 3.75)	> 15	≤ 80 (>70)	≤ 90 (>80)
KOL558	≤ 7.5 (> 3.75)	≤ 15 (> 7.5)	≤ 80 (>70)	≤ 90 (>80)

E.5.ii. TLC-Bioautography:

The TLC-Bioautography combines TLC with both biological and chemical detection (Balouiri et al., 2016). Here, developed TLC plates were soaked with viable bacterial culture and incubated overnight at 37 °C in humid conditions. Upon incubation, plates were sprayed with MTT. MTT is a yellow dye converted to purple formazan by the metabolic enzyme of living cells. In the experiment, it was observed that a distinct, clear white zone surrounded by a blue area appeared on developed TLC plates of *Scoparia dulcis* root extract, which indicated inhibition of *S.* Typhi growth by the extract (**Figure E.5.ii.α**). It was also observed that the clear zone appeared in the middle to the upper portion of the plate, indicating inhibition by low to intermediate polarity compounds present in the *Scoparia dulcis* root extract. But in the case of *Senna occidentalis* leaf extract, there was no distinct, clear white zone observed (**Figure E.5.ii.β**).

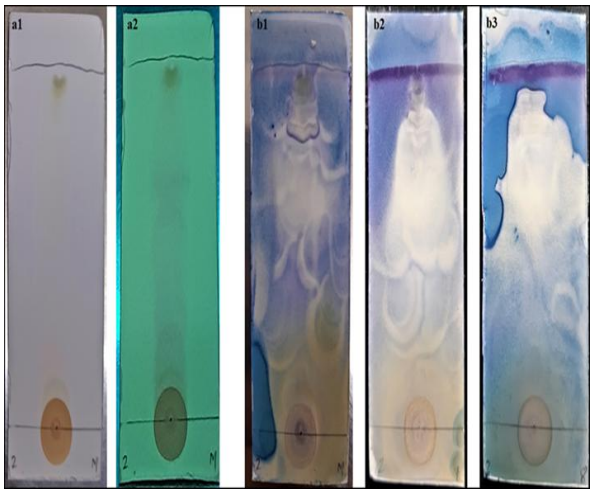


Figure E.5.ii.α: TLC plates; a1, a2: showing separated bands of *S. dulcis* root extract under visual light and UV light respectively; b1, b2, b3: showing the blue area of bacterial growth (of *S.* Typhi MTCC734, KOL557, KOL558 respectively) surrounding the clear zone.

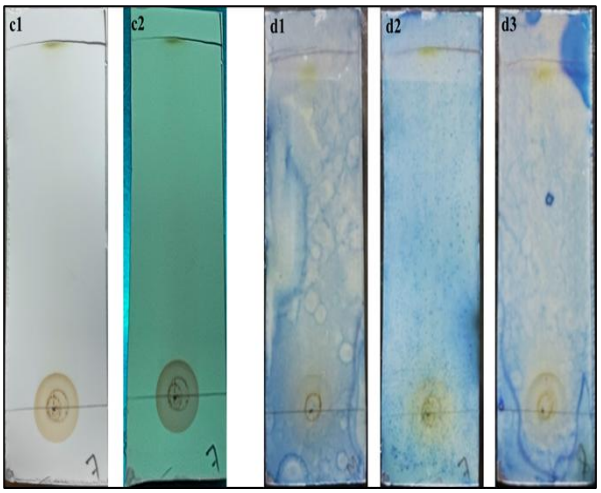


Figure E.5.ii.β: TLC plates; c1, c2: showing separated bands of *S. occidentalis* leaf extract under visual light and UV light respectively; d1, d2, d3: showing the blue area of bacterial growth (of *S.* Typhi MTCC734, KOL557, KOL558 respectively) surrounding the clear zone.

E.6. Fractionation of Crude Extracts by Column Chromatography:

The crude extract of *Scoparia dulcis* root was fractionated into thirteen fractions, whereas the crude extract of *Senna occidentalis* was fractionated into twenty-nine fractions by column chromatography. *S. dulcis* fractions were brown to blackish-brown with a strong aromatic smell, whereas S. occidentalis fractions were black to pitch-black with a pungent smell.

E.7. Selection of the Most Active Fractions:

The collected fractions from **Section E.6** were tested against 3 *S.* Typhi strains (MTCC734, KOL557, KOL558) by performing microbroth dilution to determine their MIC and MBC. Most of the fractions had MIC and MBC values of $\leq$ 15 (>7.5) mg/ml and >15 mg/ml against the test strains (**Table E.7**). The fraction SDFRC2 was most effective in comparison to other fractions which had MIC value of $\leq$3.75 (>1.8) mg/ml against the test strains and MBC of $\leq$3.75 (>1.8) mg/ml against MTCC734 and of $\leq$7.5 (>3.75) against clinical isolates KOL557 and KOL558. Two other fractions, i.e. SDFRC4 and SDFRC3 also showed comparatively better effect against the test strains. The fraction SDFRC4 had MIC and MBC of $\leq$3.75 (>1.8) mg/ml against MTCC734 and $\leq$7.5 (>3.75) mg/ml against KOL557 and KOL558. The fraction SDFRC3 had MIC and MBC of $\leq$3.75 (>1.8) mg/ml and $\leq$7.5 (>3.75) mg/ml, respectively, against MTCC734 and against clinical isolates KOL557 and KOL558 the MIC and MBC were $\leq$7.5 (>3.75) mg/ml. The two more fractions which had considerably improved activity against test strains were SDFRC5 and SDFRC1.

Table E.7: MIC and MBC of each collected fraction against control MTCC734 and 2 clinical isolates of *S.* Typhi, i.e. KOL557 and KOL558

	Fractions	MTCC 734		KOL557		KOL558	
		MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
1	SDFRC1	≤3.75 (>1.8)	≤15 (>7.5)	≤7.5 (>3.75)	≤ 15 (>7.5)	≤7.5 (>3.75)	≤ 15 (>7.5)
2	SDFRC2	≤3.75 (>1.8)	≤3.75 (>1.8)	≤3.75 (>1.8)	≤7.5 (>3.75)	≤3.75 (>1.8)	≤7.5 (>3.75)
3	SDFRC3	≤3.75 (>1.8)	≤7.5 (>3.75)	≤7.5 (>3.75)	≤7.5 (>3.75)	≤7.5 (>3.75)	≤7.5 (>3.75)
4	SDFRC4	≤3.75 (>1.8)	≤3.75 (>1.8)	≤7.5 (>3.75)	≤7.5 (>3.75)	≤7.5 (>3.75)	≤7.5 (>3.75)
5	SDFRC5	≤7.5 (>3.75)	≤7.5 (>3.75)	≤7.5 (>3.75)	≤7.5 (>3.75)	≤7.5 (>3.75)	≤ 15 (>7.5)
6	SDFRC6	≤7.5 (>3.75)	≤ 15 (>7.5)	≤ 15 (>7.5)	> 15	≤ 15 (>7.5)	≤ 15 (>7.5)
7	SDFRC7	≤ 15 (>7.5)	≤ 15 (>7.5)	≤ 15 (>7.5)	≤ 15 (>7.5)	≤ 15 (>7.5)	≤ 15 (>7.5)
8	SDFRC8	≤15 (>7.5)	> 15	≤15 (>7.5)	> 15	≤ 15 (>7.5)	> 15
9	SDFRC9	> 15	> 15	> 15	> 15	> 15	> 15
10	SDFRC10	≤ 15 (>7.5)	≤ 15 (>7.5)	> 15	> 15	≤ 15 (>7.5)	≤ 15 (>7.5)
11	SDFRC11	≤ 15 (>7.5)	≤ 15 (>7.5)	≤ 15 (>7.5)	≤ 15 (>7.5)	≤ 15 (>7.5)	≤ 15 (>7.5)
12	SDFRC12	≤15 (>7.5)	≤ 15 (>7.5)	≤ 15 (>7.5)	> 15	≤ 15 (>7.5)	≤ 15 (>7.5)
13	SDFRC13	≤15 (>7.5)	≤ 15 (>7.5)	≤ 15 (>7.5)	≤ 15 (>7.5)	≤ 15 (>7.5)	≤ 15 (>7.5)
14	SOFRC1	≤7.5 (>3.75)	≤7.5 (>3.75)	≤7.5 (>3.75)	≤ 15 (>7.5)	≤7.5 (>3.75)	≤ 15 (>7.5)
15	SOFRC2	≤ 15 (>7.5)	> 15	> 15	> 15	> 15	> 15
16	SOFRC3	> 15	> 15	> 15	> 15	> 15	> 15
17	SOFRC4	≤ 15 (>7.5)	> 15	≤ 15 (>7.5)	> 15	≤ 15 (>7.5)	> 15
18	SOFRC5	> 15	> 15	> 15	> 15	> 15	> 15
19	SOFRC6	≤ 15 (>7.5)	≤ 15 (>7.5)	≤ 15 (>7.5)	> 15	≤ 15 (>7.5)	> 15
20	SOFRC7	> 15	> 15	> 15	> 15	> 15	> 15
21	SOFRC8	≤ 15 (>7.5)	≤ 15 (>7.5)	≤ 15 (>7.5)	> 15	≤ 15 (>7.5)	> 15
22	SOFRC9	≤ 15 (>7.5)	> 15	> 15	> 15	> 15	> 15
23	SOFRC10	≤15 (>7.5)	> 15	> 15	> 15	> 15	> 15
24	SOFRC11	≤15 (>7.5)	≤15 (>7.5)	≤ 15 (>7.5)	> 15	≤ 15 (>7.5)	> 15
25	SOFRC12	≤15 (>7.5)	> 15	≤ 15 (>7.5)	> 15	≤ 15 (>7.5)	> 15
26	SOFRC13	> 15	> 15	> 15	> 15	> 15	> 15
27	SOFRC14	≤7.5 (>3.75)	> 15	≤ 15 (>7.5)	> 15	≤ 15 (>7.5)	> 15
28	SOFRC15	≤ 15 (>7.5)	≤ 15 (>7.5)	≤ 15 (>7.5)	> 15	≤ 15 (>7.5)	> 15
29	SOFRC16	≤7.5 (>3.75)	≤ 15 (>7.5)	≤ 15 (>7.5)	> 15	≤7.5 (>3.75)	≤ 15 (>7.5)
30	SOFRC17	≤7.5 (>3.75)	≤ 15 (>7.5)	≤ 15 (>7.5)	> 15	≤ 15 (>7.5)	> 15
31	SOFRC18	≤ 15 (>7.5)	≤ 15 (>7.5)	≤ 15 (>7.5)	≤ 15 (>7.5)	≤ 15 (>7.5)	≤ 15 (>7.5)
32	SOFRC19	≤7.5 (>3.75)	≤ 15 (>7.5)	≤ 15 (>7.5)	≤ 15 (>7.5)	≤ 15 (>7.5)	≤ 15 (>7.5)
33	SOFRC20	≤7.5 (>3.75)	≤ 15 (>7.5)	≤ 15 (>7.5)	> 15	≤ 15 (>7.5)	≤ 15 (>7.5)
34	SOFRC21	> 15	> 15	> 15	> 15	> 15	> 15
35	SOFRC22	≤ 15 (>7.5)	> 15	≤ 15 (>7.5)	> 15	≤ 15 (>7.5)	> 15
36	SOFRC23	> 15	> 15	> 15	> 15	> 15	> 15
37	SOFRC24	> 15	> 15	> 15	> 15	> 15	> 15
38	SOFRC25	> 15	> 15	> 15	> 15	> 15	> 15
39	SOFRC26	> 15	> 15	> 15	> 15	> 15	> 15
40	SOFRC27	> 15	> 15	> 15	> 15	> 15	> 15
41	SOFRC28	> 15	> 15	> 15	> 15	> 15	> 15
42	SOFRC29	> 15	> 15	> 15	> 15	> 15	> 15

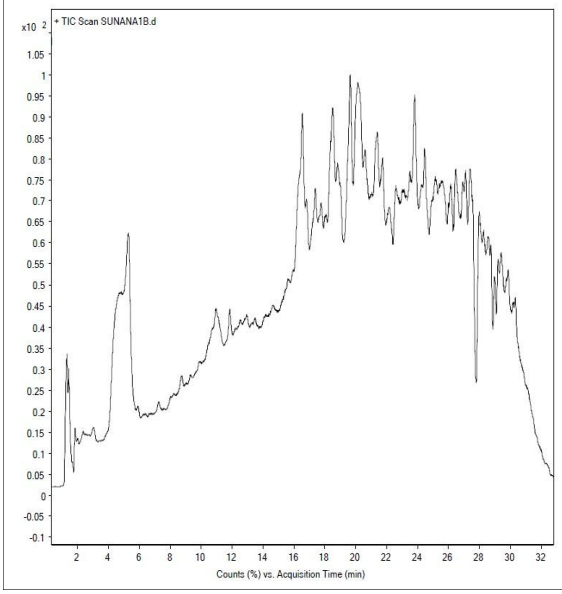
Out of those collected fractions, the five most active fractions, SDFRC2, SDFRC4, SDFRC3, SDFRC5, and SDFRC1, were selected for further study.

E.8. Identification of Phytochemicals:

The selected 5 most active fractions from **Section E.7** were subjected to phytochemical profiling by LC-MS. A total of forty-three unique phytochemicals were identified from five fractions. Most of the identified phytochemicals were flavonoids (14) and terpenoids (13), a few were benzoxazinoids (5), with others. In fraction **SDFRC1**, eighteen phytochemicals were identified (**Figure E.8a**), where Cirsimaritin, Betulinic acid, and Scoparic acid had the highest area in Total Ion Chromatography (TIC). The fraction **SDFRC2**, which is described in **Section E.7** was found to be of highest effective, consisted of twenty phytochemicals (**Figure E.8b**), where 6-Methoxybenzoxazolinone, Benzoxazolinone, and Palmitic acid had the highest area in TIC. The fraction **SDFRC3** consisted of twenty-three phytochemicals (**Figure E.8c**), and 6-Methoxybenzoxazolinone, coxicol, and 5,7,4'-trihydroxy-3-methoxyisoflavone had the highest TIC area. On the other hand, in the fraction **SDFRC4**, eighteen phytochemicals were identified (**Figure E.8d**), and its TIC graph had the highest area of Trihydroxyoctadecadienoic acid, Coxicol and Scopanolal. Lastly, the fraction **SDFRC5** consisted of 11 phytochemicals (**Figure E.8e**), and 6-Methoxybenzoxazolinone, coxicol, and Apigenin had the highest TIC area. Of these total fourty-three phytochemicals, 6-methoxybenzoxazolinone and betulinic acid were present in all five fractions and in total 6-methoxybenzoxazolinone and Benzoxazolinone had the highest abundances (highest TIC area).

Some of these phytochemicals have been previously reported to exhibit antibacterial properties, although not specifically against *S.* Typhi. Phan et al. 2006 highlighted the selective inhibition of

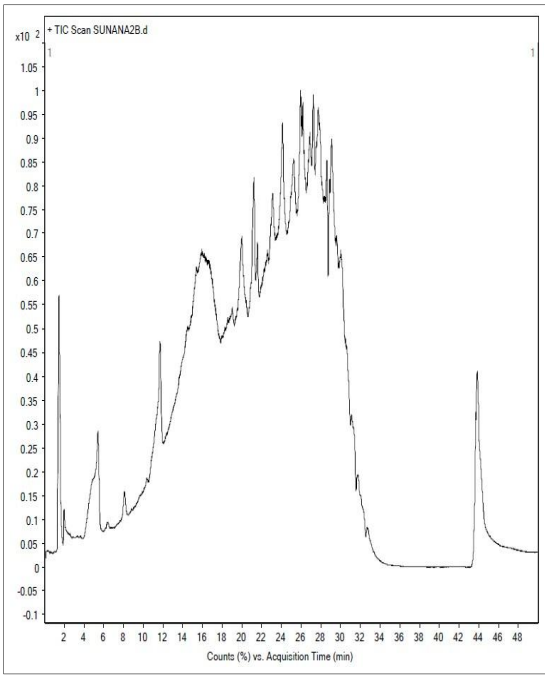
4-epi-scopadulcic acid B (a diterpenoid) against MRSA. Along with this, another diterpenoid, dulcidiol, demonstrated moderate inhibition of multidrug resistance-associated protein (MRP) with IC50 values of 15 µg/ml and 30 µg/ml, respectively. 6-methoxybenzoxazolinone (benzoxazinoid), has shown antifungal and antibacterial activity against *E. coli, P. vulgaris* and *A. tumefaciens*, as well as inhibitory effects on the HIV (Wang et al., 2001; Wang and Ng, 2002). The flavonoid acacetin has been reported to enhance the efficacy of antibiotics such as norfloxacin, oxacillin, and ampicillin, while also exhibiting inhibitory effects against MRSA, *E. coli, E. faecalis,* and *P. aeruginosa* (Alibi et al., 2021; Cha et al., 2014). Similarly, apigenin has been recognized for its antibacterial properties (Nayaka et al., 2014; Osonga et al., 2019). Nayaka et al. (2014) specifically reported its activity against *S.* Typhimurium*, P. aeruginosa, K. pneumoniae*, *P. mirabilis* and *E. aerogenes*, suggesting its potential for antibacterial drug development. Several researchers have also noted the antibacterial effects of other compounds, including benzoxazolinone (Bravo et al., 1997), Betulinic acid (Oloyede et al., 2017), luteolin (Adamczak et al., 2020; Guo et al., 2020; Gutiérrez-Venegas et al., 2019), p-coumaric acid (Mitani et al., 2018; Tlak Gajger et al., 2017) stigmasterol (Edilu et al., 2015; Yusuf et al., 2018) particularly against *S. aureus*. Flavonoids like nevadensin and nicotiflorin have also been reported to possess antibacterial activity (Kröner et al., 2012; Özçelik et al., 2008; Shadrina et al., 2021; Suksamrarn et al., 2003).



IDENTIFICATION OF COMPOUNDS IN SAMPLE 1

Formula	m/z	Mass	RT	Width	Height	Area	Name
C16 H12 O6	301.0707	300.0633	23.4	0.89	9543	322711	5,7,4'-trihydroxy -3'-methoxyisoflavone
C7 H6 O4	155.0337	154.0264	3.02	0.25	50087	1037634	Gentisic acid
C7 H5 N O2	136.0394	135.0329	10.75	0.48	19028	280305	Benzoxazolinone
C17 H14 O6	315.0859	314.0816	12.25	0.42	59035	1646892	Cirsimaritin
C30 H46 O3	455.3527	454.3452	18.71	0.15	91020	845119	Ifflaionic acid
C8 H7 N O3	166.0499	165.0425	14.96	0.22	17160	197201	6-methoxybenzoxazolinone
C16 H12 O6	301.0713	300.0641	9.75	0.41	39382	1247233	5,7,4'-trihydroxy -3'-methoxyisoflavone
C27 H36 O4	425.2701	424.2647	22.55	0.19	114540	1352296	Scopanolal
C30 H48 O3	457.3696	456.3612	20.83	0.14	182276	1544764	Betulinic acid
C8 H7 N O4	182.0451	181.0383	18.98	0.14	4366	17974	3-hydroxy -6-methoxy -2-benzoxazolinone
C19 H18 O7	359.1128	358.1054	16.08	0.22	12722	171113	Hydroxy -tetramethoxyflavone
C18 H16 O7	345.0973	344.0901	14.13	0.32	45024	951174	Nevadensin
C9 H8 O3	165.0545	164.0468	24.08	0.19	11289	123074	p-coumaric acid
C16 H26 O4	305.1753	282.1785	7.41	0.48	48768	1509500	Scoparic acid D
C27 H30 O14	579.1741	578.1667	20.97	0.16	4462	20185	isorhoifolin
C27 H36 O4	447.2495	424.2555	20.06	0.09	25049	46411	Scopanolal
C18 H28 O9	411.1654	388.1757	1.88	0.3	7125	75255	Tuberonic acid glucoside
C27 H34 O6	477.2312	454.242	2.29	0.14	8461	71535	Scopadulcic acid A

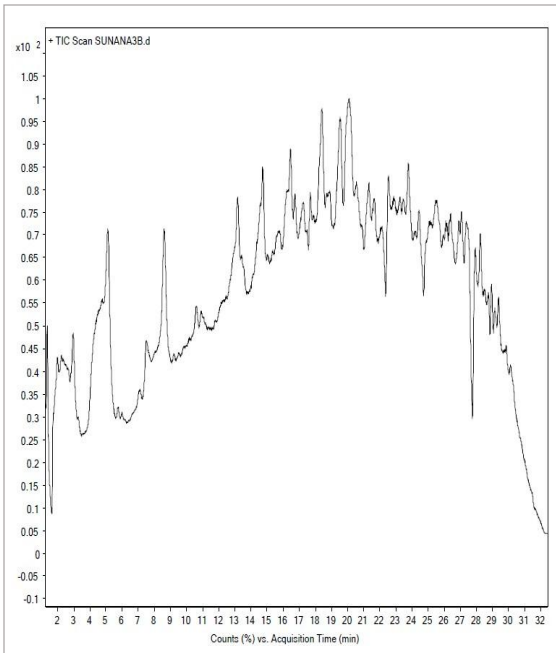
Figure E.8.a: Total Ion Chromatography (TIC) and list of identified phytochemicals in SDFRC1



Sample 2

Formula	m/z	Mass	RT	Width	Height	Area	Name
C15 H10 O5	271.0604	270.0531	9.3	0.35	3868	62915	Apigenin
C15 H10 O6	287.0546	286.0589	19.9	7.77	1522	14080	Luteolin
C9 H7 N O3	178.0496	177.0426	3.28	0.17	35027	801356	Coxicol
C27 H36 O5	463.1713	440.1864	14.67	0.1	4892	12405	Scoparic acid A
C16 H12 O5	285.0754	284.0682	22.33	0.63	3311	88194	acacetin
C25 H32 O5	413.2323	412.2252	13.15	0.15	51647	459789	Scoparic acid B
C30 H48 O3	457.3675	456.3588	26.52	0.23	25813	330544	Betulinic acid
C17 H14 O7	353.0667	330.0759	5.38	0.3	9132	189897	Cirsiliol
C18 H16 O7	345.0961	344.0905	13.33	0.58	3047	51428	Nevadensin
C16 H33 N O	256.2634	255.2559	29.1	0.15	146057	1620166	Palmitic amide
C27 H38 O4	427.281	426.2742	15.75	0.34	12230	116605	Dulcidiol
C30 H50 O	427.3925	426.3855	25.44	0.14	61972	414030	Glutinol
C16 H12 O6	301.0709	300.0635	9.79	0.57	7545	226049	5,7,4'-trihydroxy -3'-methoxyisoflavone
C7 H5 N O2	136.0394	135.0321	4.76	0.41	163163	2922726	Benzoxazolinone
C8 H7 N O3	166.0501	165.0428	5.38	0.84	5221801	245402962	6-methoxybenzoxazolinone
C8 H7 N O	134.0595	133.0835	4.11	1.37	3056	64478	Benzoxazine
C26 H27 O14	586.1315	563.1356	1.33	0.14	4406	32331	Apigenin 6 -C-pentosyl-8-C-hexoside
C26 H32 O5	447.2132	424.2238	1.46	0.03	8788	15250	Scoparic acid C
C27 H30 O15	595.168	594.1619	1.93	0.07	4449	21340	Nicotiflorin
C18 H32 O5	351.214	328.2244	12.09	0.58	12957	428720	Trihydroxyoctadecadienoic acid

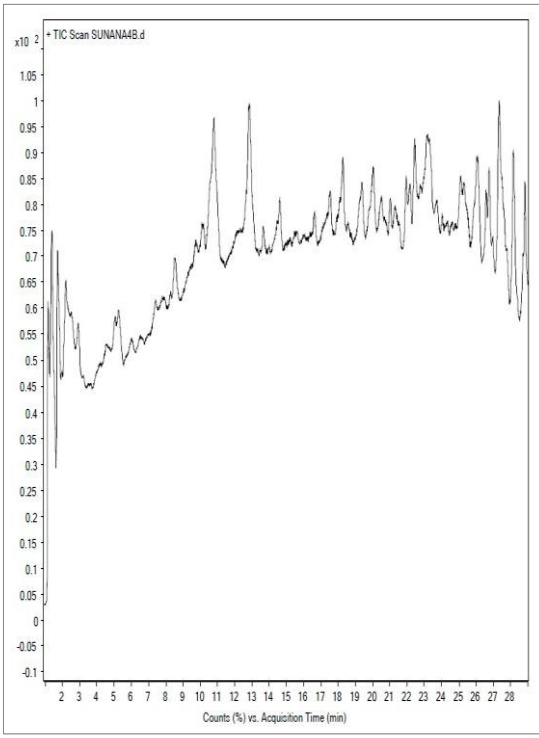
Figure E.8.b: Total Ion Chromatography (TIC) and list of identified phytochemicals in SDFRC2



IDENTIFICATION OF COMPOUNDS in sample 3

Formula	m/z	Mass	RT	Width	Height	Area	Name
C15H10O5	271.0601	270.0528	9.25	0.25	275729	6257676	Apigenin
C9 H7 N O3	178.0504	177.0431	2.96	0.2	2543268	68742265	Coxicol
C27 H36 O5	463.2446	440.2582	24.27	0.09	22970	105453	Scoparic acid A
C16 H12 O5	285.0756	284.0739	13.62	0.23	34775	645112	Acacetin
C27 H36 O4	425.2686	424.2612	22.46	0.2	48492	429554	Scopanolal
C25 H32 O5	413.2348	412.2759	18.59	1.92	17623	54684	Scoparic acid B
C30 H48 O3	457.3681	456.3607	17.76	0.08	22249	103793	Betulinic acid
C27 H30 O14	601.1545	578.1648	17.93	0.16	6010	50429	Isorhoifolin
C17 H14 O6	315.0862	314.0789	14.25	0.23	87682	1441232	Cirsimaritin
C18 H16 O6	329.1019	328.0951	19.15	0.21	43590	608343	Gonzalitosin I
C16 H33 N O	256.2641	255.2567	27.91	0.13	1229313	11124834	Palmitic amide
C9 H8 O3	165.0551	164.0477	3.27	0.17	286651	3373599	P-coumaric acid
C16 H12 O6	301.0717	300.0645	9.79	0.45	629868	21563950	5,7,4'-trihydroxy -3'-methoxyisoflavone
C7 H5 N O2	136.0392	135.0319	10.6	0.17	93802	1444279	Benzoxazolinone
C8 H7 N O3	166.0502	165.0429	5.11	1.13	8219065	499685242	6-methoxybenzoxazolinone
C7 H6 O4	155.0336	154.0262	11.72	0.52	5656	116013	Gentisic acid
C26 H52 O2	419.3733	396.3833	31.46	0.11	6768	35677	Hexacosonol
C36 H62 O9	661.4275	638.4403	26.96	0.1	24288	100822	Ginsenoside F1
C15 H19 N O9	380.0963	357.1074	1.96	0.11	53135	488152	(2R)-7-methoxy-2H-1,4-benzoxazin-3(4H)-one 2-O-β-galactopyranoside
C27 H34 O6	477.2303	454.2398	2.16	0.12	12843	124125	Scopadulcic acid A
C27 H30 O15	595.1671	594.1582	1.72	0.09	6799	35015	Nicotiflorin
C6 H5 N O2	124.0389	123.0673	5.47	0.17	25011	247748	Nicotinic acid
C18 H32 O5	351.2141	328.2247	14.93	0.26	183395	3578364	Trihydroxyoctadecadienoic acid

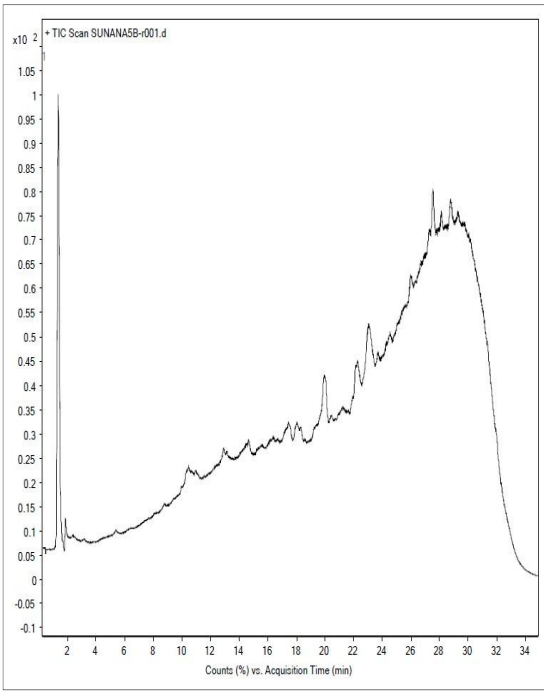
Figure E.8.c: Total Ion Chromatography (TIC) and list of identified phytochemicals in SDFRC3



Identification of compounds in sample 4

Formula	m/z	Mass	RT	Width	Height	Area	Name
C15 H10 O5	271.0598	270.0525	9.14	0.21	49694	1057972	Apigenin
C27 H34 O5	439.25	438.2394	10.45	0.02	16643	23481	4-epi-scopadulcic acid
C15 H10 O6	287.0546	286.0526	7.39	0.2	38258	896749	Luteolin
C9 H7 N O3	178.0497	177.0426	25.63	0.15	128031	1553275	Coxicol
C27 H36 O5	463.2492	440.2451	20.24	0.58	11533	204042	Scoparic acid A
C28 H32 O14	593.1867	592.1793	4.23	0.15	10723	83259	Linarin
C19 H18 O8	375.1073	374.1001	12.56	0.38	6444	96483	Hymenoxin
C27 H36 O4	425.2715	424.2644	8.28	0.42	61800	1305285	Scopanolal
C30 H48 O3	457.3679	456.3604	19.49	0.24	38845	468325	Betulinic acid
C37 H50 O19	821.285	798.2939	15.09	0.09	11318	50689	Ferruginoside C
C17 H14 O6	315.0859	314.081	14.12	0.38	10590	154286	Cirsimaritin
C18 H16 O6	329.1015	328.0965	18.7	0.12	23481	193226	Gonzalitosin I
C8 H7 N O3	166.0499	165.0426	26.34	0.14	84179	782107	6-methoxybenzoxazolinone
C29 H48 O	413.3784	412.3712	20.23	0.28	4728	33913	Stigmasterol
C29 H36 O15	625.2099	624.2025	8.61	0.14	67570	586952	trans Acteoside
C26 H32 O5	447.217	424.23	29.29	0.53	2724	13583	Scoparic acid C
C27 H30 O15	595.1658	594.1581	1.68	0.06	10000	28834	Nicotiflorin
C18 H32 O5	351.2139	328.2248	14.81	0.5	63636	2115556	Trihydroxyoctadecadienoic acid

Figure E.8.d: Total Ion Chromatography (TIC) and list of identified phytochemicals in SDFRC4



IDENTIFICTATION OF COMPOUNDS IN SAMPLE 5

Formula	m/z	Mass	RT	Width	Height	Area	Name
C15 H10 O5	271.0592	270.052	9.36	0.59	7502	237197	Apigenin
C9 H7 N O3	178.0498	177.0424	3.15	0.16	40280	492835	Coxicol
C27 H36 O4	425.2726	424.2633	1.38	0.12	6970	55607	Scopanolal
C30 H48 O3	457.3695	456.3409	19.4	0.58	6544	81712	Betulinic acid
C17 H14 O6	315.0852	314.0781	12.1	0.43	4786	108541	Cirsimaritin
C29 H32 O15	621.1814	620.1744	25.19	0.15	3628	40670	Apigenin 7 -O-R-L-3-O-acetylrhamnopyranosyl -(1-6)--D-glucopyranoside
C8 H7 N O3	166.0502	165.0429	5.37	0.25	145913	2604723	6-methoxybenzoxazolinone
C29 H48 O	413.3746	412.3664	25.57	0.18	3480	18543	Stigmasterol
C29 H36 O15	627.213	624.2068	1.37	0.13	5557	50784	trans Acteoside
C26 H32 O5	425.232	424.2276	23.47	0.53	2086	12279	Scoparic acid C
C18 H32 O5	351.2141	328.2249	10.01	0.84	5001	207767	Trihydroxyoctadecadienoic acid

Figure E.8.e: Total Ion Chromatography (TIC) and list of identified phytochemicals in SDFRC5

E.9. Computational Screening of Identified Phytochemicals:

The physicochemical properties of identified phytochemicals were screened virtually to compute their lipophilicity, water solubility, pharmacokinetics, druglikeness, medicinal chemistry, and toxicity (**Table E.9**). For lipophilicity, the Log $P_{o/w}$ value was predicted which was the logarithm of the octanol-water partition coefficient indication the tendency of a molecule to dissolve in lipids rather than water. The Log $P_{o/w} > 0$ indicates a lipophilic compound, Log $P_{o/w} = 0$ indicates a compound that dissolves equally in lipid and aqueous phases, whereas Log $P_{o/w} < 0$ indicates a hydrophilic compound. Of all the screened phytochemicals, eighteen were lipophilic, nine were hydrophilic, and fourteen showed Log Po/w value close to 0. Then, Log S was also calculated to predict the water solubility class. Here, four phytochemicals were classified under the class "very soluble", seventeen under "soluble", fifteen under "moderately soluble" and six under "poorly

soluble". Log S value between -1 and -5 is common for drugs; values greater than -1 may indicate low membrane permeability, whereas values lower than -6 are rare for drugs. In Gastrointestinal (GI) absorption and Blood-Brain-Barrier (BBB) permeability prediction, twenty-seven phytochemicals showed high GI absorption, and nine showed BBB permeability. GI absorption indicates a drug's ability to be absorbed from the digestive system to the bloodstream, whereas BBB permeability indicates a drug's ability to enter the brain. The log K_p was also calculated, where a high value indicates greater skin permeability. The log K_p prediction is important in transdermal drug delivery. Here, all the screened phytochemicals showed negative log K_p values, which indicated limited skin permeability. In Lipinski's rule of five, thirty-five phytochemicals showed druglike properties. Out of all the phytochemicals screened, only nine showed leadlikeness. In LD_{50} and GHS (globally harmonized system) toxicity class prediction, four phytochemicals were listed under toxicity class VI, twenty-seven were listed under class V, ten were listed under class IV and one was listed under class III.

Table E.9: Computational screening of identified phytochemicals

Serial no	Phytochemicals	Physicochemical Properties										Lipophilicity	Water Solubility		Pharmacokinetics			Druglikeness	Medicinal Chemistry		Toxicity	
		Formula	Molecular weight	Num. heavy atoms	Num. arom. Heavy atoms	Fraction Csp3	Num. rotatable bonds	Num. H-bond acceptors	Num. H-bond donors	Molar Refractivity	Topological Polar Surface Area	Log Po/w	Log S	Solubility Class	GI absorption	BBB permeant	Log Kp	Lipinski's rule of five	Leadlikeness	Synthetic accessibility	LD_{50}	GHS Toxicity Class
1	(2R)-7-methoxy-2H-1,4-benzoxazin-3(4H)-one 2-O-β-galactopyranoside	$C_{15}H_{19}NO_9$	357.1074 g/mol	25	6	0.53	4	9	5	83.30	146.94 Å^2	-2.04	-0.80	Very Soluble	Low	No	-9.79 cm/s	Yes; 0 violation	No	4.63	1380mg/kg	4

11	10	9	8	7	6	5	4	3	2
Benzoxazolinone	Benzoxazine	Apigenin 7-O-R-L-3-O-acetylrhamnopyranosyl-(1f6)-α-D glucopyranoside	Apigenin 6-C-pentosyl-8-C-hexoside	Apigenin	Acacetin	6-methoxybenzoxazolinone	5,7,4´-trihydroxy-3´-methoxyisoflavone	4-epi-scopadulcic acid B	3-hydroxy-6-methoxy-2-benzoxazolinone
$C_7H_5NO_2$	C_8H_7NO	$C_{29}H_{32}O_{15}$	$C_{26}H_{27}O_{14}$	$C_{15}H_{10}O_5$	$C_{16}H_{12}O_5$	$C_8H_7NO_3$	$C_{16}H_{12}O_6$	$C_{27}H_{34}O_5$	$C_8H_7NO_4$
135.12 g/mol	133.15 g/mol	620.1744 g/mol	563.1356 g/mol	270.0531 g/mol	284.0682 g/mol	165.15 g/mol	300.0635 g/mol	438.2394 g/mol	181.0383 g/mol
10	10	44	40	20	21	12	22	32	13
9	6	16	16	16	16	9	16	6	9
0.00	0.00	0.45	0.42	0.00	0.06	0.12	0.06	0.67	0.12
0	0	8	5	1	2	1	2	4	1
2	1	15	14	5	5	3	6	5	4
1	1	7	10	3	2	1	3	1	1
36.83	42.82	147.07	133.26	73.99	78.46	43.33	80.48	121.53	44.51
46.00 Å²	21.26 Å²	235.04 Å²	250.97 Å²	90.90 Å²	79.90 Å²	55.23 Å²	100.13 Å²	80.67 Å²	64.60 Å²
0.83	1.54	-2.56	-3.16	0.52	0.77	0.58	0.22	4.28	0.83
-2.07	-2.58	-3.89	-2.32	-3.94	-4.14	-2.06	-4.06	-5.73	-1.91
Soluble	Soluble	Soluble	Soluble	Soluble	Moderately Soluble	Soluble	Moderately Soluble	Moderately Soluble	Very Soluble
High	High	Low	Low	High	High	High	High	High	Low
Yes	Yes	No	No	No	No	Yes	No	No	No
-6.30 cm/s	-5.45 cm/s	-9.56 cm/s	-10.85 cm/s	-5.80 cm/s	-5.66 cm/s	-6.51 cm/s	-5.93 cm/s	-5.25 cm/s	-6.84 cm/s
Yes; 0 violation	Yes; 0 violation	No; 3 violations: MW>500, NorO>10,NHorOH >5	No; 3 violations: MW>500, NorO>10,NHorO H>5	Yes; 0 violation	Yes; 0 violation	Yes; 0 violation	Yes; 0 violation	Yes; 1 violation: MLOGP>4.15	Yes; 0 violation
No	No	No	No	Yes	Yes	No	Yes	No	No
2.34	3.21	6.47	6.18	2.96	2.98	2.34	3.03	5.68	2.64
890 mg/kg	1000mg/kg	5000mg/kg	5000mg/kg	2500mg/kg	4000mg/kg	1310mg/kg	2500mg/kg	3200mg/kg	1310mg/kg
4	4	5	5	5	5	4	5	5	4

20	19	18	17	16	15	14	13	12
Glutinol	Ginsenoside F1	Gentisic acid	Ferruginoside C	Dulcidiol	Coxicol	Cirsimaritin	Cirsiliol	Betulinic acid
$C_{30}H_{50}O$	$C_{36}H_{62}O_9$	$C_7H_6O_4$	$C_{37}H_{50}O_{19}$	$C_{27}H_{38}O_4$	$C_9H_7NO_3$	$C_{17}H_{14}O_6$	$C_{17}H_{14}O_7$	$C_{30}H_{48}O_3$
426.3855 g/mol	638.4403 g/mol	154.0264 g/mol	798.2939 g/mol	426.2742 g/mol	177.0426 g/mol	314.0816 g/mol	330.0759 g/mol	456.3588 g/mol
31	45	11	56	31	13	23	24	33
0	0	6	12	6	6	16	16	0
0.93	0.94	0.00	0.59	0.74	0.11	0.12	0.12	0.90
0	7	1	15	4	1	3	3	2
1	9	4	19	4	4	6	7	3
1	7	3	9	2	1	2	3	2
134.88	173.42	37.45	188.58	121.88	46.07	84.95	86.97	136.91
20.23 Å²	160.07 Å²	77.76 Å²	282.21 Å²	66.76 Å²	59.38 Å²	89.13 Å²	109.36 Å²	57.53 Å²
6.92	1.77	0.40	-3.40	4.47	0.31	0.47	-0.07	5.82
-8.25	-6.02	-2.23	-3.36	-5.91	-2.06	-4.20	-4.12	-7.71
Poorly soluble	Poorly soluble	Soluble	Soluble	Moderately soluble	Soluble	Moderately Soluble	Moderately Soluble	Poorly Soluble
Low	Low	High	Low	High	High	High	High	Low
No	No	No	No	Yes	Yes	No	No	No
-2.42 cm/s	-7.17 cm/s	-6.00 cm/s	-11.85 cm/s	-4.90 cm/s	-6.42 cm/s	-5.86 cm/s	-6.14 cm/s	-3.26 cm/s
Yes; 1 violation: MLOGP>4.15	No; 2 violations: MW>500,NHorOH >5	Yes; 0 violation	No; 3 violations: MW>500, NorO>10,NHorOH >5	Yes; 1 violation: MLOGP>4.15	Yes; 0 violation	Yes; 0 violation	Yes; 0 violation	Yes; 1 violation: MLOGP>4.15
No	No	No	No	No	No	Yes	Yes	No
6.24	8.12	1.10	7.68	5.84	2.16	3.27	3.32	5.63
2000 mg/kg	4000 mg/kg	4500 mg/kg	5000 mg/kg	6330 mg/kg	1295mg/kg	4000mg/kg	4000mg/kg	2610mg/kg
4	5	5	5	6	4	5	5	5

29	28	27	26	25	24	23	22	21
Nevadensin	Luteolin	Linarin	Isorhoifolin	Ifflaionic acid	Hymenoxin	Hydroxy-tetramethoxyflavone	Hexacosonol	Gonzalitosin I
$C_{18}H_{16}O_7$	$C_{15}H_{10}O_6$	$C_{28}H_{32}O_{14}$	$C_{27}H_{30}O_{14}$	$C_{30}H_{46}O_3$	$C_{19}H_{18}O_8$	$C_{19}H_{18}O_7$	$C_{26}H_{52}O_2$	$C_{18}H_{16}O_6$
344.0905 g/mol	286.0589 g/mol	592.1793 g/mol	578.1667 g/mol	454.3452 g/mol	374.1001 g/mol	358.1054 g/mol	396.3833 g/mol	328.0951 g/mol
25	21	42	41	33	27	26	28	24
16	16	16	16	0	16	16	0	16
0.17	0.00	0.46	0.44	0.87	0.21	0.21	0.96	0.17
4	1	7	6	1	5	5	24	4
7	6	14	14	3	8	7	2	6
2	4	7	8	1	2	1	1	1
91.44	76.01	141.80	137.33	135.95	97.93	95.91	128.46	89.42
98.36 Å²	111.13 Å²	217.97 Å²	228.97 Å²	54.37 Å²	107.59 Å²	87.36 Å²	37.30 Å²	78.13 Å²
0.17	-0.03	-2.76	-2.96	5.73	-0.12	0.40	5.60	0.70
-4.03	-3.71	-3.65	-3.43	-7.16	-4.10	-3.89	-8.00	-4.47
Moderately soluble	Soluble	Soluble	Soluble	Poorly soluble	Moderately soluble	Soluble	Poorly soluble	Moderately soluble
High	High	Low	Low	Low	High	High	Low	High
No	No	No	No	No	No	No	No	Yes
-6.32 cm/s	-6.25 cm/s	-9.56 cm/s	-9.71 cm/s	-3.93 cm/s	-6.52 cm/s	-6.56 cm/s	-0.51 cm/s	-5.64 cm/s
Yes; 0 violation	Yes; 0 violation	No; 3 violations: MW>500, NorO>10,NHorOH >5	No; 3 violations: MW>500, NorO>10,NHorOH >5	Yes; 1 violation: MLOGP>4.15	Yes; 0 violation	Yes; 0 violation	Yes; 1 violation: MLOGP>4.15	Yes; 0 violation
Yes	Yes	No	No	No	No	No	No	No
3.47	3.02	6.43	6.31	5.97	3.62	3.64	3.53	3.31
3919 mg/kg	3919 mg/kg	5000 mg/kg	5000 mg/kg	5000 mg/kg	5000 mg/kg	4000 mg/kg	3400 mg/kg	4000 mg/kg
5	5	5	5	5	5	5	5	5

38	37	36	35	34	33	32	31	30
Scoparic acid C	Scoparic acid B	Scoparic acid A	Scopanolal	Scopadulcic acid A	P-coumaric acid	Palmitic amide	Nicotinic acid	Nicotiflorin
$C_{26}H_{32}O_5$	$C_{25}H_{32}O_5$	$C_{27}H_{36}O_5$	$C_{27}H_{36}O_4$	$C_{27}H_{34}O_6$	$C_9H_8O_3$	$C_{16}H_{33}NO$	$C_6H_5NO_2$	$C_{27}H_{30}O_{15}$
424.53 g/mol	412.2252 g/mol	440.1864 g/mol	424.2555 g/mol	454.242 g/mol	164.0468 g/mol	255.2559 g/mol	123.0673 g/mol	594.1619 g/mol
31	30	32	31	33	12	18	9	42
6	6	6	6	6	6	0	6	16
0.50	0.56	0.56	0.56	0.67	0.00	0.94	0.00	0.44
8	7	8	8	5	2	14	1	6
5	5	5	4	6	3	1	3	15
1	1	2	1	2	2	1	1	9
120.52	116.19	126.29	124.72	122.69	45.13	81.93	31.20	139.36
80.67 Å²	80.67 Å²	83.83 Å²	63.60 Å²	100.90 Å²	57.53 Å²	43.09 Å²	50.19 Å²	249.20 Å²
3.92	3.79	4.20	4.22	3.47	1.28	3.79	-1.13	-3.43
-5.43	-4.87	-5.75	-5.79	-5.00	-2.02	-4.61	-1.26	-3.42
Moderately soluble	Moderately soluble	Moderately soluble	Moderately soluble	Moderately soluble	Soluble	Moderately soluble	Very soluble	Soluble
High	High	High	High	High	High	High	High	Low
Yes	No	No	No	No	Yes	Yes	Yes	No
-5.12 cm/s	-5.68 cm/s	-4.97 cm/s	-4.72 cm/s	-6.22 cm/s	-6.26 cm/s	-3.23 cm/s	-6.80 cm/s	-9.91 cm/s
Yes; 0 violation	Yes; 0 violation	Yes; 1 violation: MLOGP>4.15	Yes; 0 violation	Yes; 0 violation	Yes; 0 violation	Yes; 0 violation	Yes; 0 violation	No; 3 violations: MW>500, NorO>10,NHorOH >5
No	No	No	No	No	No	No	No	No
4.72	4.68	5.03	4.96	5.81	1.61	2.12	1.00	6.48
5500 mg/kg	5500 mg/kg	5000 mg/kg	5500 mg/kg	3200 mg/kg	2850 mg/kg	1000 mg/kg	3700 mg/kg	5000 mg/kg
6	6	5	6	5	5	4	5	5

39	**Scoparic acid D**	**$C_{16}H_{26}O_4$**	**424.2238 g/mol**	**20**	**0**	**0.81**	**3**	**4**	**3**	**77.90**	**77.76 Å²**	**1.96**	**-2.56**	**Soluble**	**High**	**No**	**-6.71 cm/s**	**Yes; 0 violation**	**Yes**	**4.14**	**2500 mg/kg**	**5**
40	**Stigmasterol**	**$C_{29}H_{48}O$**	**282.1785 g/mol**	**30**	**0**	**0.86**	**5**	**1**	**1**	**132.75**	**20.23 Å²**	**6.62**	**-7.46**	**Poorly soluble**	**Low**	**No**	**-2.74 cm/s**	**Yes; 1 violation: MLOGP>4.15**	**No**	**6.21**	**890 mg/kg**	**4**
41	**Trans Acteoside**	**$C_{29}H_{36}O_{15}$**	**412.3712 g/mol**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
42	**Trihydroxyoctadecadienoic acid**	**$C_{18}H_{32}O_5$**	**624.2025 g/mol**	**23**	**0**	**0.72**	**15**	**5**	**4**	**92.99**	**97.99 Å²**	**2.72**	**-3.89**	**Soluble**	**High**	**No**	**-4.92 cm/s**	**Yes; 0 violation**	**No**	**3.86**	**3389 mg/kg**	**4**
43	**Tuberonic acid glucoside**	**$C_{18}H_{28}O_9$**	**328.2244 g/mol**	**27**	**0**	**0.78**	**9**	**9**	**5**	**92.73**	**153.75 Å²**	**-1.26**	**-0.89**	**Very soluble**	**Low**	**No**	**-9.54 cm/s**	**Yes; 0 violation**	**No**	**5.57**	**55 mg/kg**	**3**

[- *: Screening not done due to non-ability of Data*]

Out of 43 identified phytochemicals, 3 flavonoids, *i.e.,* Acacetin, Apigenin, and Luteolin, and 2 benzoxazinoids, namely 6-methoxybenzoxazolinone (MBOA) and Benzoxazolinone (BOA), were selected to delve deeper.

Acacetin, apigenin, and luteolin are flavonoids, natural plant compounds known for their antioxidant, anti-inflammatory, and antimicrobial properties. They are also found in various herbs, fruits, and vegetables. Acacetin is known for its neuroprotective, anticancer, and antimicrobial effects. It inhibits bacterial enzymes and has been studied for its ability to reduce oxidative stress (etchem, 2024; Yao et al., 2021). Apigenin is recognized for its ability to modulate immune responses, combat oxidative damage, and protect against neurological disorders. It also inhibits

bacterial biofilm formation, making it useful in antimicrobial applications (Ginwala et al., 2021; Kovacs, 2025; Pei et al., 2023; Zhang et al., 2020). Luteolin is demonstrates anti-inflammatory, anti-cancer, and antibacterial properties, particularly against multidrug-resistant *E. coli*. It can interfere with bacterial metabolism and weaken pathogens (Almatroodi et al., 2024; Ding et al., 2024; El-Shiekh et al., 2023; Singh and Shukla, 2025).

MBOA plays a role in plant defense mechanisms, particularly against herbivores and insects. MBOA has been studied for its effects on animal reproduction, microbial communities, and even potential health benefits in humans. It can inhibit digestion and growth in herbivores and has shown antimicrobial properties (Dai et al., 2022; Shi et al., 2024). BOA is known for its phytotoxic properties. It can inhibit the growth of competing plants (allelopathy), and its degradation products have been studied for their effects on soil organisms and microbial communities (Chiapusio et al., 2004; Coja et al., 2006).

E.10. Procurement of Selected Phytochemicals:

All five selected phytochemicals were purchased from SIGMA-ALDRICH at their highest purity (**Table E.10**). Acacetin was whitish yellow powder with ≥ 97.0% (HPLC) purity, Apigenin was yellow powder with ≥ 95.0% (HPLC) purity, Luteolin was whitish yellow powder with ≥ 98% (TLC) purity, MBOA was white crystal with 97% purity and BOA was chalky white with 98% purity.

Table E.10: List of purchased phytochemicals procured for the study

Serial no.	Phytochemical	Manufacturer	Catalogue no.	Purity
1	Acacetin	SIGMA-ALDRICH	00017	≥ 97.0% (HPLC)
2	Apigenin	SIGMA-ALDRICH	10798	≥ 95.0% (HPLC)
3	Luteolin	SIGMA-ALDRICH	L9283	≥ 98% (TLC)
4	MBOA	SIGMA-ALDRICH	543551	97%
5	BOA	SIGMA-ALDRICH	157058	98%

E.11. Cytotoxicity Test of Selected Phytochemicals:

The cytotoxicity of each 5 selected phytochemicals was tested against RAW264.7 macrophage cells by performing MTT assay. The yellow MTT dye is converted to purple formazan by the mitochondrial activity of viable cells. The greater the intensity of formazan (OD_{595}) higher the number of viable cells. The test data showed that luteolin and MBOA had less cytotoxicity in comparison to other three phytochemicals (**Figure E.11**). More the 80% cells remained viable up to 0.02 mg/ml when treated with acacetin, apigenin and BOA whereas in case of luteolin and MBOA up to 0.1 mg/ml concentration more than 80% cells remained viable.

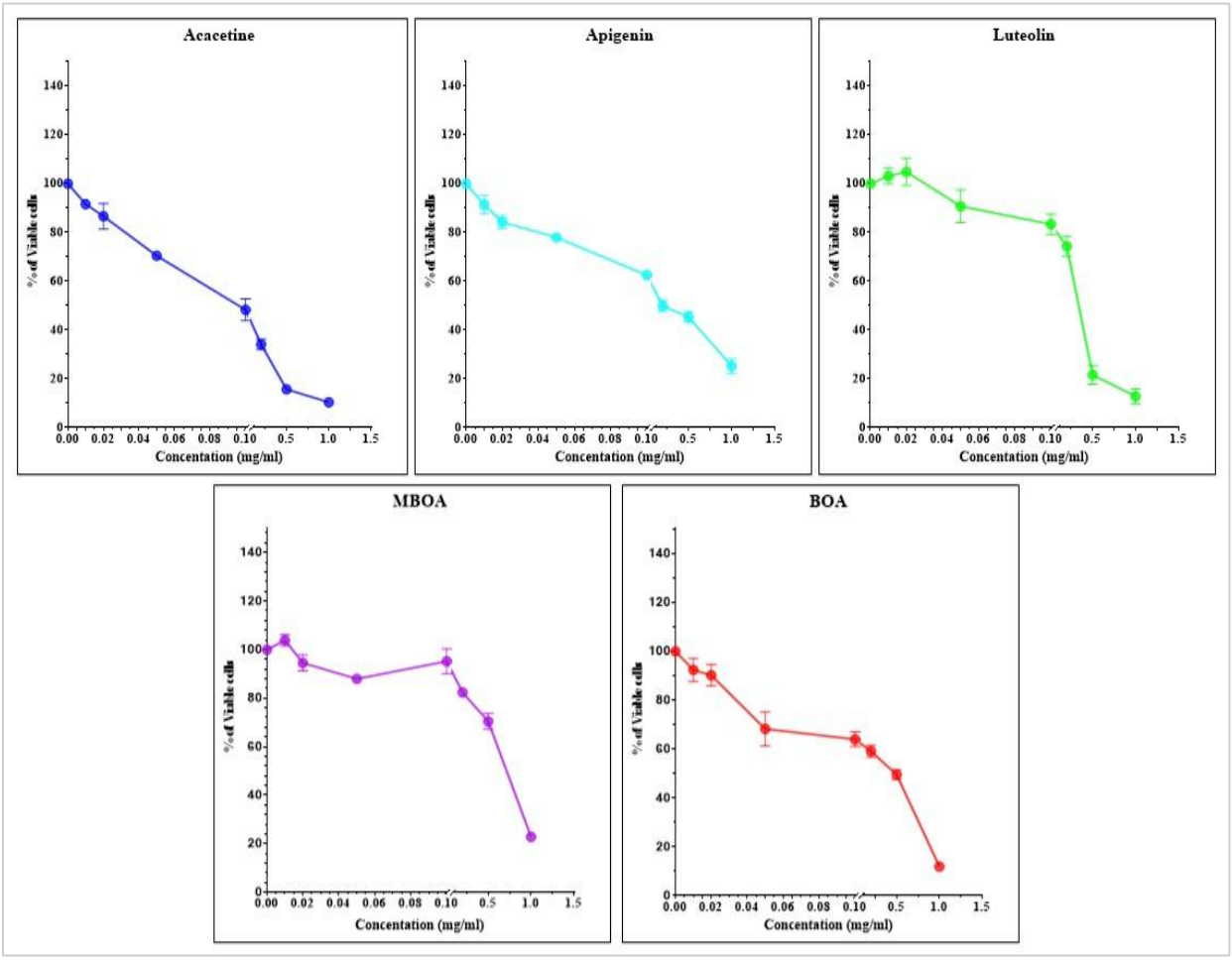


Figure E.11: Graphical representation of cytotoxicity of 5 selected phytochemicals

E.12. Evaluation of Antibacterial Activity of Selected Phytochemicals:

E.12.i. Detection of MICs and MBCs:

The MICs and MBCs of five selected phytochemicals were determined against the S. Typhi MTCC734 strain and 15 drug-resistant clinical isolates (**Table E.12.i**). Among the five phytochemicals, luteolin exhibited the lowest MIC and MBC of $\leq$ 0.03 (> 0.01) mg/ml and $\leq$ 0.25 (> 0.12) mg/ml, respectively, against KOL42, which was resistant to chloramphenicol, trimethoprim-sulfamethoxazole, nalidixic acid, norfloxacin, ciprofloxacin, and streptomycin (**Table I.1**), followed by $\leq$ 0.06 (> 0.03) mg/ml and $\leq$ 0.25 (> 0.12) mg/ml against KOL148, which was resistant to nalidixic acid, norfloxacin, ciprofloxacin, ofloxacin, levofloxacin, streptomycin, and gemifloxacin (**Table I.1**). Against most strains, luteolin recorded an MIC of $\leq$ 0.5 (> 0.25) mg/ml and an MBC of $\leq$ 2 (> 1) mg/ml. Acacetin had an MIC of $\leq$ 0.5 (> 0.25) mg/ml against most strains, except KOL343, which was resistant to nalidixic acid, ciprofloxacin, ofloxacin, levofloxacin, and streptomycin, and an MBC of $\leq$ 2 (> 1) mg/ml against all the test strains. Apigenin demonstrated the lowest MIC of $\leq$ 0.25 (> 0.125) mg/ml against KOL18 (resistant to ampicillin, chloramphenicol, trimethoprim-sulfamethoxazole, nalidixic acid, norfloxacin, ciprofloxacin, streptomycin, azithromycin, and amoxicillin-clavulanic acid) and KOL42, whereas against most strains, it was $\leq$ 0.5 (> 0.25) mg/ml and the MBC was $\leq$ 2 (> 1) mg/ml. The two benzoxazinoids, MBOA and BOA, had MIC and MBC values of $\leq$ 1 (> 0.5) mg/ml and $\leq$ 2 (> 1) mg/ml, respectively, against most of the test strains. In comparison, the three flavonoid phytochemicals were more effective against the test strains than the two benzoxazinoids.

Table E.12.i: MICs and MBCs of 5 selected phytochemicals against S. Typhi strains

S. Typhi Strains	Acacetin		Apigenin		Luteolin		MBOA		BOA	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
MTCC734	≤ 0.25 (> 0.12)	≤ 2 (> 1)	≤ 0.25 (> 0.12)	≤ 4 (> 2)	≤ 0.25 (> 0.12)	≤ 2 (> 1)	≤ 0.5 (>0.25)	≤ 2 (> 1)	≤ 1(>0.5)	≤ 4 (> 2)
KOL5	≤ 0.5 (>0.25)	≤ 2 (>1)	≤ 0.5 (>0.25)	≤ 2 (>1)	≤ 0.25 (> 0.12)	≤ 2 (>1)	≤ 1(>0.5)	≤ 2 (>1)	≤ 1(>0.5)	≤ 2 (>1)
KOL18	≤ 0.5 (>0.25)	≤ 2 (>1)	≤ 0.25 (> 0.12)	≤ 1(>0.5)	≤ 0.25 (> 0.12)	≤ 2 (>1)	≤ 1(>0.5)	≤ 2 (>1)	≤ 2 (>1)	≤ 2 (>1)
KOL31	≤ 0.25 (> 0.12)	≤ 2 (>1)	≤ 0.5 (>0.25)	≤ 2 (>1)	≤ 0.12 (>0.06)	≤ 2 (>1)	≤ 1(>0.5)	≤ 1(>0.5)	≤ 2 (>1)	≤ 2 (>1)
KOL42	≤ 0.5 (>0.25)	≤ 2 (>1)	≤ 0.25 (> 0.12)	≤ 2 (>1)	≤ 0.03 (>0.01)	≤ 0.25 (> 0.12)	≤ 1(>0.5)	≤ 1(>0.5)	≤ 1(>0.5)	≤ 2 (>1)
KOL89	≤ 0.5 (>0.25)	≤ 2 (>1)	≤ 0.5 (>0.25)	≤ 2 (>1)	≤ 0.12 (>0.06)	≤ 2 (>1)	≤ 1(>0.5)	≤ 2 (>1)	≤ 1(>0.5)	≤ 2 (>1)
KOL112	≤ 0.5 (>0.25)	≤ 2 (>1)	≤ 0.5 (>0.25)	≤ 2 (>1)	≤ 0.12 (>0.06)	≤ 2 (>1)	≤ 1(>0.5)	≤ 1(>0.5)	≤ 1(>0.5)	≤ 2 (>1)
KOL148	≤ 0.5 (>0.25)	≤ 2 (>1)	≤ 0.5 (>0.25)	≤ 2 (>1)	≤ 0.06 (>0.03)	≤ 0.25 (> 0.12)	≤ 1(>0.5)	≤ 1(>0.5)	≤ 1(>0.5)	≤ 2 (>1)
KOL162	≤ 0.5 (>0.25)	≤ 2 (>1)	≤ 0.5 (>0.25)	≤ 2 (>1)	≤ 0.5 (>0.25)	≤ 2 (>1)	≤ 1(>0.5)	≤ 2 (>1)	≤ 1(>0.5)	≤ 2 (>1)
KOL180	≤ 0.5 (>0.25)	≤ 2 (>1)	≤ 0.5 (>0.25)	≤ 2 (>1)	≤ 0.5 (>0.25)	≤ 2 (>1)	≤ 1(>0.5)	≤ 2 (>1)	≤ 1(>0.5)	≤ 2 (>1)
KOL244	≤ 0.5 (>0.25)	≤ 2 (>1)	≤ 0.5 (>0.25)	≤ 2 (>1)	≤ 0.5 (>0.25)	≤ 2 (>1)	≤ 2 (>1)	≤ 2 (>1)	≤ 1(>0.5)	≤ 2 (>1)
KOL288	≤ 0.25 (> 0.12)	≤ 2 (>1)	≤ 0.5 (>0.25)	≤ 2 (>1)	≤ 0.5 (>0.25)	≤ 2 (>1)	≤ 2 (>1)	≤ 2 (>1)	≤ 1(>0.5)	≤ 2 (>1)
KOL301	≤ 0.25 (> 0.12)	≤ 2 (>1)	≤ 0.5 (>0.25)	≤ 2 (>1)	≤ 0.5 (>0.25)	≤ 2 (>1)	≤ 2 (>1)	≤ 2 (>1)	≤ 1(>0.5)	≤ 2 (>1)
KOL343	≤ 0.12 (>0.06)	≤ 2 (>1)	≤ 1(>0.5)	≤ 2 (>1)	≤ 0.5 (>0.25)	≤ 2 (>1)	≤ 2 (>1)	≤ 2 (>1)	≤ 1(>0.5)	≤ 2 (>1)
KOL557	≤ 0.25 (> 0.12)	≤ 2 (>1)	≤ 0.25 (> 0.12)	≤ 4 (> 2)	≤ 0.5 (> 0.25)	≤ 2 (>1)	≤ 1(>0.5)	≤ 1(>0.5)	≤ 1(>0.5)	≤ 2 (>1)
KOL558	≤ 0.25 (> 0.12)	≤ 2 (>1)	≤ 0.25 (> 0.12)	≤ 4 (> 2)	≤ 0.25 (> 0.12)	≤ 2 (>1)	≤ 1(>0.5)	≤ 1(>0.5)	≤ 1(>0.5)	≤ 2 (>1)

E.12.ii. Time-kill Assay:

The time-kill assay of the selected 5 phytochemicals was performed against 3 selected *S.* Typhi strains, viz. MTCC734, KOL557, KOL558 using 3 concentrations i.e. MIC×0.5, MIC, and MIC×2. Time-kill assay is a method that measures the rate at which an antibacterial agent kills a bacterial population over time. This method helps to understand the bactericidal or bacteriostatic effect of the test agent and whether the effect is concentration-dependent or time-dependent. The time-kill curve of test strains in the presence or absences of phytochemical concentrations were plotted in **Figure E.12.ii**. By analyzing the curves, it was observed that the MIC×0.5 concentrations of the flavonoids had a non-significant effect on the test strains' growth curve, but the MIC and MIC×2 concentrations significantly caused a slow decrease in growth rate. The effect of the flavonoids on the growth curve was bacteriostatic and both concentration and time-dependent. The two benzoxazinoids also caused a significant decrease in growth rate, but the rate of killing was comparatively rapid, with chosen concentrations indicating a bactericidal activity. Here also the effect of concentration was concentration-dependent as well as time-dependent.

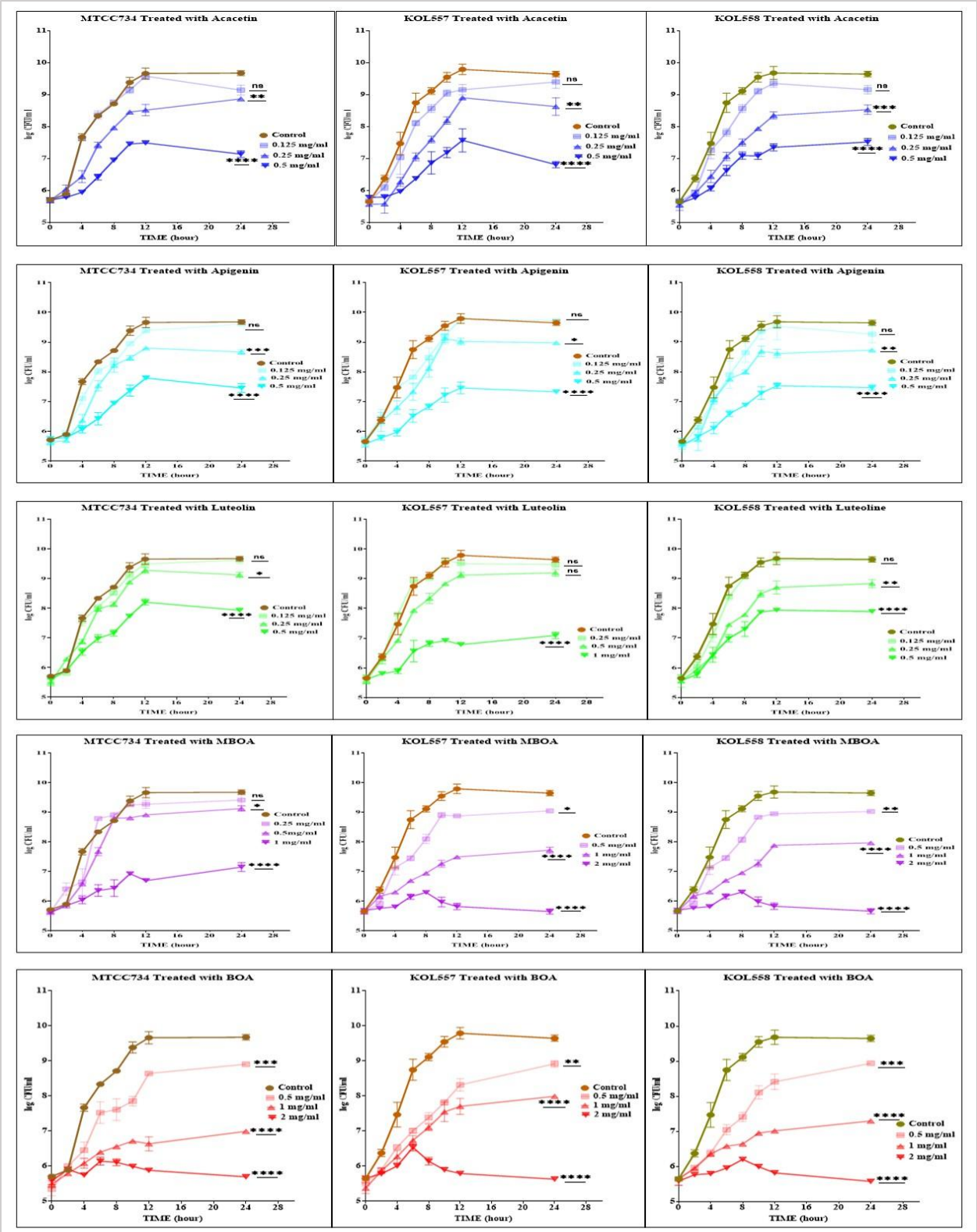


Figure E.12.ii: Time-kill curves of 5 selected phytochemicals against *S.* Typhi MTCC734, KOL557, KOL558

E.12.iii. Antibiofilm Assay:

Biofilms form when microorganisms adhere to any surface and each other and become encased in a slimy, self-generated matrix composed of extracellular polymeric substances (EPS). Biofilms are a protective mechanism for microorganisms from environmental stress. Microorganisms within biofilm become more resistant to antibiotics than free-floating ones (Donlan, 2002; Shree et al., 2023). Here, the effect of the 5 phytochemicals was evaluated on the biofilm formation and disruption of *S.* Typhi (MTCC734, KOL558, KOL558) and observed that each phytochemical had a more negative effect on biofilm formation than biofilm disruption (**Figure E.12.ii**). All the tested concentrations of the phytochemicals significantly decreased the biofilm formation with a dose-dependent effect. In biofilm disruption, the 2×MIC concentration of each phytochemical had a comparatively more significant effect than the MIC and 0.5×MIC concentrations.

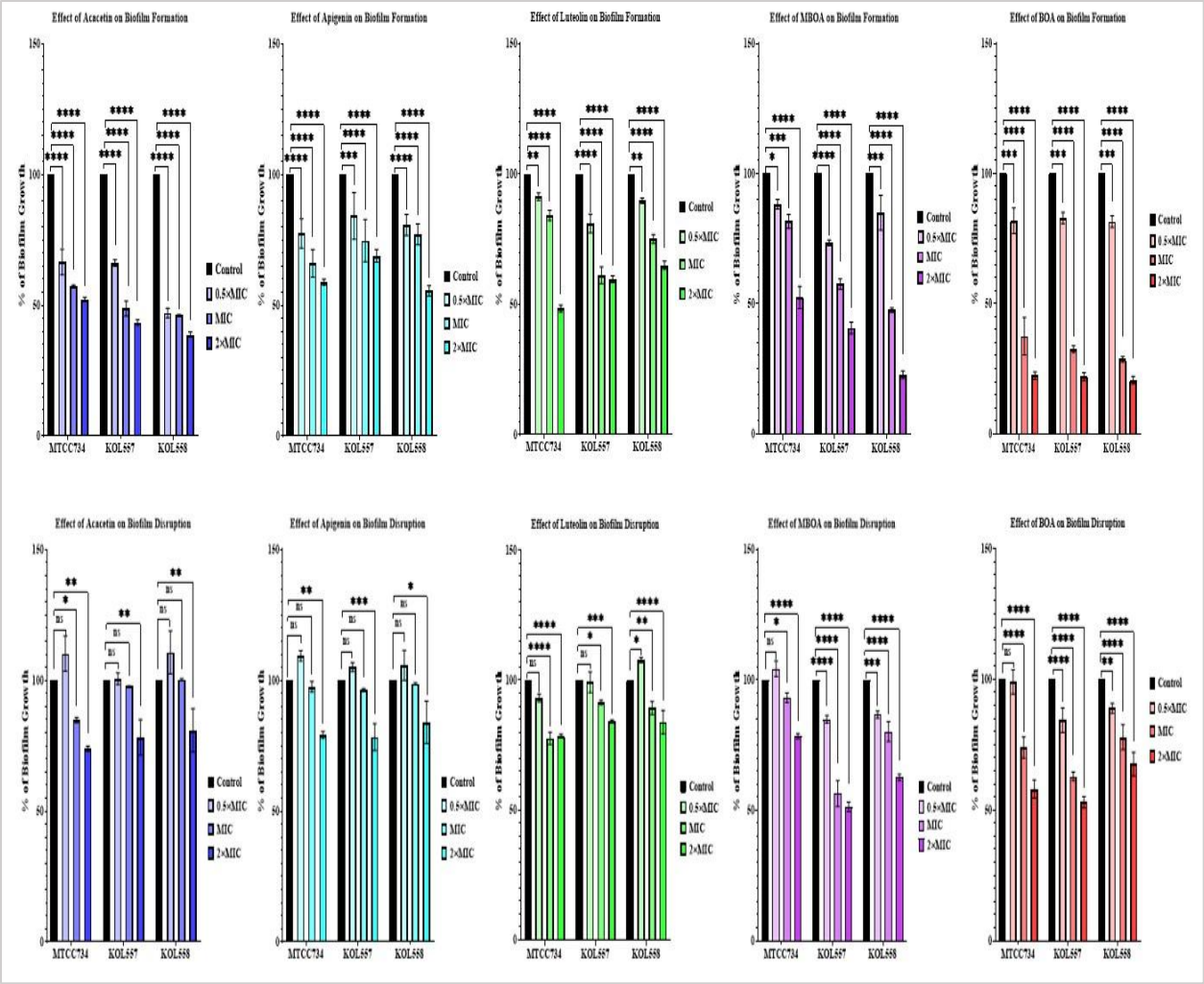


Figure E.12.iv: Graphical representation of the effect of selected phytochemicals on biofilm formation and disruption

E.12.iv. Invasion Assay:

Salmonella Typhi invade macrophage cells as a part of their pathogenesis, which plays a crucial role in establishing a systemic infection and evading the host immune system. *S.* Typhi inside macrophage cells reside in a specialized compartment called the Salmonella-containing vacuole (SCV) to prevent fusion with lysosomes. Macrophage cell helps *S.* Typhi to transport to various tissues like the liver, spleen and bone marrow. Preventing the invasion of *S.* Typhi into the

macrophage may decrease the severity of infection. In this invasion assay, where RAW264.7 macrophage cells were treated with different concentrations of the phytochemicals after being infected with *S.* Typhi (MOI 20), it was observed that the treated cells had a significantly decreased number of invaded *S.* Typhi infection than non-treated cells (**Figure E.12.iv**).

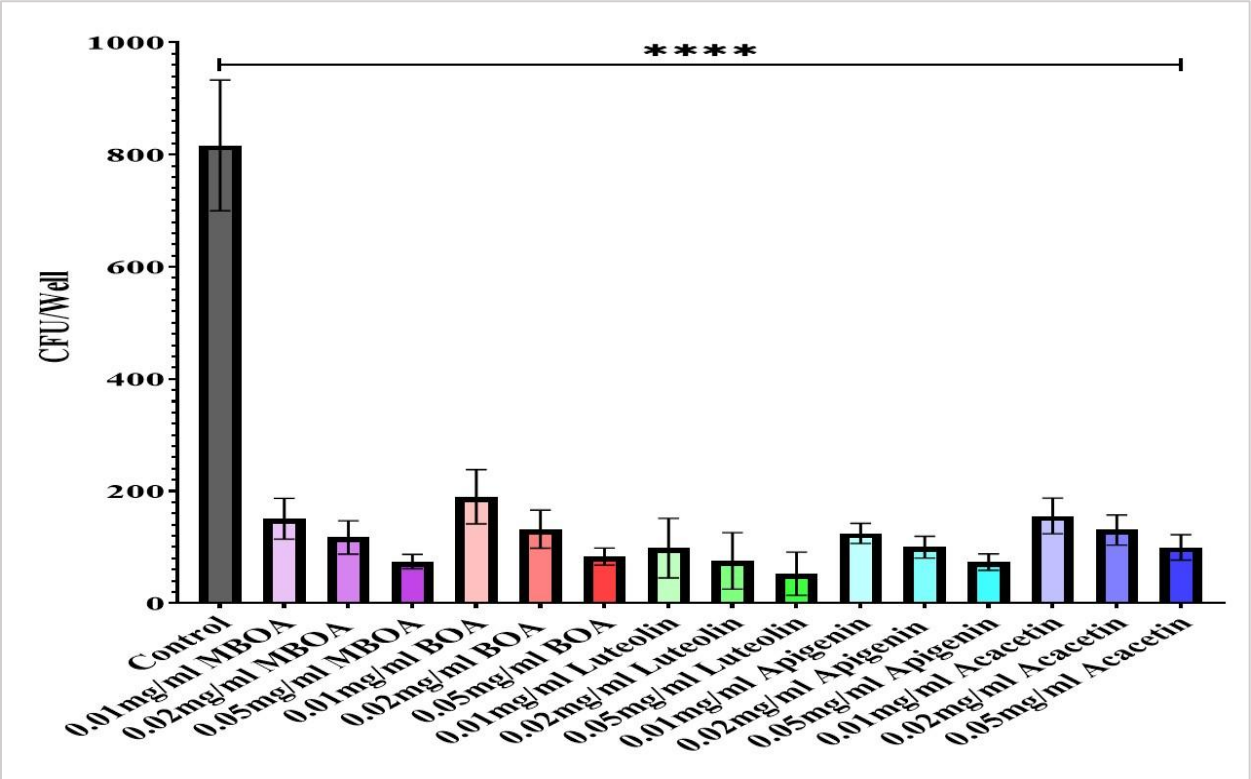


Figure E.12.iv: Graphical representation invaded *S.* Typhi cells in the presence or absence of the phytochemicals' concentrations.

E.13. Computational Docking:

Computational docking is a virtual simulation-based prediction of the binding affinity of small molecules (ligands) to larger molecules (receptors). Computational docking is a great tool for understanding how small molecules, here phytochemicals, bind with specific targets.

In this study, the Dihydrofolate Reductase (DHFR) protein of *S.* Typhi was chosen as a target for the phytochemicals. DHFR is an essential protein in both eukaryotic and prokaryotic cells. It is a crucial enzyme involved in folate metabolism, catalysing a vital reaction required for the de novo synthesis of glycine and purine, as well as DNA precursors, and inhibition of the protein causes cell death. DHFR is a good species-specific target due to its structural differences between different organisms.

E.13.i. Retrieval of SMILES and SDF of the Phytochemicals:

SMILES, or Simplified Molecular-Input Line-Entry System, is a streamlined chemical notation that represents the 3D structure of molecules, facilitating efficient computer processing, whereas SDF, or Structural Data File, is a file format to store or exchange information about chemical structure. The SMILES of selected phytochemicals are presented below, and the 3D structures are shown in **Figure E.13.i**.

Acacetin: COC1=CC=C(C=C1)C2=CC(=O)C3=C(C=C(C=C3O2)O)O

Apigenin: C1=CC(=CC=C1C2=CC(=O)C3=C(C=C(C=C3O2)O)O)O

Lueolin: C1=CC(=C(C=C1C2=CC(=O)C3=C(C=C(C=C3O2)O)O)O)O

MBOA: COC1=CC2=C(C=C1)NC(=O)O2

BOA: C1=CC=C2C(=C1)NC(=O)O2

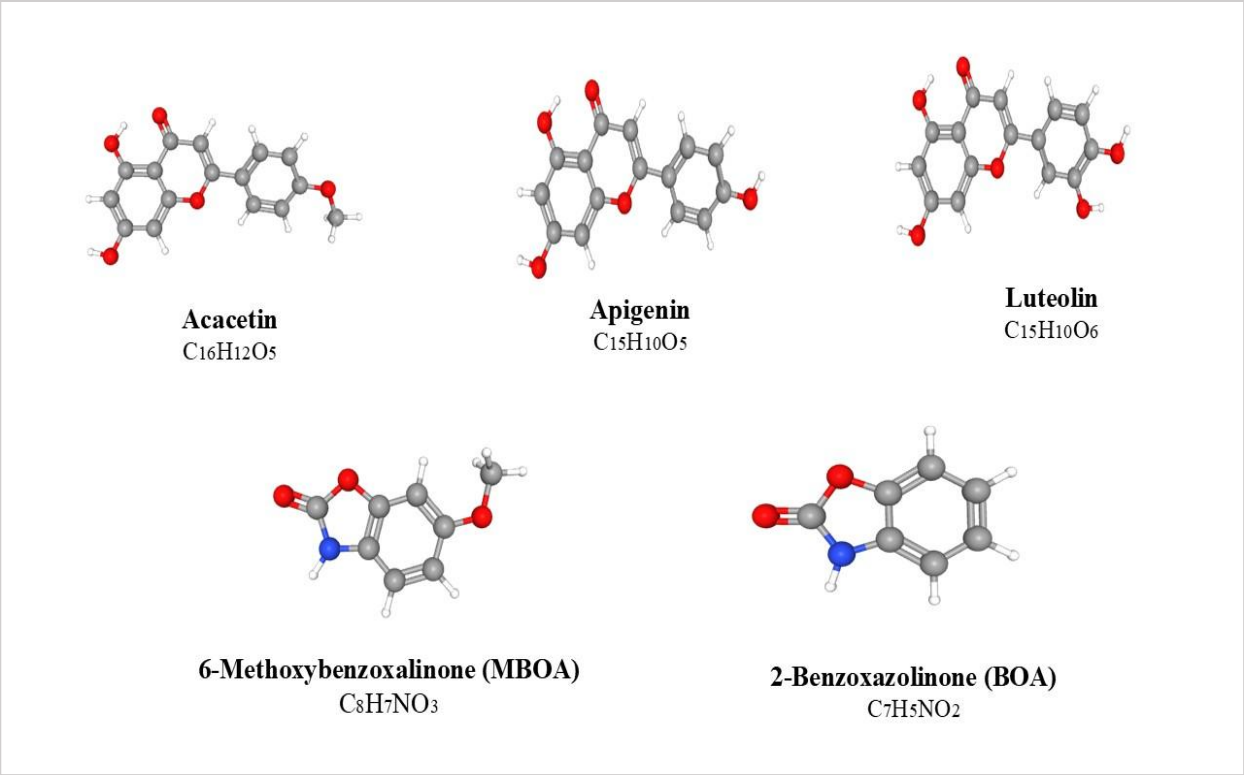


Figure E.13.i: Three-dimensional (3D) structure of selected 5 phytochemicals.

E.13.ii. Retrieval of DHFR Protein Sequence:

The retrieved FASTA sequence of the *Salmonella* Typhi DHFR protein (Q8Z9J9) was 159 amino acids long and had a molecular weight of 17.98 kDa, and when aligned with Human DHFR (P00374), which is 187 amino acids long, the protein had only 28.30% identity (**Figure E.13.ii**). The FASTA sequence of the protein is presented below.

MISLIAALAVDRVIGMENAMPWNLPADLAWFKRNTLNKPVVMGRHTWESIGRPLPGRK
NIIISSQPGTDDRVQWVKSVDEAIAACGDAPEIMVIGGGRVYEQFLPKAQKLYLTHIDAE
VEGDTHFPDYEPDDWESVFSEFHDADAQNSHSYCFEILERR

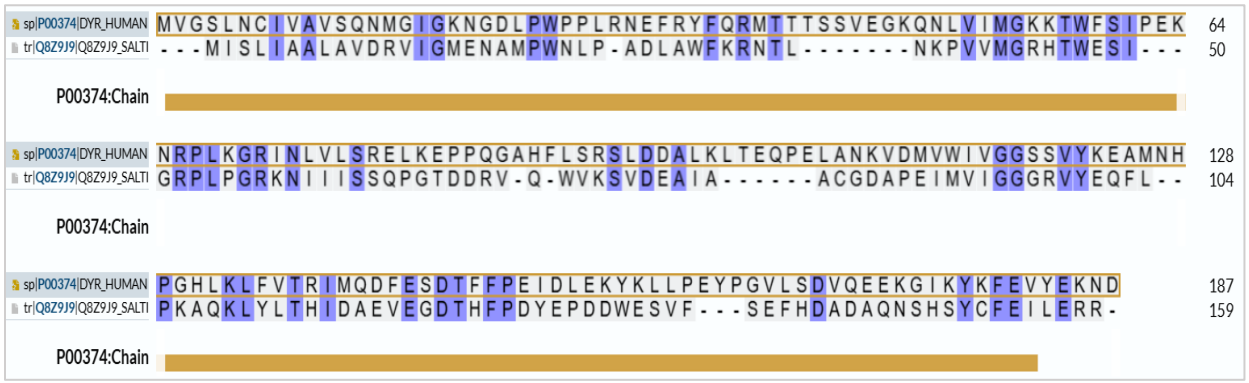


Figure E.13.ii: Sequence alignment of between Human DHFR (P00374) and *Salmonella* Typhi DHFR (Q8Z9J9)

E.13.iii. Generation of DHFR's 3D Structure:

The DHFR's 3D structure was predicted by homology modeling on the SWISS-MODEL web server, where the FASTA sequence of *Salmonella* Typhi DHFR was aligned with a template sequence, here 4nx6.1.A. The *Salmonella* Typhi DHFR had 97.48% sequence identity in a 1.00 coverage value. The PDB file generated 3D model was retrieved and visualized in Pymol software (**Figure E.13.iii.a**). The generated model contains one folic acid molecule as a ligand. In quality assessment, the model showed 0.98 GMQE (Global Model Quality Estimate) and 0.88 ± 0.07 QMEANDisCo Global with a QMEAN Z score of < 1 (**Figure E.13.iii.b**). The GMQE and QMEANDisCo Global are metrics used to evaluate overall model quality, ranging from 0 to 1, where higher values denote better expected quality. Additionally, the QMEAN Z-score serves as an indicator of protein stability (Benkert et al., 2011; Studer et al., 2020). The model's structure was also assessed on the Ramachandran Plot, and it was computed that with a Clash Score of 0.39, 100% residuals fall under Ramachandran Favoured and 2.96% (118 GLU, 148 SER, 119 VAL, 52 ARG) Rotamer outliers (**Figure E.13.iii.b**). Further quality was assessed by ERRAT2 (Colovos

and Yeates, 1993) and got an overall quality factor of 98.630, which was higher than the standard value (**Figure E.13.iii.b**).

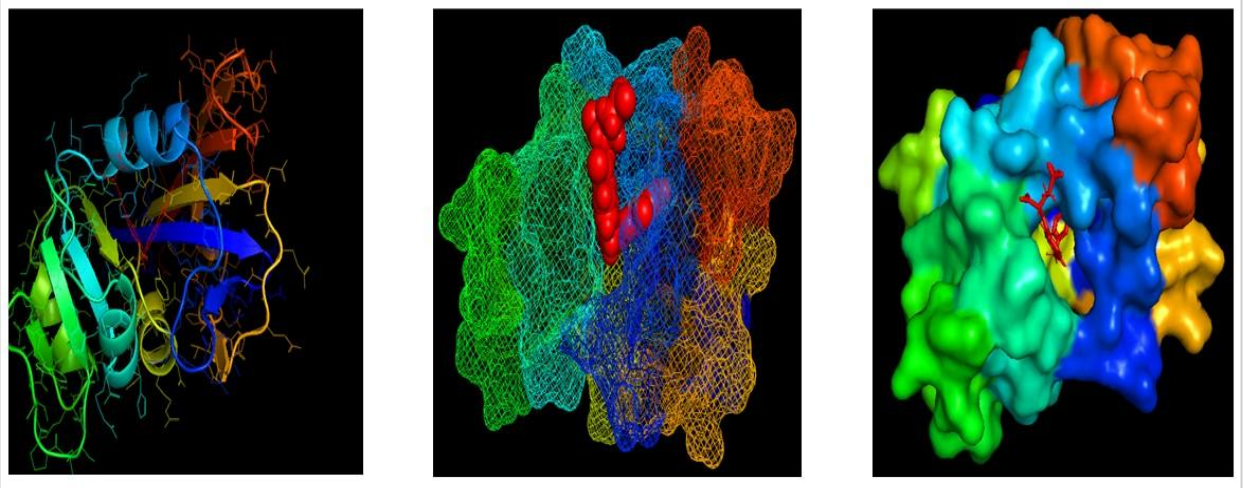


Figure E.13.iii.a: Cartoon, mesh and surface view of generated 3D model of *Salmonella* Typhi DHFR

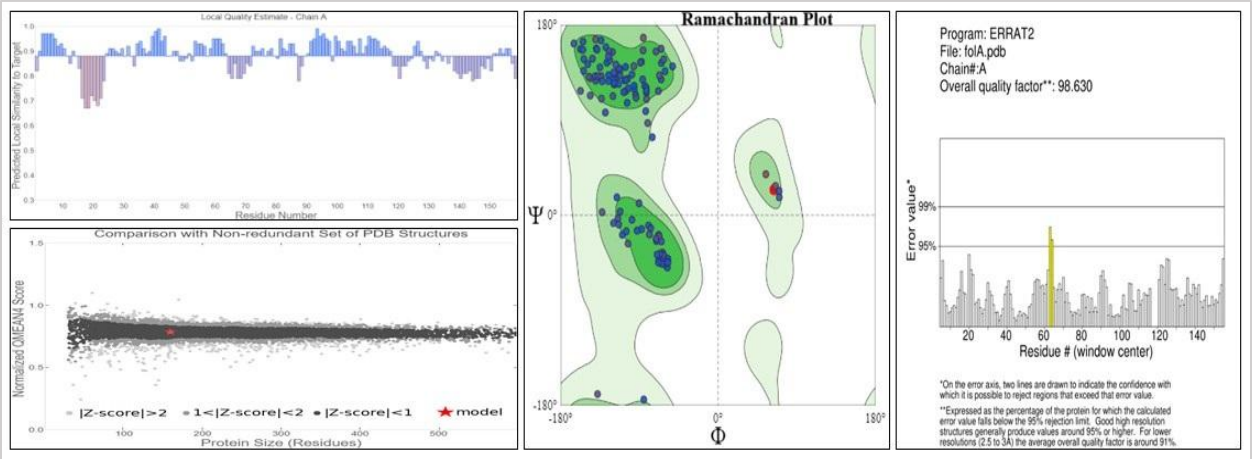


Figure E.13.iii.b: Quality assessment of the generated model

E.13.iv. Protein-Ligand Interaction:

The phytochemicals were docked with *Salmonella* Typhi DHFR to evaluate their binding affinity and interactions (**Figure E.13.iv**). The binding site of folic acid, which was attached to the generated model (see **Section E.13.iii**), was chosen as the binding site for phytochemicals. Out of 5 phytochemicals, luteolin had the highest binding affinity with the protein, with a docking score of -8.35, and interacted with the 7th ALA and 18th ASN amino acids by forming hydrogen bonds. Apigenin had the second-highest binding affinity with a docking score of -7.07 and also formed hydrogen bonds with the same 7th ALA and 18th ASN amino acids. Acacetin had a docking score of -6.91 and interacted with the 27th ASP and 31st PHE amino acids by hydrogen bond and Pi-Pi interaction, respectively. MBOA and BOA had docking scores of -6.01 and -5.75, respectively, and both interacted with the 7th ALA by hydrogen bond.

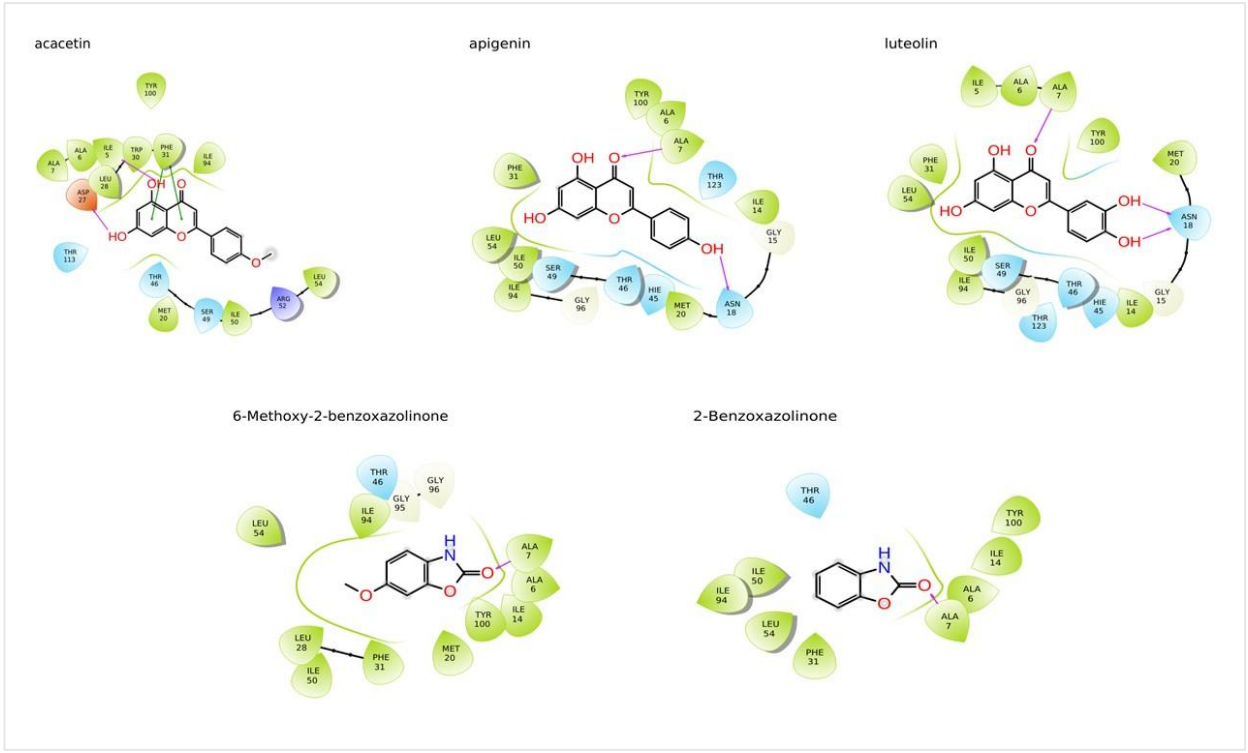


Figure E.13.iv: 2D representation of interactions between amino acids of *Salmonella* Typhi DHFR and the phytochemicals [→ : Hydrogen bond; → Pi-Pi interaction]

E.14. Inhibition of DHFR protein:

E.14.i. Isolation of Genomic DNA of *Salmonella* Typhi:

The isolated genomic DNA had a concentration of 247 ng/µl with an OD_{260}/OD_{280} value of 1.75 and an OD_{260}/OD_{230} value of 2.31.

E.14.ii. Amplification of the dhfr Gene by PCR:

The *dhfr* gene is 480 bp long, and the incorporated restriction digestion sites of NdeI and XhoI restriction enzymes on each end were 14 bp and 9 bp, respectively. The final PCR amplicon with restriction digestion sites was 503 bp. The PCR-amplified *dhfr* genes were visualized by gel electrophoresis, where in lanes 2 and 3 (replicates) DNA bands appeared at the 500 bp position, which indicates successful amplification of the *dhfr* gene.

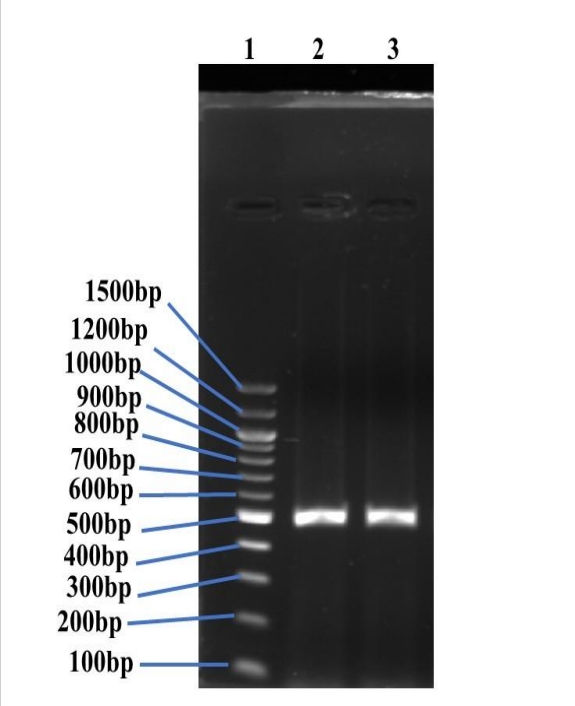


Figure E.14.ii: DNA band of PCR amplified *dhfr* gene (503 bp) on agarose

E.14.iii. Purification of PCR Product:

The successful PCR amplified *dhfr* genes were purified, pulled together and then quantified. The purified gene had a concentration of 142.84 ng/µl with an OD_{260}/OD_{280} value of 1.82 and an OD_{260}/OD_{230} value of 2.44.

E.14.iv. Isolation of Vector Plasmid:

The isolated pET-20b (+) plasmid on quantification showed a concentration of 400 ng/µl with an OD_{260}/OD_{280} value of 1.89 and an OD_{260}/OD_{230} value of 2.19. The gel image of the plasmid is shown in Figure E.14.iv.

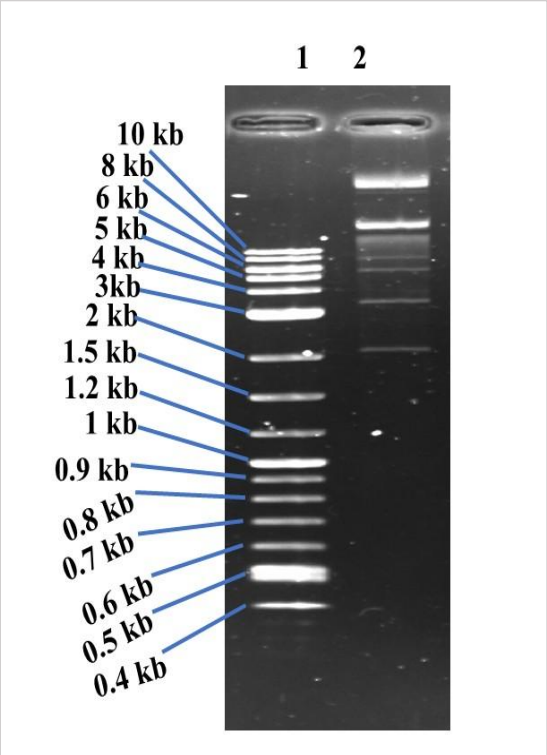


Figure E.14.iv: Band image of pET-20b (+) plasmid on agarose gel

E.14.v. Enzymatic Restriction Digestion of *dhfr* Gene and pET-20b (+) Plasmid:

Both the *dhfr* gene and pET-20b (+) plasmid were successfully digested by NdeI and XhoI. The digested pET-20b (+) plasmid is about 3.7 kb long (**Figure E.14.v**), similar to the expected length. The digested *dhfr* was untraceable on agarose gel imaging, so on successful pET-20b (+) plasmid digestion, it was assumed that successful digestion of *dhfr* gene.

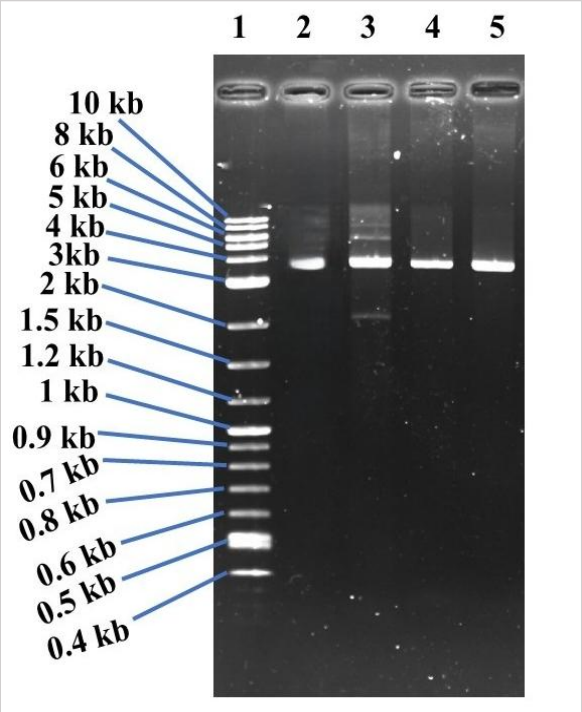


Figure E.14.v: Band image of digested pET-20b (+) plasmids (~3.7 kb) on agarose

E.14.vii. Purification of Digested dhfr Gene and pET-20b (+) Plasmid:

The digested *dhfr* and pET-20b (+) were purified by gel extraction. The purified digested *dhfr* had a concentration of 25 ng/µl with an OD_{260}/OD_{280} value of 1.76 and an OD_{260}/OD_{230} value of 1.99 whereas the purified digested pET-20b (+) had a concentration of 125 ng/µl with an OD_{260}/OD_{280} value of 1.81 and an OD_{260}/OD_{230} value of 2.08.

E.14.viii. Ligation and Transformation:

The NdeI and XhoI digested *dhfr* was inserted into the NdeI and XhoI digested pET-20b (+) plasmid by ligation reaction, and then the hybrid plasmid was transferred to competent *E. coli* cells. The *E. coli* cells were then subcultured on LB_{Amp} agar plate. The pET-20b (+) plasmid carries a resistance gene for ampicillin; hence, only the cells carrying the pET-20b (+) plasmid can grow on LB_{Amp} agar plates. After the subculture of *E. coli* cells on LB_{Amp} agar plate, 2×10^2 CFU/ml transformed cell colonies appeared.

E.14.ix. Confirmation by PCR and Plasmid DNA Mapping Using Restriction Enzymes:

The colonies of *E. coli* cells from LB_{Amp} agar plate were collected, and the plasmid was isolated. The successful insertion of *dhfr* gene into the pET-20b (+) plasmid was confirmed by PCR amplification of the gene (**Figure E.14.ixa**). The successful amplification of *dhfr* by using pET-20b (+) plasmid as a template suggests the successful insertion of *dhfr* gene into the pET-20b (+) plasmid. The insertion was also confirmed by DNA mapping, where the isolated plasmid was digested by NdeI and XhoI. The appearance of two separate DNA bands of *dhfr* (480 bp) and pET-20b (~3.7 kb) indicated the successful insertion of *dhfr* gene into the pET-20b (+) plasmid (**Figure E.14.ixb**).

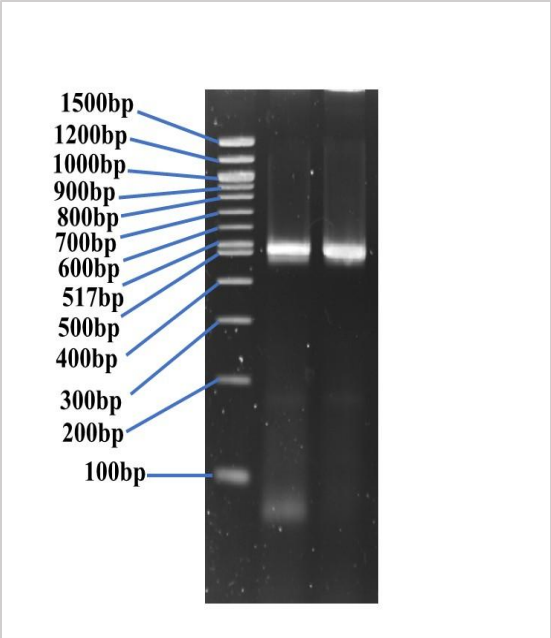


Figure E.14.ix.a: Band image of *dhfr* (503bp) on agarose gel

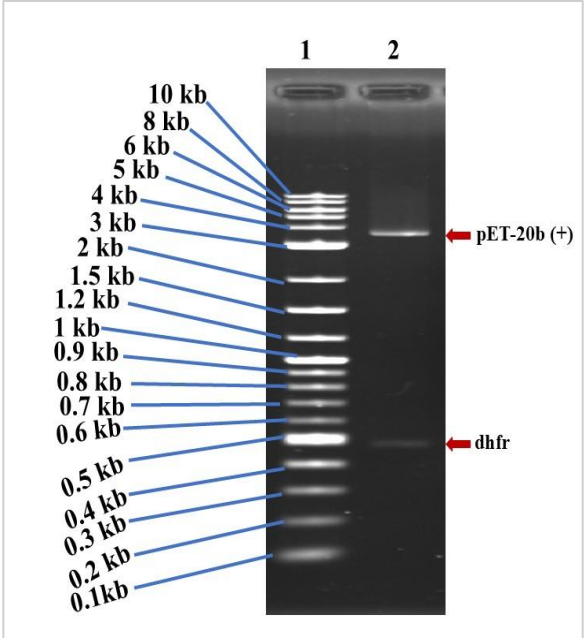


Figure E.14.ix.b: Band image of *dhfr* (480bp) and pET-20b (~3.7kb) agarose

E.14.x. DHFR protein Expression and Purification:

The hybrid pET-20b-dhfr plasmid was transferred to an *E. coli* BL21 competent cell, where the DHFR protein was overexpressed and purified. The purified DHFR protein was quantified and analyzed by SDS-PAGE. On quantification, the protein showed a concentration of 291 µg/ml, and on SDS-PAGE, a band appeared below the 18 kDa position (**Figure E.14.x**), which confirms the successful purification of DHFR protein (17.98 kDa).

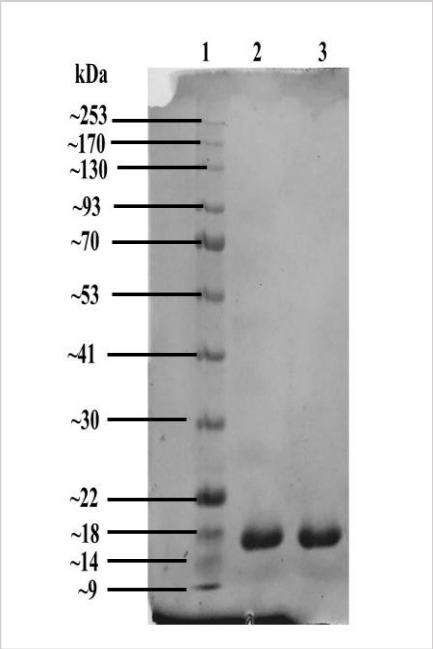


Figure E.14.x: Band image of purified DHFR protein (~17.98 kDa)

E.14.xi. Enzyme Assay:

The purified *Salmonella* Typhi DHFR was used in the enzyme assay, where its activity, along with the activity of Human DHFR, was evaluated under the influence of the five selected phytochemicals. All the tested phytochemicals significantly reduced the activity of *Salmonella* Typhi DHFR with a dose-dependent effect (**Figure D.14.xi**). This reduction in activity level indicates the inhibition of the protein by the phytochemicals. The 3 phytochemicals, *i.e.* BOA, MBOA and luteolin, had comparatively greater inhibition than acacetin and apigenin. In further comparison, under the cytotoxic concentration (see **Section E.11**), luteolin had a better effect than the others, followed by MBOA. The activity of Human DHFR was also checked under the influence of the phytochemical, but no significant decrease was observed (**Figure D.14.xi**), indicating no inhibition.

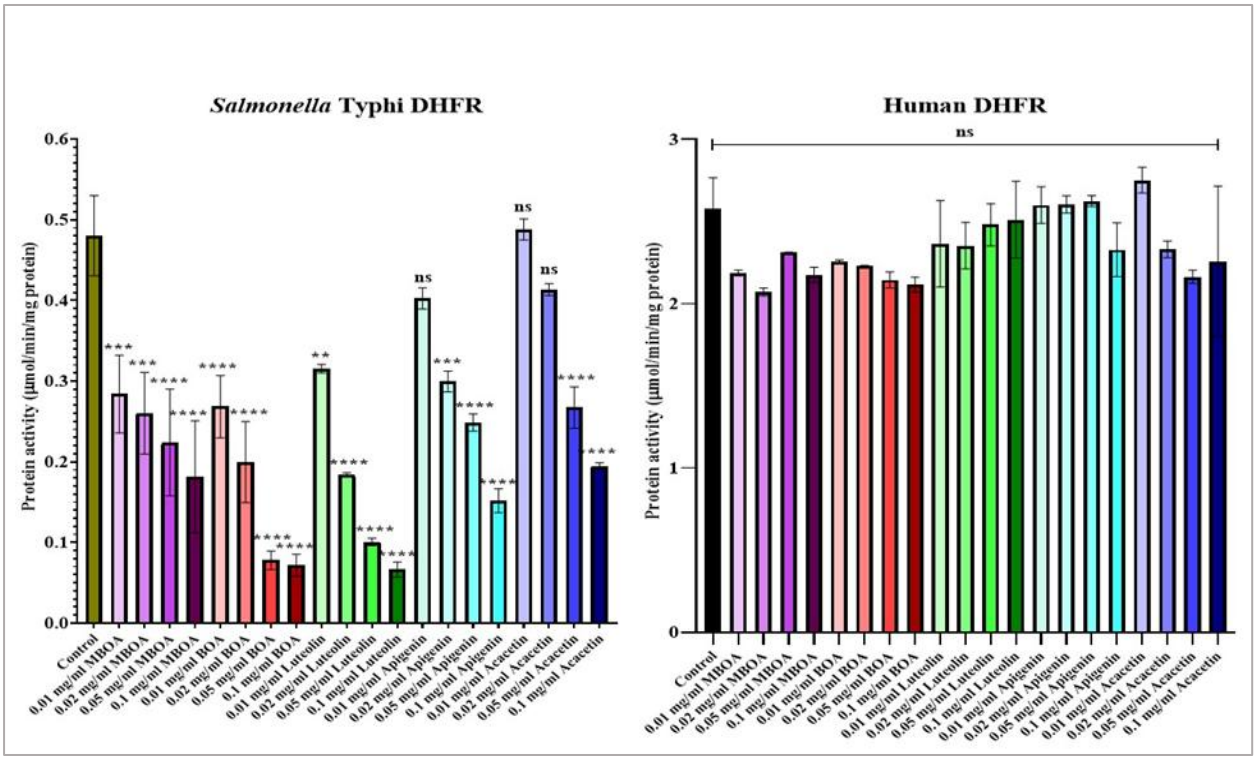


Figure E.14.xi: Graphical representation of activity of *Salmonella* Typhi DHFR and Human DHFR in the presence or absence of phytochemical concentrations

E.15. Determination of *in vivo* Therapeutic Potential of Selected Phytochemicals:

The therapeutic potential of selected 5 phytochemicals was evaluated in *in vivo* mice model where group of animals were infected with *S.* Typhimurium to develop typhoid-like condition (non-typhoidal salmonellosis) and different doses of phytochemicals were orally administered for five days. During course of the treatment 100% of animals from phytochemical treated groups survived, in chloramphenicol treated group survival percentage drop to 83% whereas only 50% animals survived in non-treated group (infection control) (**Figure E.15.a**). Bacterial shedding through stool was also observed, and found that in MBOA and BOA treated groups, there were no significant change in bacterial shedding from that of non-treated group as similar in chloramphenicol treated group. However, there was significantly less bacterial shedding in the

luteolin, apigenin, and acacetin treated groups, and a gradual increase was observed with passing days (**Figure E.15.b**). In terms of bacterial colonization in the intestine and liver, it was observed that there was significantly less colonization in the treatment groups and a dose dependent effect was observed in all group except for BOA where lower dose showed better efficacy (**Figure E.15.c**). The groups treated with the high dose of apigenin and luteolin had the lowest bacterial colonization in the intestine, almost similar to that of the chloramphenicol treated group. In case of colonization in the liver, all 3 doses of MBOA had the lowest colonization, lower than chloramphenicol treated group, whereas the high dose of luteolin and apigenin and the high and middle doses of acacetin had almost similar load of colonization to that of chloramphenicol treated group. No bacterial growth in blood was observed in any of the groups, including the non-treated group.

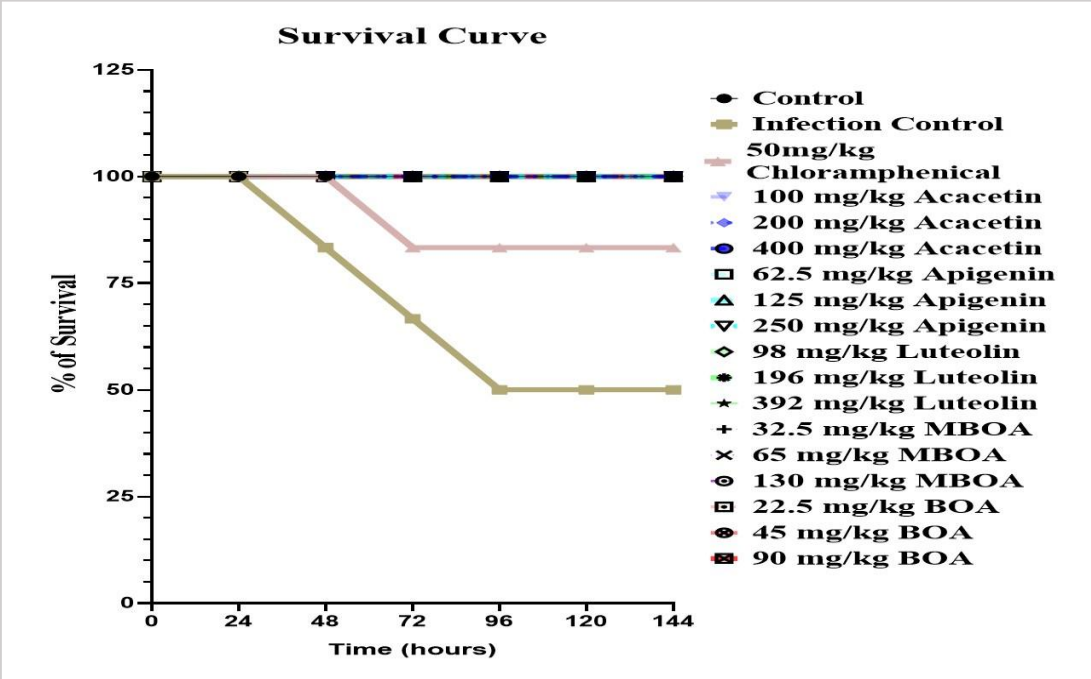


Figure E.15.a: Graphical representation of % of survival in the treated and non-treated animal groups.

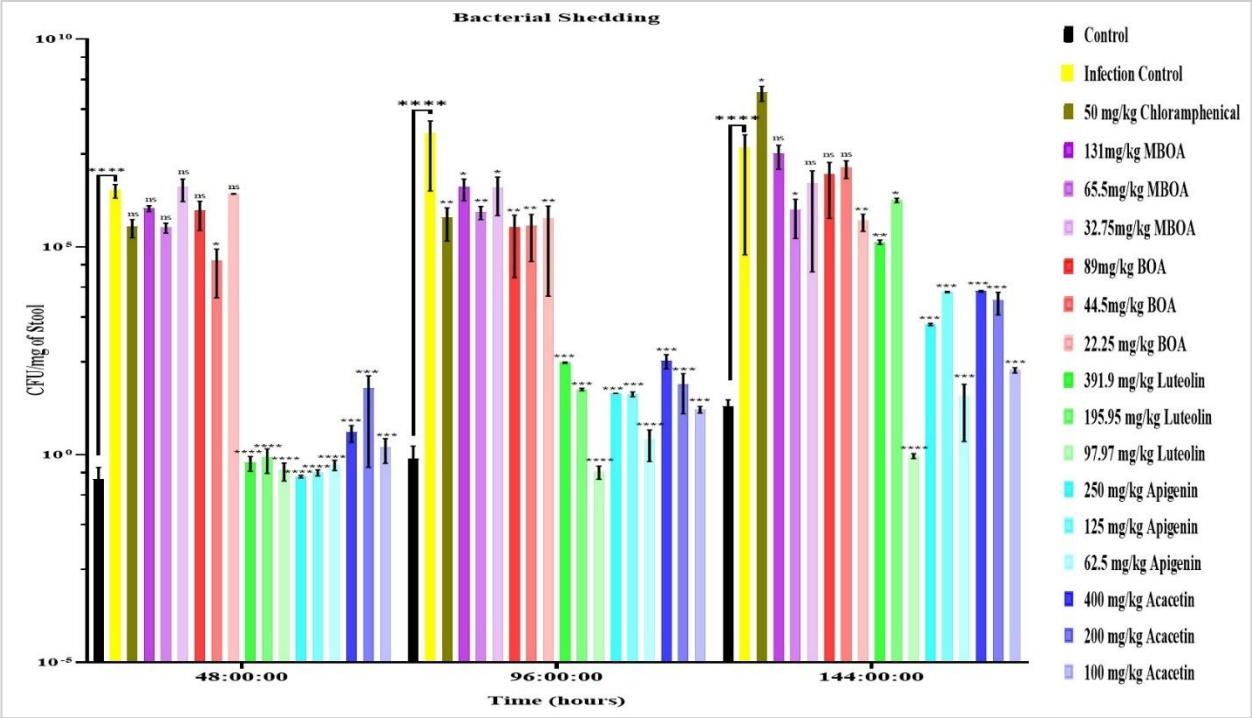


Figure E.15.b: Graphical representation of bacterial shedding through stool during courses of treatment.

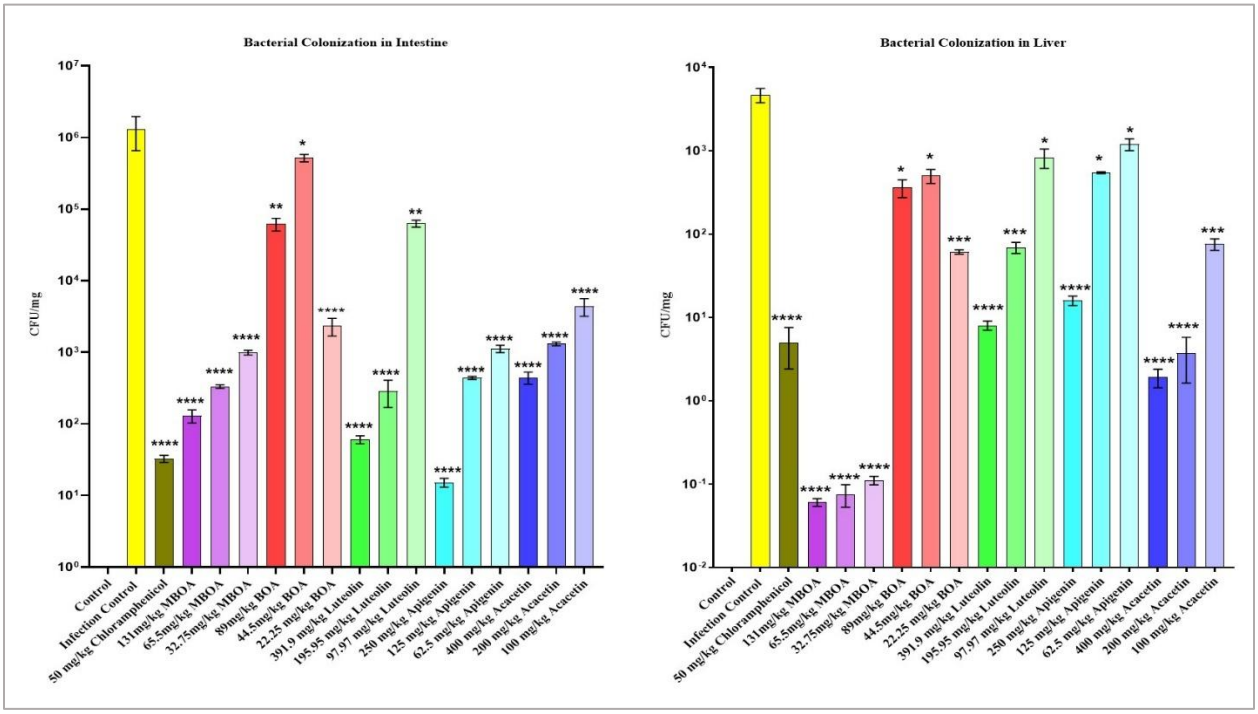


Figure E.15.c: Graphical representation of bacterial colonization in the intestine (left) and liver (right).

F. Highlight and Conclusion:

- Leaves of *S. occidentalis* and roots of *S. dulcis* were traditionally used by the Indian tribal communities to treat typhoid symptoms.
- The methanolic leaf extract of *S. occidentalis* and the methanolic root extract of *S. dulcis,* which were rich in secondary metabolites like flavonoids, alkaloids, terpenoids, tannins, *etc*.
- In comparison, the methanolic root extract of *S. dulcis* had higher efficacy with MIC of ≤ 7.5 (>3.75) mg/ml than the methanolic leaf extract of *S. occidentalis,* which did not show any inhibition of growth in the TLC-autobiography test.
- Both crude extracts were fractionated by column chromatography and tested against *S.* Typhi isolates.
- Out of forty-two collected fractions, five that were fractionated from the methanolic root extract of *S. dulcis* showed an improved activity against *S.* Typhi isolates.
- A total of forty-three phytochemicals were identified from the selected five fractions in phytochemical profiling through LC-MS; most of which were flavonoids (14) and terpenoids (13), a few were benzoxazinoids (5), with others.
- All the identified phytochemicals were computationally screened to study their physicochemical properties and predict their drug likeness and toxicity.
- Thirty-five of them showed druglikeness, and most of them (27) fall under the toxicity class V.
- Out of those identified phytochemicals, three flavonoids *i.e.,* acacetin, apigenin and luteolin and two benzoxazinoids *i.e.*, 6-methoxybenzoxazolinone (MBOA) and benzoxazolinone (BOA) were chosen to dive deeper into their effectiveness against *S.* Typhi.
- All five chosen phytochemicals possess drug-like properties. The three flavonoids fall under the toxicity class V, whereas the two benzoxazinoids fall under the toxicity class IV.

- In the cytotoxicity test, luteolin and 6-methoxybenzoxazolinone showed comparatively less toxicity than the other three.
- In the activity against *S.* Typhi isolates, luteolin showed the lowest MIC of $\leq$ 0.03 (> 0.01) mg/ml, followed by acacetin and apigenin, whereas 6-methoxybenzoxazolinone and Benzoxazolinone had the MIC of $\leq$ 1 (> 0.5) mg/ml against most of the test isolates.
- All the phytochemicals at their $\geq$ MICs caused a decrease in growth rate and inhibited biofilm formation.
- The effect of flavonoids was bacteriostatic, whereas it was bactericidal for the benzoxazinoids.
- All five phytochemicals showed effective inhibition of the invasion of *S.* Typhi into the macrophage cell.
- The phytochemicals were virtually docked with *S.* Typhi DHFR protein, which is an essential protein for DNA synthesis, where luteolin showed the highest binding affinity, followed by apigenin and acacetin.
- In *in vitro* enzyme assay, all the phytochemicals significantly inhibited the activity of *S.* Typhi DHFR protein, but no inhibition was observed in human DHFR by the influence of the phytochemicals.
- The phytochemicals had also shown a significant inhibition in the colonization of infection in *S.* Typhimurium challenged mice and showed 100% survival in mice treated with phytochemicals compared to 50% in the non-treated group.

Both the plant extracts showed inhibitory effects against *S.* Typhi, which validated their traditional use against the pathogen, but comparatively, the root extract of *S. dulcis* showed higher efficacy, whereas the leaf extract of *S. occidentalis* was effective at higher concentrations. The study with chosen phytochemicals suggested that the flavonoids, particularly luteolin, had more effectiveness

than the chosen benzoxazinoids against the drug-resistant *S.* Typhi isolates. In the *S.* Typhimurium induced non-typhoidal salmonellosis mouse model, all the chosen phytochemicals showed therapeutic potential, but the two benzoxazinoids were effective at low doses. This observation suggests that the root of *S. dulcis* can be a herbal remedy for *S.* Typhi infection and a source for new antibacterial agents, and those five studied phytochemicals, acacetin, apigenin, luteolin, 6-methoxybenzoxazolinone and benzoxazolinone possess great potential to become anti-typhoid drugs and may lead to further clinical studies.

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I. Supplements:

Table I.1: The antimicrobial-resistant profiles (CLSI, 2018) of collected clinical isolates of *S.* Typhi and *S.* Typhimurium

Serial no.	Strain ID	Date of isolation	Ampicillin (A)	Chloramphenicol (C)	Tetracycline (T)	Trimethoprim-Sulfamethoxazole (Q)	Nalidixic acid (Na)	Norfloxacin (Nor)	Ciprofloxacin (Cip)	Ofloxacin (Ofx)	Levofloxacin (Lvx)	Amikacin (An)	Gentamicin (G)	Streptomycin (S)	Cefotaxime (Ctx)	Anthramycin (Atm)	Ceftriaxone (Cro)	Ceftazidime (Caz)	Azithromycin (Azm)	Amoxicillin-Clavulanic acid (Ac)	Gemifloxacin (GAT)	Cefixime (Cfm)	Pefloxacin (Pfx)
1	KOL5	15.5.12	S	S	S	S	R	R	R	R	I	S	I	S	S	S	S	S	R	S	I	_	-
2	KOL18	1.6.12	R	R	S	R	R	I	I	S	S	S	S	R	S	S	S	S	I	R	S	_	_
3	KOL31	17.9.12	R	R	S	R	R	S	R	R	I	S	S	R	S	S	S	S	I	R	S	_	_
4	KOL42	19.2.13	S	R	S	R	R	I	I	S	S	S	S	I	S	S	S	S	S	S	S	_	_
5	KOL89	17.9.13	S	S	S	S	R	R	R	R	R	S	S	I	S	S	S	S	S	S	R	_	_
6	KOL112	9.11.13	S	S	S	S	R	R	R	R	R	S	S	I	S	S	S	S	S	S	R	_	_
7	KOL148	24.4.14	S	S	S	S	R	R	R	R	R	S	S	I	S	S	S	S	S	S	R	_	_
8	KOL162	16.5.14	S	S	R	R	R	R	R	R	R	S	S	R	S	S	S	S	S	S	R	_	_
9	KOL180	24.7.14	S	S	S	S	R	_	I	I	I	S	S	I	S	S	S	S	S	S		S	_
10	KOL244	1.5.15	S	S	S	S	R	_	R	R	R	S	S	S	S	_	S	S	S	_	S	S	_
11	KOL288	3.9.15	S	S	S	S	R	_	R	R	R	_	S	S	_	_	S	S	S	_		S	_
12	KOL301	8.9.15	S	S	S	S	R	_	R	R	R	_	_	S	_	_	S	S	S	_	S	S	_
13	KOL343	18.1.16	S	S	S	S	R	_	R	R	R	S	S	I	S	_	S	S	S	_	S	S	_
14	KOL557	18.9.18	S	S	S	_	R	_	R	R	R	S	S	_	S	_	S	S	S	S	_	_	R
15	KOL558	27.9.18	S	S	S	_	R	_	I	I	I	S	S	_	S	_	S	S	S	S	_	_	R
16	OSS535	18.3.19	R	S	R	_	R	R	R	_	_	R	S	S	R	R	R	R	R	S	_	_	_

[*S: Sensitive; R: Resistant; I: Intermediate; - : Not tested*]

Table I.2: Interaction scores of phytochemicals (ligand) with DHFR protein

Rank	Ligand	Score	GScore	Lipo	HBond	Metal	Rewards	vdW	Coul	RotB	Site	Emodel	CvdW	Intern	Configu	Pose
1	Luteolin	-8.35	-8.35	--	-2.2	--	--	-37.9	-9.6	--	--	-64.0	-47.5	0.6	1	1
2	Apigenin	-7.07	-7.07	--	-1.3	--	--	-38.8	-5.7	--	--	-61.6	-44.6	0.1	1	1
3	Acacetin	-6.91	-6.91	--	-1.3	--	--	-33.6	-3.2	--	--	-51.3	-36.9	0.5	1	3
4	2-Benzoxazolinone	-6.01	-6.01	--	-0.7	--	--	-21.4	-1.4	--	--	-27.6	-22.8	0.0	1	14
5	6-Methoxy-2-benzoxazolinone	-5.75	-5.75	--	-0.2	--	--	-24.2	-1.0	--	--	-28.9	-25.2	3.7	1	7

GScore = a * vdW + b * Coul + Lipo + Hbond + Metal + Rewards + RotB + Site,
where:
vdW = van der Waals interaction energy
Coul = Coulomb interaction energy
Lipo = Lipophilic-contact plus phobic-attractive term
HBond = Hydrogen-bonding term
Metal = Metal-binding term (usually a reward)
Rewards = Various reward or penalty terms
RotB = Penalty for freezing rotatable bonds
Site = Polar interactions in the active site,
and the coefficients of vdW and Coul are:
a = 0.050, b = 0.150 for Glide 5.0 (the contribution from the Coulomb term is capped at -4 kcal/mol)
a = 0.063, b = 0.120 for Glide 4.5 (the contribution from the Coulomb term is capped at -4 kcal/mol)
a = 0.080, b = 0.100 for Glide 4.0

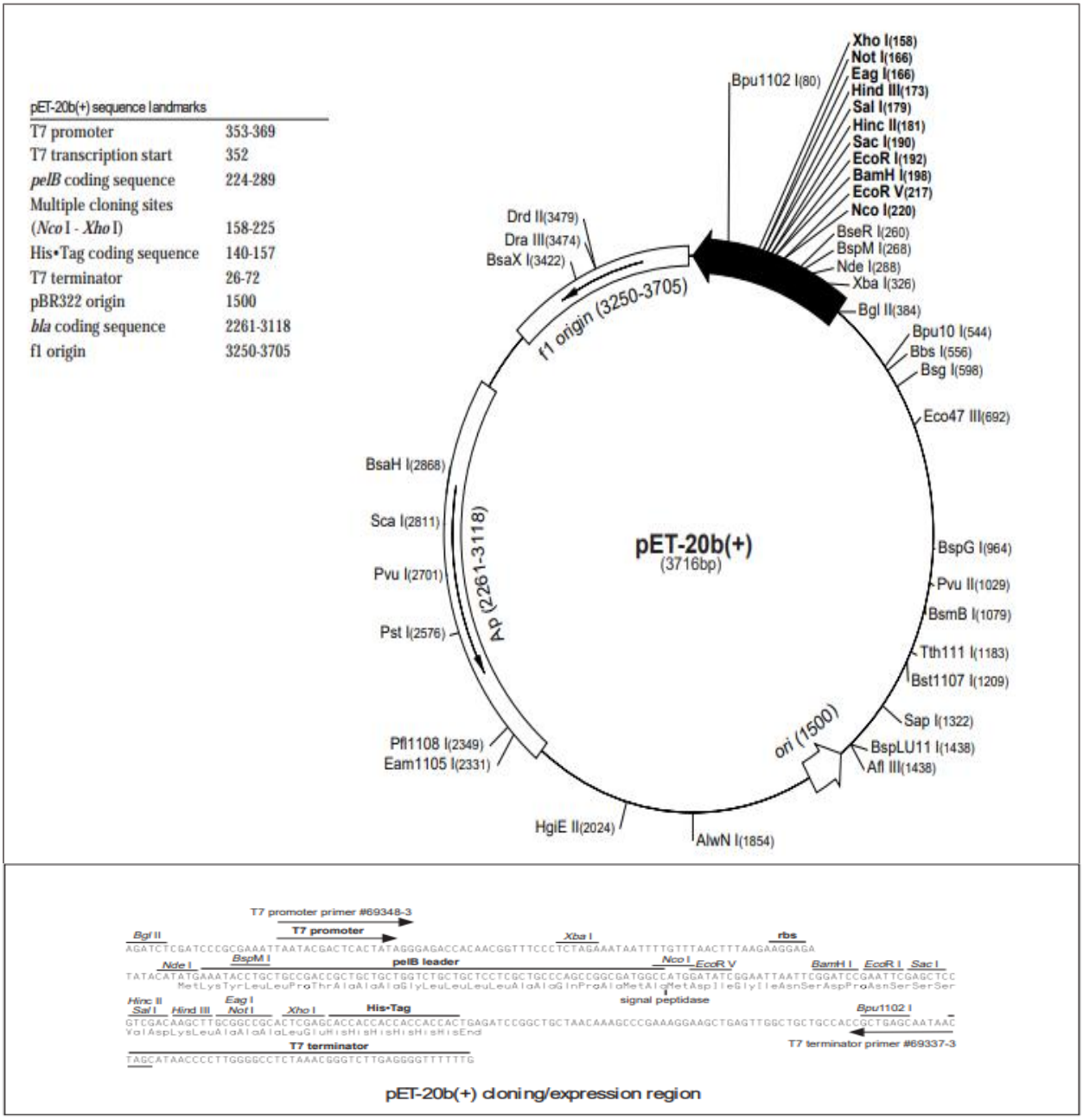

Figure I: p ET-20b(+) Vector plasmid map

J. Appendix:

J.1. List and composition of different culture media used in the study:

J.1.i. Mac Conkey Agar (MAC) (Difco, USA):

Peptone: 17.0g
Proteose peptone: 3.0g
Bile salt: 1.5g
Sodium chloride: 5.0g
Neutral red: 0.03g
Crystal violet: 0.001g
Agar: 13.5g
pH: 7.1±0.2 at 25°C

50g of dehydrated agar was suspended in 1 litre of sterile double-distilled water and boiled to dissolve. It is autoclaved at 1210 C for 15 minutes. After autoclaving media was distributed in sterile disposable petri plates under laminar flow.

J.1.ii. Xylose lysine deoxycholate (XLD) agar (Difco, USA):

Yest extract: 3.0g
Sodium chloride: 5.0g
Xylose: 3.75g
Lactose: 7.50g
Sucrose: 7.50g
L-lysine hydrogen chloride: 5.0g
Sodium thiosulphate: 6.8g
Ferric ammonium citrate: 0.8g
Phenol red: 0.08g
Sodium deoxycholate: 1.0g
Agar: 15.0g
pH: 7.4±0.2 at 25°C

55 g of dehydrated agar was suspended in 1 litre of sterile double-distilled water, mixed thoroughly, and heated with agitation until the medium boiled. Do not overheat nor autoclave. The medium was distributed in sterile disposable petri plates under laminar flow.

J.1.iii. Nutrient agar (NA) (Difco, USA):

Beef extract: 3.0g
Peptone: 5.0g
Agar: 15.0g
pH: 6.8±0.2 at 25°C

23g of dehydrated medium was dissolved in 1000 mL of sterile double-distilled water by heating and autoclaved at 121oC for 15 min. The medium was distributed in sterile disposable petri plates under laminar flow.

J.1.iv: Mueller-Hinton agar (MHA) (Merck, Germany):

Beef extract: 2.0g
Acid digest of casein: 17.5g
Starch: 1.5g
Agar: 17.0g
pH: 7.3±0.1 at 25°C

38g of dehydrated medium was dissolved in the 1000ml of sterile double-distilled water by heating and autoclaved at 121oC for 15 min. The medium was distributed in sterile disposable petriplates under laminar flow.

J.1.v: Mueller-Hinton broth (MHB) (Merck, Germany):

Beef extract: 2.0g
Acid digest of casein: 17.5g
Starch: 1.5g
pH: 7.3±0.1 at 25°C

21g of dehydrated medium was dissolved in 1000 mL of sterile double-distilled water by heating and autoclaved at 121oC for 15 min. 3 ml of the medium was dispensed in sterile test tubes under laminar flow.

J.1.vi: Luria-Bertani (LB) broth (Difco, USA):

Tryptone: 10.0g
Yeast extract: 5.0g
Sodium chloride: 10.0g
pH: 7.0±0.2 at 25°C

25g of dehydrated medium was dissolved in 1000 mL of sterile double-distilled water by heating and autoclaved at 121oC for 15 min. 3 ml of the medium was dispensed in sterile test tubes under laminar flow.

J.1.vii: Trypticase soy broth (TSB) (Difco, USA):

Pancreatic digest of casein: 17.0g
Papaic digest of soybean: 3.0g
Dextrose: 2.5g
Sodium chloride: 5.0g
Dipotassium phosphate: 2.5g
pH: 7.3±0.2 at 25°C

30g of dehydrated medium was dissolved in 1000 mL of sterile double-distilled water by heating and autoclaved at 121oC for 15 min. 3 ml of the medium was dispensed in sterile test tubes under laminar flow.
Lactose 12.0g

J.1.viii: Triple Iron sugar agar (TSI) (Difco, USA):

Beef extract: 3.0g
Yeast extract: 3.0g
Pancreatic digest of casein: 15.0g
Proteose peptone no.: 3 5.0g
Sodium chloride: 5.0g

Lactose: 10.0g

Sucrose: 10.0g

Dextrose: 1.0g

Ferrous sulphate: 0.2g

Sodium thiosulphate: 0.3g

Phenol red: 0.024g

Agar: 12.0g

pH: 7.4±0.2 at 25°C

65g of dehydrated medium was dissolved in 1000 mL of sterile double-distilled water by heating and autoclaved at 121oC for 15 min. 3 ml of the medium was dispensed in sterile test tubes under laminar flow and allowed to cool in slanted position so that deep butt is formed.

J.1.ix: Salmonella-Shigella (SS) agar (BD, USA):

Beef Extract: 5.0 g

Pancreatic Digest of Casein: 2.5 g

Peptic Digest of Animal Tissue: 2.5 g

Lactose: 10.0 g

Bile Salts Mixture: 8.5 g

Sodium Thiosulfate: 8.5 g

Ferric Citrate: 1.0 g

Neutral Red: 0.025

Agar: 13.5 g

Brilliant Green: 0.330 g

pH: 7.0 ± 0.2 at 25°C

Suspend 60g of the powder in 1000 ml of purified water. Mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder. do not autoclave. Cool to 45 to 50 °C and use immediately. Allow the plates to dry for about 2 h.

J.2. List and Composition of Different Buffer Used in The Study:

J.2.i.1X standard PCR buffer:

100mM Tris-HCl

5mM MgCl2

500mM KCl

pH 8.3

J.2.ii. Phosphate Buffer Solution (PBS):

1 PBS tablet was dissolved in 200 mL of distilled water and autoclaved

J.2.iii. 1X TBE electrophoresis buffer (pH 8.0):

First, 10X stock solution was made by adding

Tris base: 54.0g

Boric acid: 27.5g

0.5M EDTA (pH-8.0): 20ml

Milli-Q water: Volume made up to 500ml

1X working buffer was made by adding 100ml 10X TBE to 900ml Milli-Q water

J.3. List of Reagents:

Table J.3: List of Reagent used in the study

Reagents	**Manufacturers**
Acetic acid, glacial	SRL, India
Agarose	Sigma-Aldrich, USA
Antibiotic disk	BD, USA
Antibiotic powder	Sigma-Aldrich, USA
Boric acid	Sigma-Aldrich, USA
Bromothymol blue	Sigma-Aldrich, USA
Restriction enzyme	New England Biolabs, USA
DNA ladder (100bp, 500bp)	New England Biolabs, USA

DNA taq polymerase	New England Biolabs, USA
dNTPs	New England Biolabs, USA
Ethanol (100% pure)	Merck, Darmstadt, Germany
Ethidium bromide Roche	Applied Sciences, Germany
Ethylenedinitrotetraacetic acid (EDTA)	Sigma-Aldrich, USA
Ferric chloride	HiMedia, India
Formaldehyde (37%)	Sigma-Aldrich, USA
Glycerol (≥99%)	Sigma-Aldrich, USA
Hydrochloric Acid, HCl (32%)	SRL, India
Kovac's reagent	HiMedia, India
McFarland (0.5) N-Lauroyl Sarcosine	HiMedia, India
PCR primers	IDT, USA
Phosphate Buffered Saline (PBS) tablets	Sigma-Aldrich, USA
Sodium acetate, 3M, pH 5	Amresco, USA
Sodium azide	Sigma-Aldrich, USA
Sodium chloride	SRL, India
Sodium hydroxide	SRL, India
Sodiumdodecyl sulfate (SDS) Thio urea	Sigma-Aldrich, USA
Tris base	Sigma-Aldrich, USA

J.4. List of Consumables

Table J.3: List of Consumables used in the study

Consumables	**Manufacturers**
Beakers (glass)	Riviera, India
Beakers (plastic)	Tarsons, India
Cragie's tube	Riviera, India
Conical flasks (glass)	Riviera, India
Cryovials	Tarsons, India
Cryo 1°C cooler	Tarsons, India
Cryovial racks	Tarsons, India

Cuvettes (disposable)	Eppendorf, Germany
Filter paper	Whatman
Nichrome loop and straight wires	HiMedia, India
Measuring cylinders	Riviera, India
McCartney bottles	Borosil, India
Microcentrifuge Tubes	Tarsons, India
Micropipette plastic tips	Tarsons, India
Micropipettes	Eppendorf, Germany
Microscope glass slides	LOMB Scientific Co., Australia
Pasteur pipettes	Roth, Karlsruhe
Parafilms	Tarsons, India
Petriplates	HiMedia, India
Sterile cotton swab sticks	HiMedia, India
Sterile filters	Millipore, Germany
Syringe	BD, USA
Test tubes (glass)	Riviera, India
Test tube racks	Tarsons, India

J.5. List of laboratory Equipment and Instruments

Equipments	**Manufacturers**	**Model**
Autoclave	Tomy, Germany	ES-215
Biophotometer	Eppendorf, Germany	Biophotometer plus
Biosafety Cabinet Class II	ESCO, Singapore	AC-2, 3-F1 (Gen 3)
Centrifuge	Eppendorf, Germany	Mini spin plus
Centrifuge	Heraeus, Germany	Biofuge Primo R
Digital balance	Scientech Technologies, India	ZSP 250
DNA analyzer	Applied Biosystems, USA	3730

Electronic precision weighing balance	Mettler Toledo, USA	New Classic ML
Electrophoresis tank	Bio-Rad, USA	Wide Mini-sub cell GT
Freezer −20°C	ThermoFisher Scientific, USA	ULT-1340-3-V40
Freezer −80°C	ThermoFisher Scientific, USA	ULT-1386-3-Y37
Gel-Doc System	Bio-Rad, USA	Gel-Doc EQ
Heating block	Eppendorf, Germany	Thermomixer compact
Ice flake machine	Simag, Singapore	SPR 80
Incubator 37°C	Sanyo, India	MIR 262
Magnetic stirrer	Remi equipments, India	2 MLH
Mili-Q water dispenser	Millipore, Germany	
PCR GeneAmp	Applied Biosystem, USA	PCR System 9700
PCR Veriti	Applied Biosystem, USA	96 well thermocycler
pH-Meter	Seshin, India	
Refrigerator (4°C)	Sayno, India	MPR 1410
Rocker (gel rocker)	Genei, India	
Shaker incubator	Bibby Stuart Scientific, USA	SI 500
Vortex	ThermoFisher Scientific, USA	Genei 2
Water bath	Bibby Stuart Scientific, USA	SBS 30

K. Publications:

Thesis Related Publication:

1. **Saren, S**., Samajpati, S., Mohan, U., Chattopadhyay, D., Velayutham, R., Dutta, S., 2024. Antibacterial activity of Scoparia dulcis L. root extract against Salmonella typhi and identification of active phytochemicals. J. Med. Plants Stud. 12, 01–09. https://doi.org/10.22271/plants.2024.v12.i4a.1685

Others:

2. Pramanik, K., **Saren, S.**, Mitra, S., Ghosh, P.K., Maiti, T.K., 2018. Computational elucidation of phylogenetic, structural and functional characteristics of Pseudomonas Lipases. Computational Biology and Chemistry 74, 190–200. https://doi.org/10.1016/j.compbiolchem.2018.03.018

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Antibacterial activity of *Scoparia dulcis* L. root extract against *Salmonella* typhi and identification of active phytochemicals

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Abstract
The root of *Scoparia dulcis* was consumed in its raw form by the Santali community inhabited in the tribal areas of West Bengal, India for treating loose stools and/or stomach pain due to ingestion of contaminated food. This observation led us to determine and validate the antibacterial activity of the root against common bacteria contaminating food like *Salmonella* Typhi. The extract was tested against 15 clinical isolates of *Salmonella* Typhi along with the standard MTCC734 strain and observed that the MIC values ranged from ≤7.5 mg/ml to >3.7 mg/ml. The time-kill assay showed decrease in growth rate of the bacteria in presence of the extract. TLC-Bioautography showed presence of phytochemicals in the extract which can inhibit the growth of *Salmonella* Typhi. A total of 43 phytochemicals were identified by LC-MS from the 5 selected fractions of the crude. Further research is necessary to identify most potent phytochemicals, which may lead to the development of anti-typhoid drug.

Keywords: *Scoparia dulcis* L., *Salmonella* typhi, antibacterial activity, phytochemicals, LC-MS

1. Introduction
The *Scoparia dulcis* L. (Plantaginaceae) is an annual herb of the tropical and subtropical regions; commonly known as “licorice weed”, “mithipatti” in Hindi, “bondhonya” in Bengali. Since ancient days, it was observed that the root of this plant was consumed in its raw form by the Santali community inhabited in one tribal area of East Burdwan, West Bengal, India for treating of loose stools and/or stomach pain due to ingestion of contaminated food. Locally this plant is known as “Chinidaare”, the name derived from Santali word ‘Chini’ (sugar) and ‘Daare’ (plant) as the leaves of this plant is sweet in taste. In the past, a number of works has been undertaken with the leaves and aerial parts of this plant to validate its use in treating bronchitis, cough, cancer, diabetes, diarrhea, fever, haemorrhoids, hepatosis, inflammation, skin rash, stomach aches, tuberculosis, ulcer, wounds etc. [1-5]. Scientific literature focusing on activities of roots of this plant especially the antimicrobial activity was very rare. So, in this study, we focused on the activities of root extract of *Scoparia dulcis* on food contaminant bacteria like *Salmonella* Typhi.
Salmonella enterica serovar Typhi (ST) is a Gram-negative flagellated rod-shaped bacterium which causes human-restricted typhoid or enteric fever. Typhoid may progress to life-threatening conditions, if not treated with antibacterial agents. Over the years, ST disappeared from high-income countries, while increasing antimicrobial-resistant (AMR) variants remain as endemic strains in low-to-middle-income countries (LMICs). In late eighties to early nineties, multidrug-resistant (MDR) ST outbreaks were reported from India, Pakistan, and Bangladesh [6-8]. The International Vaccine Institute estimated 11.9 million cases and 129,000 deaths in LMICs in 2010 [9]. The emergence of AMR-ST not only hindered effective treatment but demand new antimicrobials.
In this particular research paper, we discussed the efficacy of root extract of *Scoparia dulcis* by testing it against clinical isolates *Salmonella* Typhi and then identified the present phytochemicals from its most active fractions.

2. Material and methods

2.1 Collection of plant samples

The root samples of *Scoparia dulcis* L. were collected from a tribal village of East Burdwan, West Bengal, India. The plant was identified and authenticated at the Department of Botany, the University of Calcutta, and a voucher specimen (no. 20100) was deposited in the Calcutta University Herbarium (CUH), Kolkata and also in the host institute.

Fig 1: Map of West Bengal

Fig 2: *Scoparia dulcis*

2.2 Preparation of Plant Extract

The freshly collected root samples of *S. dulcis* were thoroughly washed and shade dried. The dried roots were powered by a mechanical grinder, and the powdered root were extracted in methanol following a standard maceration technique [10]. The root samples were soaked in methanol with continuous stirring at room temperature for 24 h, and the supernatant was collected. The remaining residuals were resoaked in methanol, and the previous steps were repeated until the supernatant became colourless. The methanol from the collected supernatant then completely evaporated at room temperature.

2.3 Detection of Secondary Metabolites

The presence of secondary metabolites like Flavonoids, Tannins, Phenolics compounds, Alkaloids, Terpenoids, Saponins in the crude extracts were tested by following standard traditional methods [11, 12].

2.4 Bacteria used

The ST strains used in this study includes the reference strain MTCC734, and 15 clinical isolates, collected from the Bacterial repository of ICMR-National Institute of Cholera and Enteric Diseases (ICMR-NICED). All strains were reconfirmed as ST, following standard biochemical tests and serotyping by slide-agglutination test. The antimicrobial profile of all isolates is represented in supplementary file (Table 1).

2.5 Evaluation of antibacterial activity

2.5.1 Determination of MIC and MBC

The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal concentration (MBC) were determined by microbroth dilution method [13, 14]. The extract was dissolved into 10% Dimethyl sulfoxide (DMSO) to make working solutions. The standard anti-typhoid drug chloramphenicol was used as the control, while 10% DMSO was used as the positive control, and sterile Mueller Hinton broth (MHB) was used as the negative control.

2.5.2 TLC-Bioautography

The crude methanolic extract was spotted on Thin Layer Chromatography (TLC) plates and separated by chloroform and ethyl acetate (5:4). The direct bioautography technique was applied to investigate the antibacterial activity of crude extract against *S.* Typhi test strains. After separation, dry TLC plates were soaked with bacterial inoculum (1×10^8 CFU/ml) and incubated at 37 °C in humid condition for overnight. After incubation period plates were soaked with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and incubated at 37 °C for 5 to 8 hours.

2.5.3 Time-Kill Assay

The time-kill assay was performed following the standard method [13]. We used the Stranded strain MTCC734 and two clinical isolated KOL557 and KOL558 as references for this experiment.

2.6 Separation and Identification of Phytochemicals

To identify the active phytochemicals from the crude extract having antityphoid activity, the extract was first fractionated by column chromatography (CC) and then MIC and MBC of each collated fraction were determined against test strains to identify the most active fraction(s). The selected fractions were chosen for the identification of phytochemicals by Liquid chromatography-mass spectrometry (LC-MS), which was carried out in collaboration with the National Institute of Pharmaceutical Education and Research, Kolkata (NIPER-Kolkata).

3. Result and discussion

3.1 Preparation of plant extract

The deep brown extract with a distinct aromatic odour was collected with a final yield of ≈14% (200g of powdered sample yielded approximately 28g extract).

3.2 Detection of Secondary Metabolites

The results showed that the methanolic root extract of *S. dulcis* was rich in secondary metabolites. The crude extract showed positive results for the presence of phenolics compounds, flavonoid and tannin. For the presence of

secondary metabolites like alkaloid and terpenoids the result was positive but for saponin the test result was negative. The secondary metabolites are considered as differential compounds involves in adaptive roles like functions as defense compounds or signaling compounds in response to ecological conditions, symbiosis, metal transport, competition and so on [15]. Secreted secondary metabolites were harvested by humans in past (as antibiotics, enzyme inhibitors, immunomodulators, antitumor agents) to improve their health conditions [15]. It has been established that tannins, terpenoids, alkaloids and flavonoids had *in vitro* antimicrobial activity [15]. Many alkaloids were used in medicines for their antitumor, antipyretic and antimalarial properties [16, 17]. *In vitro* studies reported that natural phenols had antimicrobial, antiviral, anti-inflammatory, and vasodilatory activities [18-20].

3.3 Evaluation of Antibacterial Activities of crude extract

3.3.1 Determination of MIC and MBC of crude extract

The MICs and MBCs of the extract against the standard strain MTCC734 and KOL148 was found to be ≤7.5 mg/ml, indicated that the crude extract was most effective (Table 1). Whereas, for most of the clinical isolates, except KOL42 and KOL89, the MIC was ≤7.5 mg/ml; but the MBC was ≤15 mg/ml. The MBC/MIC ratio (≤4) indicated that the effect of the extract was bactericidal. Against KOL42 and KOL89 the MIC was ≤15 mg/ml which indicates the crude was least effective against these two isolates.

Table 1: MIC and MBC of *S. dulcis* crude extract against 16 strains of *Salmonella* Typhi

Serial No.	Strain ID	*S. dulcis* crude extract		Chloramphenicol	
		MIC (mg/ml)	MBC (mg/ml)	MIC (µg/ml)	MBC (µg/ml)
1.	MTCC734	≤ 7.5 (> 3.75)	≤ 7.5 (> 3.75)	≥ 2 (>1)	≤ 64 (>32)
2.	KOL5	≤ 7.5 (> 3.75)	≤ 15 (> 7.5)	≥ 2 (>1)	≤ 128 (>64)
3.	KOL18	≤ 7.5 (> 3.75)	≤ 15 (> 7.5)	≤ 128 (>64)	>256
4.	KOL31	≤ 7.5 (> 3.75)	≤ 15 (> 7.5)	≤ 256 (>128)	>256
5.	KOL42	≤ 15 (> 7.5)	> 15	≤ 128 (>64)	>256
6.	KOL89	≤ 15 (> 7.5)	>15	≥ 2 (>1)	≤ 128 (>64)
7.	KOL112	≤ 7.5 (> 3.75)	≤ 15 (> 7.5)	≥ 2 (>1)	≤ 128 (>64)
8.	KOL148	≤ 7.5 (> 3.75)	≤ 7.5 (> 3.75)	≥ 1 (> 0.5)	≤ 16 (>8)
9.	KOL162	≤ 7.5 (> 3.75)	≤ 15 (> 7.5)	≥ 2 (>1)	≤ 128 (>64)
10.	KOL180	≤ 7.5 (> 3.75)	≤ 15 (> 7.5)	≥ 2 (>1)	≤ 128 (>64)
11.	KOL244	≤ 7.5 (> 3.75)	≤ 15 (> 7.5)	≥ 2 (>1)	≤ 128 (>64)
12.	KOL288	≤ 7.5 (> 3.75)	≤ 15 (> 7.5)	≥ 2 (>1)	≤ 128 (>64)
13.	KOL301	≤ 7.5 (> 3.75)	≤ 15 (> 7.5)	≥ 2 (>1)	≤ 128 (>64)
14.	KOL343	≤ 7.5 (> 3.75)	≤ 15 (> 7.5)	≥ 2 (>1)	≤ 128 (>64)
15.	KOL557	≤ 7.5 (> 3.75)	> 15	≥ 2 (>1)	≤ 128 (>64)
16.	KOL558	≤ 7.5 (> 3.75)	≤ 15 (> 7.5)	≥ 2 (>1)	≤ 128 (>64)

3.3.2 TLC-Bioautography

In TLC plate after separation of the crude extract by chloroform and ethyl acetate mixture (5:4), a number of bands appeared at different positions (Fig.2). Three selected bacterial strains (MTCC734, KOL557 and KOL558) were allowed to grow on three separate TLC plates and to visualize the viable bacterial cells MTT was used which reacts with the viable cells and give blue colour. In our test result a clear zone surrounded by the blue area appeared in TLC plates (Fig.2). This appearance of clear zone indicated that the crude root extract of *S. dulcis* might contains some phytochemicals which were able to inhibit the growth of *S.* typhi strains.

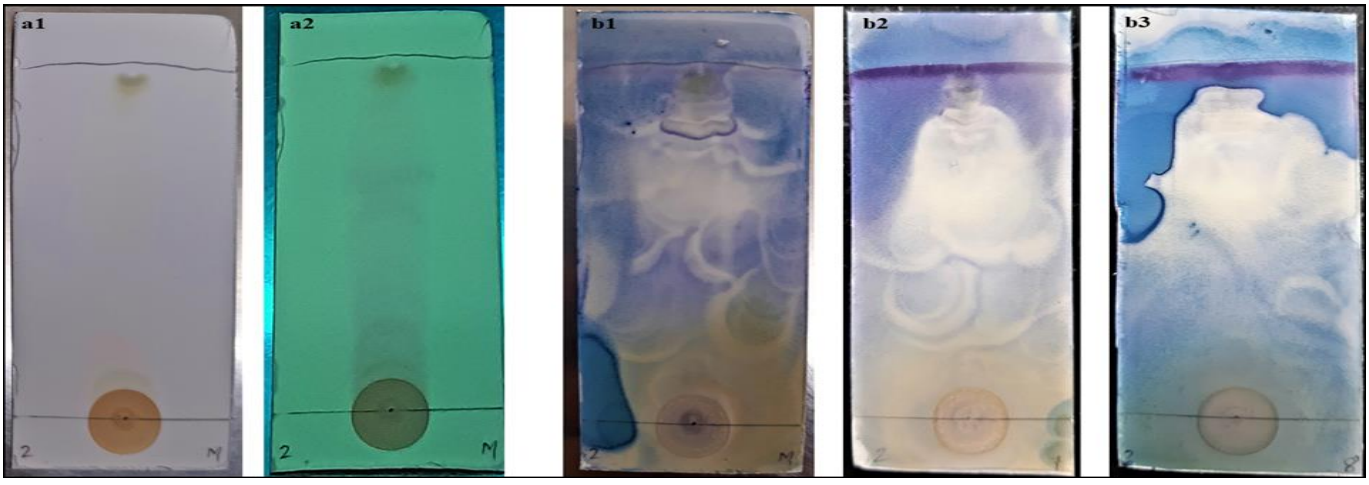


Fig 3: TLC plates; a1, a2: showing separated bands of S. dulcis root extract visualized under visual light and under UV light respectively; b.1, b.2, b.3: showing blue area of bacterial growth (of S. Typhi MTCC734, S. Typhi K557, S. Typhi K558 respectively) surrounding the clear zone

3.3.3 Time-kill assay of the Crude extract

The time-kill assay was performed with reference strain MTCC734 and two clinical isolates KOL557 and KOL558, using the MIC dose and MIC×2 dose of the extract. The result showed that there is a decrease in growth rate in the test strains when treated with the crude extract. It's also evident that the crude extract was more effective on MTCC734 and KOL558 than KOL557.

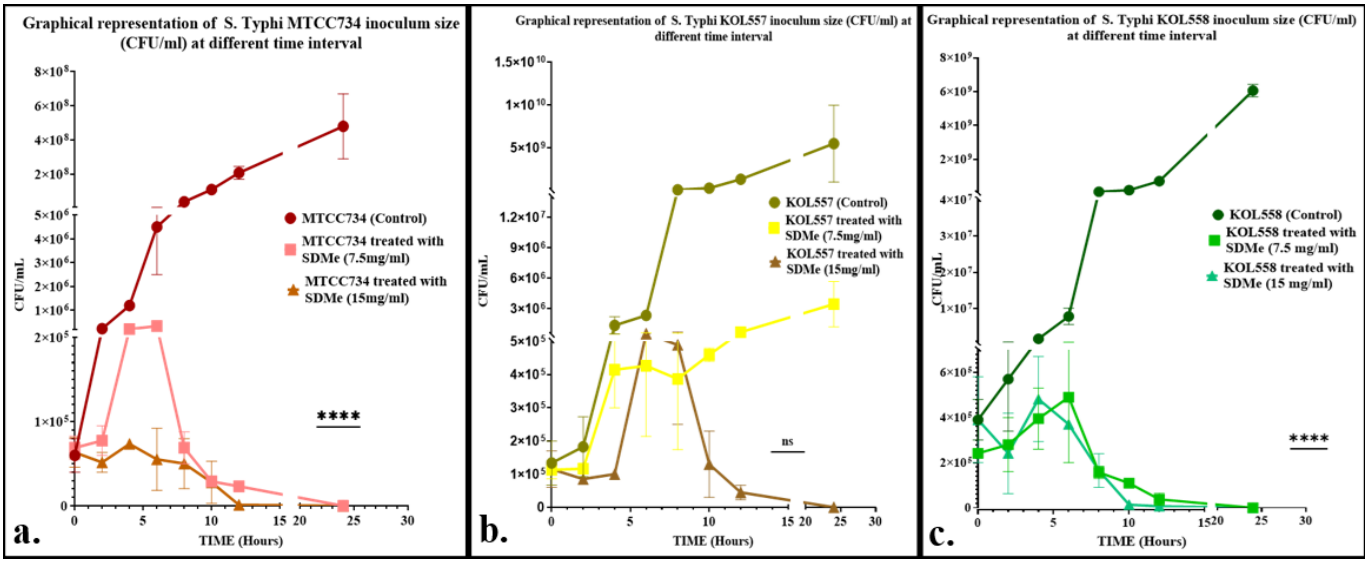


Fig 4: Time-kill curve of *S.* Typhi treated with crude extract of *S. dulcis* root (CFU/ml: Colony forming unit per milliliter; SDMe: methanolic extract of *S. dulcis* root)

3.3. Separation and identification of compounds

The crude extract was fractionated into 13 fractions by CC using solvents of different polarities. Five fractions (SDFRC1, SDFRC2, SDFRC3, SDFRC4, and SDFRC5) were collected in petroleum ether: ethyl acetate mixture (4:1, 3:2, 2:3, 1:4, 0:1, respectively), while five fractions (SDFRC6 to SDFRC10) were in 100% methanol, and three fractions (SDFRC11, SDFRC12 and SDFRC13) were collected in water. To identify the most potent fractions each collected fraction was evaluated against ST-strains MTCC734, KOL557 and KOL558 by microbroth dilution assay. Out of 13, five fractions, i.e., SDFRC2, SDFRC4, SDFRC3, SDFRC5, and SDFRC1 showed significant antibacterial activity, compared to other SDFRCs (Supplementary Table 2). The individual phytochemicals present in those five most active fractions were identified by LC-MS (Supplementary Figure 1-5). A total of 43 compounds were identified in the five fractions (Table 2), 14 of them are flavonoids and 13 are terpenoids, with five benzoxazinoids, and others. Some of these compounds were previously reported to have antibacterial activities but not specific against ST. Phan *et al.* (2006) reported a selective inhibition of 4-epi-scopadulcic acid B (diterpenoid) against *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA), and this and another diterpenoid, dulcidiol can moderately inhibit multidrug resistance-associated protein (MRP) at IC50 of 15 µg/mL and 30 µg/mL, respectively [21]. The benzoxazinoid, 6-methoxybenzoxazolinone was reported to exhibit antifungal, antibacterial activity against *E. coli*, *A. tumefaciens*, *P. vulgaris*, and inhibitory activity against human immunodeficiency virus [22, 23]. The flavonoid compounds acacetin was reported to have synergistic effects with norfloxacin, oxacillin, or ampicillin and antibacterial activity against MRSA, *E. faecalis*, *E. coli*, and *P. aeruginosa* [24, 25]. Many researchers reported apigenin as an antibacterial [26, 27]. Nayaka *et al.* (2014) reported the antibacterial activity of apigenin against *P. aeruginosa*, *S. Typhimurium*, *P. mirabilis*, *K. pneumoniae*, and *E. aerogenes*. They stated that it could be used to develop antibacterial drugs [26]. Many researchers reported compounds like benzoxazolinone [28], Betulinic acid [29], luteolin [30-32], p-coumaric acid [33, 34], stigmasterol [35, 36] had antibacterial property, especially against *S. aureus*. Flavonoid compounds such as nicotiflorin and nevadensin were also reported to show antibacterial activities [37-40]. As all these phytochemicals present in the selected fractions of S. *dulcis* showed antityphoid activity, these can be used in developing antityphoid drugs.

Table 2: List of Identified compounds from five selected fractions

Sl. No.	Compound	Molecular formula	M/Z	Mass (g·mol^{-1})	Fractions
1.	(2R)-7-methoxy-2H-1,4-benzoxazin-3(4H)-one 2-O-β-galactopyranoside	$C_{15}H_{19}NO_9$	380.0963	357.1074	SDFRC3
2.	3-hydroxy-6-methoxy-2-benzoxazolinone	$C_8H_7NO_4$	182.0451	181.0383	SDFRC1
3.	4-epi-scopadulcic acid B	$C_{27}H_{34}O_5$	439.25	438.2394	SDFRC4
4.	5,7,4´-trihydroxy-3´-methoxyisoflavone	$C_{16}H_{12}O_6$	301.0709	300.0635	SDFRC1, SDFRC2, SDFRC3
5.	6-methoxybenzoxazolinone	$C_8H_7NO_3$	166.0501	165.0428	SDFRC1, SDFRC2, SDFRC3, SDFRC4, SDFRC5
6.	Acacetin	$C_{16}H_{12}O_5$	285.0754	284.0682	SDFRC2, SDFRC3
7.	Apigenin	$C_{15}H_{10}O_5$	271.0604	270.0531	SDFRC2, SDFRC3, SDFRC4, SDFRC5
8.	Apigenin 6-C-pentosyl-8-C-hexoside	$C_{26}H_{27}O_{14}$	586.1315	563.1356	SDFRC2
9.	Apigenin 7-O-R-L-3-O-acetylrhamnopyranosyl-(1f6)-α-D glucopyranoside	$C_{29}H_{32}O_{15}$	621.1814	620.1744	SDFRC5
10.	Benzoxazine	C_8H_7NO	134.0595	133.0835	SDFRC2
11.	Benzoxazolinone	$C_7H_5NO_2$	136.0394	135.0321	SDFRC1, SDFRC2, SDFRC3
12.	Betulinic acid	$C_{30}H_{48}O_3$	457.3675	456.3588	SDFRC1, SDFRC2, SDFRC3, SDFRC4, SDFRC5
13.	Cirsiliol	$C_{17}H_{14}O_7$	353.0667	330.0759	SDFRC2

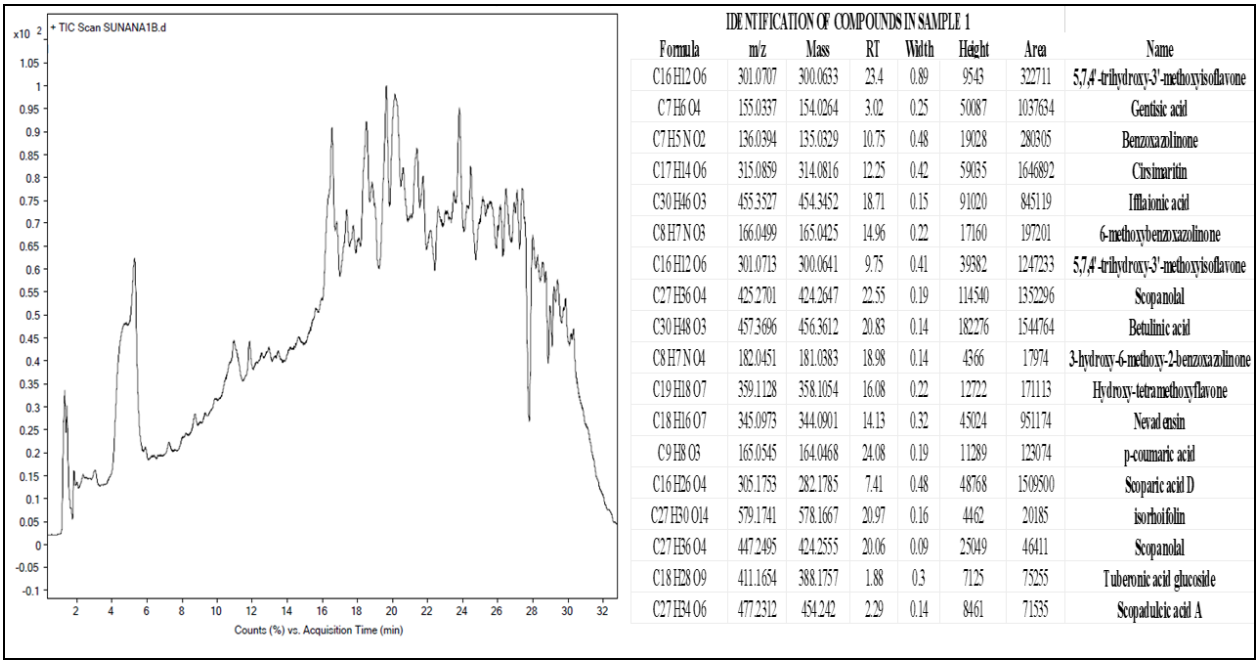
14.	Cirsimaritin	$C_{17}H_{14}O_6$	315.0859	314.0816	SDFRC1, SDFRC3, SDFRC4, SDFRC5
15.	Coxicol	$C_9H_7NO_3$	178.0496	177.0426	SDFRC2, SDFRC3, SDFRC4, SDFRC5
16.	Dulcidiol	$C_{27}H_{38}O_4$	427.281	426.2742	SDFRC2
17.	Ferruginoside C	$C_{37}H_{50}O_{19}$	821.285	798.2939	SDFRC4
18.	Gentisic acid	$C_7H_6O_4$	155.0337	154.0264	SDFRC1, SDFRC3
19.	Ginsenoside F1	$C_{36}H_{62}O_9$	661.4275	638.4403	SDFRC3
20.	Glutinol	$C_{30}H_{50}O$	427.3925	426.3855	SDFRC2
21.	Gonzalitosin I	$C_{18}H_{16}O_6$	329.1019	328.0951	SDFRC3, SDFRC4
22.	Hexacosonol	$C_{26}H_{52}O_2$	419.3733	396.3833	SDFRC3
23.	Hydroxy-tetramethoxyflavone	$C_{19}H_{18}O_7$	359.1128	358.1054	SDFRC1
24.	Hymenoxin	$C_{19}H_{18}O_8$	375.1073	374.1001	SDFRC4
25.	Ifflaionic acid	$C_{30}H_{46}O_3$	455.3527	454.3452	SDFRC1
26.	Isorhoifolin	$C_{27}H_{30}O_{14}$	579.1741	578.1667	SDFRC1, SDFRC3
27.	Linarin	$C_{28}H_{32}O_{14}$	593.1867	592.1793	SDFRC4
28.	Luteolin	$C_{15}H_{10}O_6$	287.0546	286.0589	SDFRC2, SDFRC4
29.	Nevadensin	$C_{18}H_{16}O_7$	345.0961	344.0905	SDFRC1, SDFRC2
30.	Nicotiflorin	$C_{27}H_{30}O_{15}$	595.168	594.1619	SDFRC2, SDFRC3, SDFRC4
31.	Nicotinic acid	$C_6H_5NO_2$	124.0389	123.0673	SDFRC3
32.	Palmitic amide	$C_{16}H_{33}NO$	256.2634	255.2559	SDFRC2, SDFRC3
33.	P-coumaric acid	$C_9H_8O_3$	165.0545	164.0468	SDFRC1, SDFRC3
34.	Scopadulcic acid A	$C_{27}H_{34}O_6$	477.2312	454.242	SDFRC1, SDFRC3
35.	Scopanolal	$C_{27}H_{36}O_4$	447.2495	424.2555	SDFRC1, SDFRC3, SDFRC4, SDFRC5
36.	Scoparic acid A	$C_{27}H_{36}O_5$	463.1713	440.1864	SDFRC2, SDFRC3, SDFRC4
37.	Scoparic acid B	$C_{25}H_{32}O_5$	413.2323	412.2252	SDFRC2, SDFRC3
38.	Scoparic acid C	$C_{26}H_{32}O_5$	447.2132	424.2238	SDFRC2, SDFRC4, SDFRC5
39.	Scoparic acid D	$C_{16}H_{26}O_4$	305.1753	282.1785	SDFRC1
40.	Stigmasterol	$C_{29}H_{48}O$	413.3784	412.3712	SDFRC4, SDFRC5
41.	Trans Acteoside	$C_{29}H_{36}O_{15}$	625.2099	624.2025	SDFRC4, SDFRC5
42.	Trihydroxyoctadecadienoic acid	$C_{18}H_{32}O_5$	351.214	328.2244	SDFRC2, SDFRC3, SDFRC4, SDFRC5
43.	Tuberonic acid glucoside	$C_{18}H_{28}O_9$	411.1654	388.1757	SDFRC1

Table 3: Antimicrobial resistances profile of Salmonella Typhi clinical isolates

Serial no.	Strain ID	Date of isolation	Ampicillin (A)	Chloramphenicol (C)	Tetracycline (T)	Trimethoprim-Sulfamethoxazole (Q)	Nalidixic acid (Na)	Norfloxacin (Nor)	Ciprofloxacin (Cip)	Ofloxacin (Ofx)	Levofloxacin (Lvx)	Amikacin (An)	Gentamicin (G)	Streptomycin (S)	Cefotaxime (Ctx)	Anthramycin (Atm)	Ceftriaxone (Cro)	Ceftazidime (Caz)	Azithromycin (Azm)	Amoxicillin-Clavulanic acid (Ac)	Gemifloxacin (GAT)	Cefixime (Cfm)	Peflocacin (Pfx)
1	KOL5	15.5.12	S	S	S	S	R	R	R	R	I	S	I	S	S	S	S	S	R	S	I	_	-
2	KOL18	1.6.12	R	R	S	R	R	I	I	S	S	S	S	R	S	S	S	S	I	R	S	_	_
3	KOL31	17.9.12	R	R	S	R	R	S	R	R	I	S	S	R	S	S	S	S	I	R	S	_	_
4	KOL42	19.2.13	S	R	S	R	R	I	I	S	S	S	S	I	S	S	S	S	S	S	S	_	_
5	KOL89	17.9.13	S	S	S	S	R	R	R	R	R	S	S	I	S	S	S	S	S	S	R	_	_
6	KOL112	9.11.13	S	S	S	S	R	R	R	R	R	S	S	I	S	S	S	S	S	S	R	_	_
7	KOL148	24.4.14	S	S	S	S	R	R	R	R	R	S	S	I	S	S	S	S	S	S	R	_	_
8	KOL162	16.5.14	S	S	R	R	R	R	R	R	R	S	S	R	S	S	S	S	S	S	R	_	_
9	KOL180	24.7.14	S	S	S	S	R	_	I	I	I	S	S	I	S	S	S	S	S	S		S	_
10	KOL244	1.5.15	S	S	S	S	R	_	R	R	R	S	S	S	S	_	S	S	S	_	S	S	_
11	KOL288	3.9.15	S	S	S	S	R	_	R	R	R	_	S	S	_	_	S	S	S	_		S	_
12	KOL301	8.9.15	S	S	S	S	R	_	R	R	R	_	_	S	_	_	S	S	S	_	S	S	_
13	KOL343	18.1.16	S	S	S	S	R	_	R	R	R	S	S	I	S	_	S	S	S	_	S	S	_
14	KOL557	18.9.18	S	S	S	_	R	_	R	R	R	S	S	_	S	_	S	S	S	S	_	_	R
15	KOL558	27.9.18	S	S	S	_	R	_	I	I	I	S	S	_	S	_	S	S	S	S	_	_	R

Table 4: The MIC_{90} and MBC of isolated fractions of *S. dulcis* against *Salmonella* Typhi strains

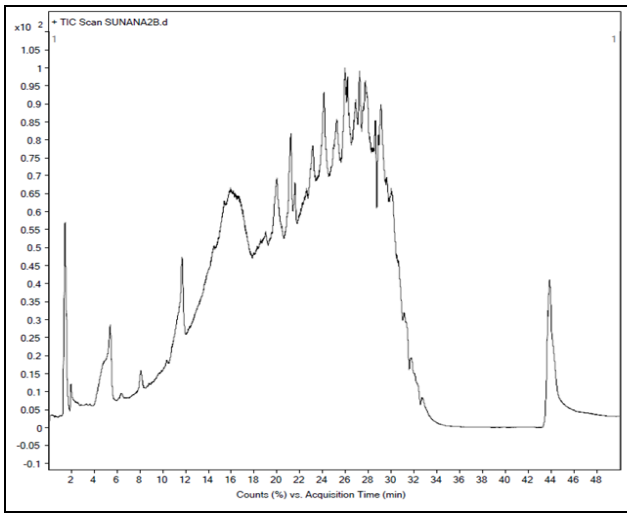
Fractions	MTCC 734		K557		K558	
	MIC_{90} (mg/ml)	MBC (mg/ml)	MIC_{90} (mg/ml)	MBC (mg/ml)	MIC_{90} (mg/ml)	MBC (mg/ml)
SDFRC1	≤3.75 (>1.8)	≤15 (>7.5)	≤7.5 (>3.75)	≤ 15 (>7.5)	≤7.5 (>3.75)	≤ 15 (>7.5)
SDFRC2	≤3.75 (>1.8)	≤3.75 (>1.8)	≤3.75 (>1.8)	≤7.5 (>3.75)	≤3.75 (>1.8)	≤7.5 (>3.75)
SDFRC3	≤3.75 (>1.8)	≤7.5 (>3.75)	≤7.5 (>3.75)	≤7.5 (>3.75)	≤7.5 (>3.75)	≤7.5 (>3.75)
SDFRC4	≤3.75 (>1.8)	≤3.75 (>1.8)	≤7.5 (>3.75)	≤7.5 (>3.75)	≤7.5 (>3.75)	≤7.5 (>3.75)
SDFRC5	≤7.5 (>3.75)	≤7.5 (>3.75)	≤7.5 (>3.75)	≤7.5 (>3.75)	≤7.5 (>3.75)	≤ 15 (>7.5)
SDFRC6	≤7.5 (>3.75)	≤ 15 (>7.5)	≤ 15 (>7.5)	> 15	≤ 15 (>7.5)	≤ 15 (>7.5)
SDFRC7	≤ 15 (>7.5)	≤ 15 (>7.5)	≤ 15 (>7.5)	≤ 15 (>7.5)	≤ 15 (>7.5)	≤ 15 (>7.5)
SDFRC8	≤15 (>7.5)	> 15	≤15 (>7.5)	> 15	≤ 15 (>7.5)	> 15
SDFRC9	> 15	> 15	> 15	> 15	> 15	> 15
SDFRC10	≤ 15 (>7.5)	≤ 15 (>7.5)	> 15	> 15	≤ 15 (>7.5)	≤ 15 (>7.5)
SDFRC11	≤ 15 (>7.5)	≤ 15 (>7.5)	≤ 15 (>7.5)	≤ 15 (>7.5)	≤ 15 (>7.5)	≤ 15 (>7.5)
SDFRC12	≤15 (>7.5)	≤ 15 (>7.5)	≤ 15 (>7.5)	> 15	≤ 15 (>7.5)	≤ 15 (>7.5)
SDFRC13	≤15 (>7.5)	≤ 15 (>7.5)	≤ 15 (>7.5)	≤ 15 (>7.5)	≤ 15 (>7.5)	≤ 15 (>7.5)



IDENTIFICATION OF COMPOUNDS IN SAMPLE 1

Formula	m/z	Mass	RT	Width	Height	Area	Name
C16 H12 O6	301.0707	300.0633	23.4	0.89	9543	322711	5,7,4'-trihydroxy-3'-methoxyisoflavone
C7 H6 O4	155.0337	154.0264	3.02	0.25	50087	1037634	Gentisic acid
C7 H5 N O2	136.0394	135.0329	10.75	0.48	19028	280305	Benzoxazolinone
C17 H14 O6	315.0859	314.0816	12.25	0.42	59035	1646892	Cirsimaritin
C30 H46 O3	455.3527	454.3452	18.71	0.15	91020	845119	Iffaionic acid
C8 H7 N O3	166.0499	165.0425	14.96	0.22	17160	197201	6-methoxybenzoxazolinone
C16 H12 O6	301.0713	300.0641	9.75	0.41	39982	1247233	5,7,4'-trihydroxy-3'-methoxyisoflavone
C27 H36 O4	425.2701	424.2647	22.55	0.19	114540	1352296	Scopanolal
C30 H48 O3	457.3696	456.3612	20.83	0.14	182276	1544764	Betulinic acid
C8 H7 N O4	182.0451	181.0383	18.98	0.14	4366	17974	3-hydroxy-6-methoxy-2-benzoxazolinone
C19 H18 O7	359.1128	358.1054	16.08	0.22	12722	171113	Hydroxy-tetramethoxyflavone
C18 H16 O7	345.0973	344.0901	14.13	0.32	45024	951174	Nevadensin
C9 H8 O3	165.0545	164.0468	24.08	0.19	11289	123074	p-coumaric acid
C16 H26 O4	305.1753	282.1785	7.41	0.48	48768	1509500	Scoparic acid D
C27 H30 O14	579.1741	578.1667	20.97	0.16	4462	20185	isorhoifolin
C27 H36 O4	447.2495	424.2555	20.06	0.09	25049	46411	Scopanolal
C18 H28 O9	411.1654	388.1757	1.88	0.3	7125	75255	Tuberonic acid glucoside
C27 H34 O6	477.2312	454.242	2.29	0.14	8461	71535	Scopadulcic acid A

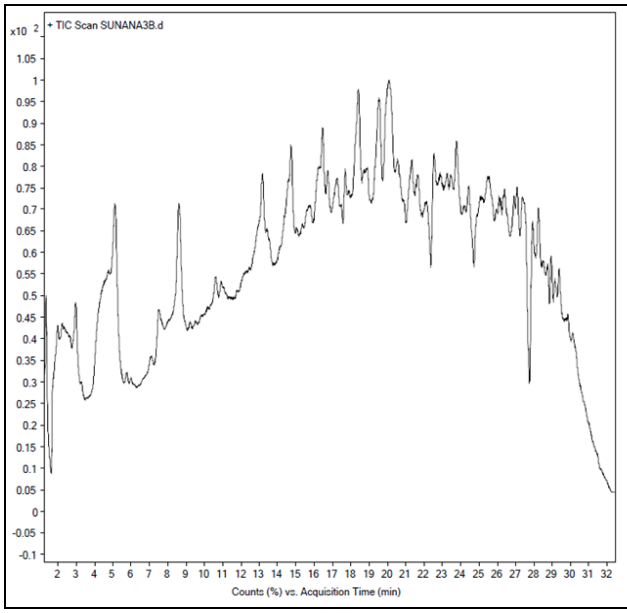
Fig 5: total lon chromatogram (TIC) and compound identification of SDFRC1



Sample 2

Formula	m/z	Mass	RT	Width	Height	Area	Name
C15 H10 O5	271.0604	270.0531	9.3	0.35	3868	62915	Apigenin
C15 H10 O6	287.0546	286.0589	19.9	7.77	1522	14080	Luteolin
C9 H7 N O3	178.0496	177.0426	3.28	0.17	35027	801356	Coxicol
C27 H36 O5	463.1713	440.1864	14.67	0.1	4892	12405	Scoparic acid A
C16 H12 O5	285.0754	284.0682	22.33	0.63	3311	88194	acacetin
C25 H32 O5	413.2323	412.2252	13.15	0.15	51647	459789	Scoparic acid B
C30 H48 O3	457.3675	456.3588	26.52	0.23	25813	330544	Betulinic acid
C17 H14 O7	353.0667	330.0759	5.38	0.3	9132	189897	Cirsiliol
C18 H16 O7	345.0961	344.0905	13.33	0.58	3047	51428	Nevadensin
C16 H33 N O	256.2634	255.2559	29.1	0.15	146057	1620166	Palmitic amide
C27 H38 O4	427.281	426.2742	15.75	0.34	12230	116605	Dulcidiol
C30 H50 O	427.3925	426.3855	25.44	0.14	61972	414030	Glutinol
C16 H12 O6	301.0709	300.0635	9.79	0.57	7545	226049	5,7,4'-trihydroxy-3'-methoxyisoflavone
C7 H5 N O2	136.0394	135.0321	4.76	0.41	163163	2922726	Benzoxazolinone
C8 H7 N O3	166.0501	165.0428	5.38	0.84	5221801	24540296	6-methoxybenzoxazolinone
C8 H7 N O	134.0595	133.0835	4.11	1.37	3056	64478	Benzoxazine
C26 H27 O14	586.1315	563.1356	1.33	0.14	4406	32331	Apigenin 6-C-pentosyl-8-C-hexoside
C26 H32 O5	447.2132	424.2238	1.46	0.03	8788	15250	Scoparic acid C
C27 H30 O15	595.168	594.1619	1.93	0.07	4449	21340	Nicotiflorin
C18 H32 O5	351.214	328.2244	12.09	0.58	12957	428720	Trihydroxyoctadecadienoic acid

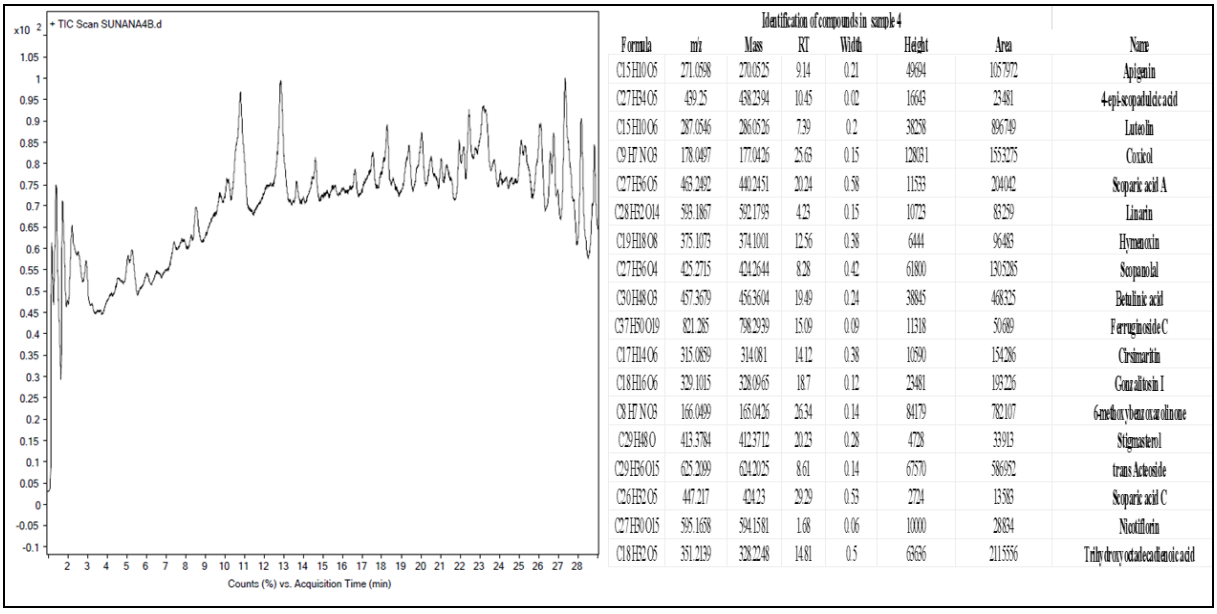
Fig 6: total lon chromatogram (TIC) and compound identification of SDFRC2



IDENTIFICATION OF COMPOUNDS in sample 3

Formula	m/z	Mass	RT	Width	Height	Area	Name
C15H10O5	271.0601	270.0526	9.25	0.25	25729	6257076	Apigenin
C9H7 N O3	178.0494	177.0421	2.96	0.2	254268	6074265	Coxicol
C27H36O5	463.2446	440.2582	24.27	0.09	23970	185453	Scoparic acid A
C16H12O5	285.0756	284.0739	13.62	0.23	34775	645112	Acacetin
C27H36O4	425.2686	424.2612	22.46	0.2	46492	429954	Scoparidol
C25H32O5	413.2348	412.2759	16.59	1.92	17623	54684	Scoparic acid B
C30H48O3	457.3681	456.3607	17.76	0.08	22249	105793	Betulinic acid
C27 H30O14	601.1585	570.1648	17.95	0.16	6010	58429	Isorhoifolin
C17H14O6	315.0862	314.0799	14.25	0.23	87302	1441232	Cirsimaritin
C18H16O6	329.1019	328.0951	19.15	0.21	45590	600343	Gonzalitosin I
C16 H33N O	256.2641	255.2567	27.91	0.13	1229213	11124834	Palmitic amide
C9H8 O3	165.0551	164.0477	3.27	0.17	206651	3375599	P-coumaric acid
C16H12O6	301.0717	300.0645	9.79	0.45	629868	21562950	5,7,4'-trihydroxy-3'-methoxyisoflavone
C7H5 N O2	136.0392	135.0319	10.6	0.17	95802	1444279	Benzoxazolinone
C8H7 N O3	166.0502	165.0429	5.11	1.13	8219065	49968542	6-methoxybenzoxazolinone
C7H6 O4	155.0336	154.0262	11.72	0.52	5656	116013	Gentisic acid
C26H32O12	439.3733	396.3933	31.46	0.11	6768	25677	Hexasesamol
C36H62O9	661.4275	650.4403	26.96	0.1	24208	100022	Ginsenoside F1
C15H19 NO9	360.0963	357.1074	1.96	0.11	53135	480152	(2R)-7-methoxy-2H-1,4-benzoxazin-3-(4H)-one 2-O-β-galactopyranoside
C27H34O6	477.2301	454.2398	2.16	0.12	12643	124125	Scopadulcic acid A
C27 H30O15	595.1671	594.1592	1.72	0.09	6799	55915	Nicotiflorin
C6H5 N O2	124.0389	123.0675	5.47	0.17	25011	247748	Nicotinic acid
C18H32O5	351.2141	328.2247	14.93	0.26	103395	3570364	Trihydroxyoctadecadienoic acid

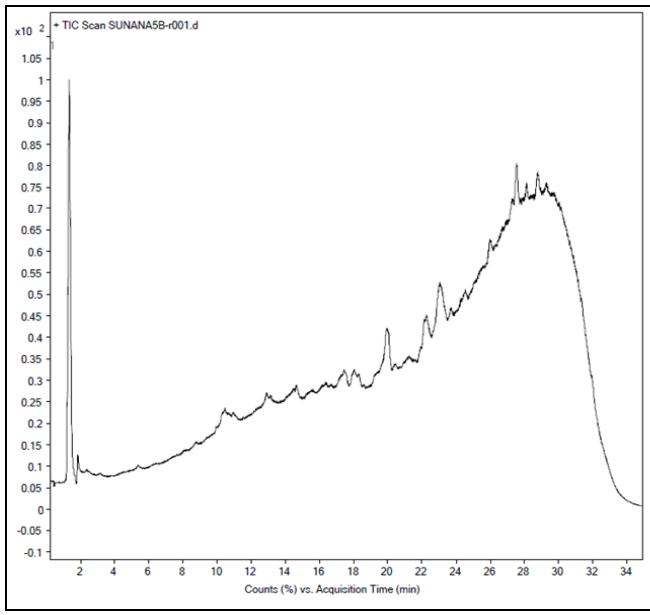
Fig 7: total lon chromatogram (TIC) and compound identification of SDFRC3



Identification of compounds in sample 4

Formula	m/z	Mass	RT	Width	Height	Area	Name
C15 H10 O5	271.0598	270.0525	9.14	0.21	49684	1057972	Apigenin
C27 H34 O5	439.25	438.2394	10.45	0.02	16643	23481	4-epi-scopadulcic acid
C15 H10 O6	287.0546	286.0526	7.39	0.2	38258	896749	Luteolin
C9 H7 N O3	178.0497	177.0426	25.63	0.15	128181	1553275	Coxicol
C27 H36 O5	463.2492	440.2451	20.24	0.58	11533	204042	Scoparic acid A
C28 H32 O14	593.1867	592.1798	4.23	0.15	10723	85259	Linarin
C19 H18 O8	375.1075	374.1001	12.56	0.38	6444	96485	Hymenoxin
C27 H36 O4	425.2715	424.2644	8.28	0.42	61800	1305265	Scopanolal
C30 H48 O3	457.3679	456.3604	19.49	0.24	38845	468325	Betulinic acid
C37 H50 O19	821.285	798.2939	15.09	0.09	11518	50689	Ferruginoside C
C17 H14 O6	315.0859	314.081	14.12	0.38	10590	154286	Cirsimaritin
C18 H16 O6	329.1015	328.0965	18.7	0.12	23481	193226	Gonzalitosin I
C8 H7 N O3	166.0499	165.0426	26.34	0.14	84179	782107	6-methoxybenzoxazolinone
C29 H48 O	413.3784	412.3712	20.23	0.28	4728	33913	Stigmasterol
C29 H36 O15	625.2089	624.2025	8.61	0.14	67570	586982	trans Acteoside
C26 H32 O5	447.217	424.23	29.29	0.53	2724	13585	Scoparic acid C
C27 H30 O15	595.1658	594.1581	1.68	0.06	10000	28854	Nicotiflorin
C18 H32 O5	351.2139	328.2248	14.81	0.5	62626	2115556	Trihydroxyoctadecadienoic acid

Fig 8: total lon chromatogram (TIC) and compound identification of SDFRC4



IDENTIFICTATION OF COMPOUNDS IN SAMPLE 5

Formula	m/z	Mass	RT	Width	Height	Area	Name
C15 H10 O5	271.0592	270.052	9.36	0.59	7502	257197	Apigenin
C9 H7 N O5	178.0498	177.0424	3.15	0.16	41020	492655	Coxicol
C27 H36 O4	425.2726	424.2653	1.38	0.12	6970	55607	Scopanwlol
C30 H48 O3	457.3685	456.3409	19.4	0.58	6544	81712	Betulinic acid
C17 H14 O6	315.0852	314.0781	12.1	0.43	4706	108541	Cirsimaritin
C29 H32 O15	621.1814	620.1744	25.19	0.15	3628	41670	Apigenin 7-O-R-L-3-O-acetyl-b-xylopyranosyl-(1-6)-D-glucopyranoside
C8 H7 N O5	166.0502	165.0429	5.37	0.25	145913	2694725	6-methoxybenzoxazolinone
C29 H48 O	413.3746	412.3664	25.57	0.18	3480	18545	Stigmasterol
C29 H36 O15	627.215	624.2068	1.37	0.13	5557	51084	trans Acteoside
C26 H32 O5	425.232	424.2276	23.47	0.53	2086	12279	Scopanic acid C
C18 H32 O5	351.2141	328.2249	10.01	0.84	5001	20767	Trihydroxyoctadecadienoic acid

Fig 9: total lon chromatogram (TIC) and compound identification of SDFRC5

4. Conclusion

The crude extract tested against ST; the causative agent of typhoid fever showed MIC range of ≤ 7.5 mg/ml to >3.75 mg/ml. The time-kill assay showed decrease in bacterial growth rate in presence of the crude extract and the killing activity was both time as well as concentration-dependent. The TLC-bioautography indicated the presence of compounds that could inhibit the growth of ST. The biological activity of any plant is generally dependent on the phytochemical constituents of the plant. Hence, phytochemical profiling is essential to know the plant's biological use. The crude root extract was separated into 13 fractions, five showing improved antibacterial activities against ST. Through LC-MS, we have identified 43 compounds. The results of this study led to the identification of compounds, that may have good antibacterial activity against ST strains. Further studies on the compounds are ongoing which may result in developing antibacterial agent against typhoid in future.

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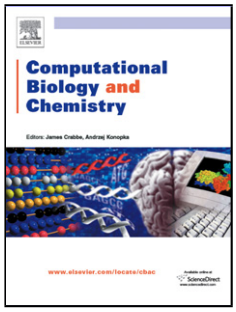

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Computational elucidation of phylogenetic, structural and functional characteristics of *Pseudomonas* Lipases

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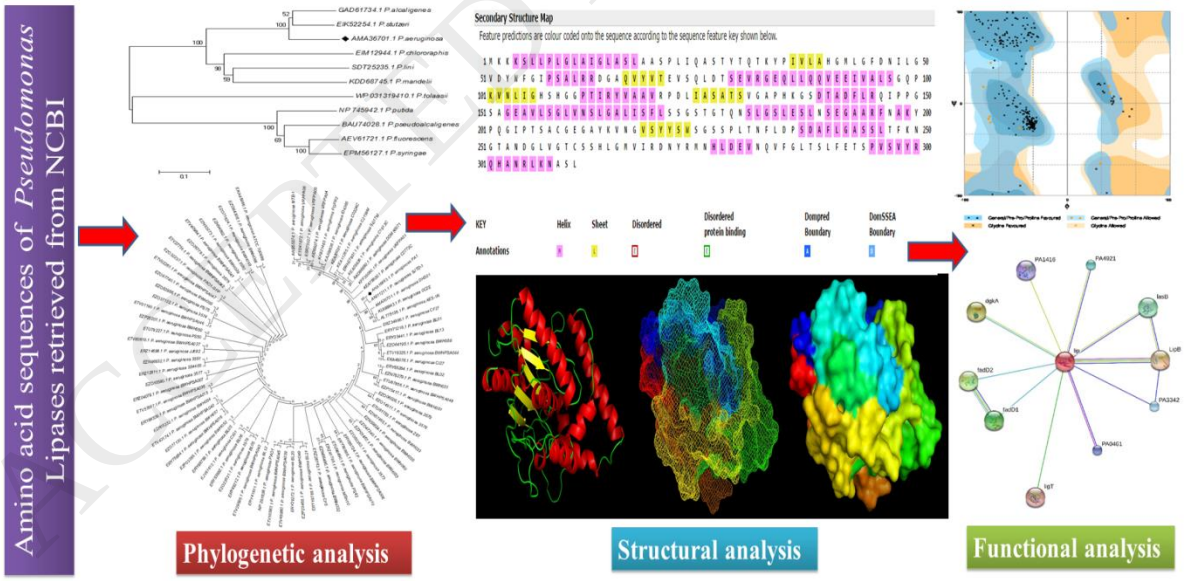
Graphical abstract



Highlights

- A total of 84 lipase sequences of *Pseudomonas aeruginosa* were retrieved from NCBI.
- A detailed phylogenetic study was done based on lipases and cDNA of *P. aeruginosa*.
- Physicochemical characterization of all selected protein sequences was performed.
- Secondary structure of selected representative protein analyzed by various tools.
- 3D structure was assessed, validated from authentic servers and functions revealed.

Abstract

Lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) catalyzes tri-, di-, and monoacyl glycerol of fat into glycerol and fatty acids. It has important roles in digestion of lipids in living organisms and industrially as laundry detergents along with proteases. The microbial lipases are more stable, active and economically feasible compared to plant and animal sources. Hence, much attention was given for maximum production of the enzyme from the microbial sources. The phylogenetic analysis revealed that the amino acid sequence of lipase protein and their corresponding cDNA of *Pseudomonas aeruginosa* clustered with *Pseudomonas stutzeri* among different species of *Pseudomonas*, while *P. aeruginosa* PA1 clustered with *P. aeruginosa* SJTD-1 among different strains of *P. aeruginosa*. The lipase of *P. aeruginosa* PA1 was monomeric, acidic and thermostable protein having molecular weight ranging in between 32.72 to 34.89 kDa. The protein was abundant with random coils and alpha helices in its secondary structure. Tertiary model showed 96.310 score as overall quality factor. Hence, this *in silico* study gives some useful information about the lipase protein without performing crystal structure assessment by X-ray Crystallography or NMR study in wet lab experiments which could be helpful for isolation and characterization of the enzyme *in vitro*.

Keywords:*Pseudomonas*; lipases; *in silico*; protein modeling; thermostability; phylogenetic analysis.

1. Introduction

Lipase is triacylglycerol acylhydrolases and classified under the enzyme class, EC 3.1.1.3. Lipase catalyzes the hydrolysis of long chain triacylglycerol at an oil-water interface where the acyl group is picked by lipase, forming lipase-acyl complex and then transfers to the –OH group of water. Lipases are serine hydrolase and they can work without support from any co-factors (Rajendran et al., 2008). In non-aqueous condition, lipase can transfer acyl group of carboxylic acid to nucleophiles (Martinelle and Hult, 1995) and carried out the ester synthesis.

Lipase enzyme has important roles in the digestion and processing of dietary lipids in living organisms. Moreover, lipase is used to remove the lipid stains as laundry detergents along with proteases. In 1995, two bacterial lipase were introduced “Lumfast” from *Pseudomonas mendocina* and “Lipomax” from *Pseudomonas alcaligenes* by Genencor International (Jaeger and Reetz, 1998) both are used in detergents. In paper industry to remove the hydrophobic components (pitch) lipase is used.

The enzyme is useful in remediation of oil spills in refinery, shore sand and processing factories, in degradation of oils from water, in degradation of polyester wastes, to purify the waste gases from factors. Immobilized lipases are used to quantitative determination of triacylglycerol as biosensor which is important in clinical diagnosis. In non-aqueous condition lipase carried out ester synthesis, this property can be used to produce biodegradable polyester and also as a surfactant in cosmetics and perfume industries (Metzger and Bornscheuer, 2006). The interestrification and transestrification property of lipase is used in food industry to modify the length of fatty acid chain which enhances the flavor.

Lipases are produced by most of the living organisms viz. animals, plants and microorganisms. But lipases from microorganisms are industrially more important than that from other sources as they have ease for cultivation and genetic manipulation (Hasan et al., 2006) and are more stable. The oil processing factories,

dairies, oil contaminated soil, decaying food particles, hot springs and compost heaps are the natural habitat for lipase producing microorganism (Wang et al., 1995). Most of the lipase producing microorganism is gram negative bacteria and most important gram negative genus is *Pseudomonas* (Gilbert, 1993) which has variety of application in biotechnology. *Pseudomonas* lipases are differing from other lipases in their thermostability and activity at alkaline pH.

In 2010, the global market for industrial enzymes is calculated at $3.3 billion. It was expected to reach $4.4 billion by 2015 and at the rate of 6% compound annual growth rate (CAGR) over the 5-year forecast period (BBC Research, 2011). It was estimated that by 2020, the industrial used enzymes will reach $6.3 billion in terms of value in global market at a CAGR of 5.8% from 2017.

It is obvious that lipase, especially *Pseudomonas* lipase, has great importance in various fields. So, the isolation and selection of new strains as sources of lipase has demand in each field. It is necessary to know the 3D structure of lipase for designing and engineering lipase and make it suitable for specific purposes. The main purpose of this study is to understand the physicochemical, structural and functional properties and phylogenetic relationship of *Pseudomonas aeruginosa* lipase protein through *in silico* analysis which is essential for application of lipase in different fields.

2. Materials and methods

2.1 Retrieval of sequences

Eighty four amino acid sequences of lipase protein of different strains of *P. aeruginosa*, eleven amino acid sequences of lipase of different species of *Pseudomonas* were retrieved from NCBI (http://www.ncbi.nlm.nih.gov/) in FASTA format. Each amino acid sequences were reverse translated to cDNA from reverse translate tool (http://www.bioinformatics.org/sms2/rev_trans.html).

2.2 Phylogenetic analysis

A total of four phylogenetic trees were constructed from retrieved amino acid and reverse translated sequences, using MEGA7 (Version 7.0.18) software (Kumar, 2016). The Evolutionary history was inferred using the neighbor-joining method (Saitou and Nei, 1987) and the evolutionary distance were computed using the p-distance method (Nei and Kumar, 2000) in each case.

2.3Physicochemical characterization

To predict the physicochemical characteristics of 84 amino acid sequences of different strains of *P. aeruginosa*, the expasyProtParam tool (Gasteiger et al., 2005) was used. The server calculates the molecular weight (MW), theoretical isoelectric point (pI), amino acid composition, extinction coefficient (EC), estimated half-life, instability index (II), aliphatic index (AI) and grand average of hydropathicity (GRAVY).

2.4 Secondary structural analysis

The expasy SOPMA tool (Geourjon and Deléage, 1995) and PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/) were used for the prediction of secondary structure which counts the number of helices, sheets, turns, and coils in the amino acid sequence.

2.5 Homology modeling and evaluation

Pseudomonas aeruginosa PA1 strain was selected as a representative of all the 84 strains for the prediction of tertiary structure of lipase protein. To obtain the 3D model and QMEAN score, the expasy SWISS-MODEL (ProMod3 version 1.0.2) workspace (Biasini et al., 2014) was used. The quality of built model was evaluated in SAVES server (http://services.mbi.ucla.edu/SAVES/) and the overall quality factor was obtained by SAVES ERRAT. The Ramachandran plot was constructed in SAVES server and RAMPAGE (http://morded.bioc.cam.ac.uk/~rapper/rampage.php) using the pdb file to visualize the energetically allowed regions for backbone dihedral angles ϕ against ψ amino acid residues in lipase protein structure.

2.6 Functional analysis

The interacting proteins of *P. aeruginosa* lipase were predicted by STRING server (http://string.db.org). The conserve domain of lipase protein was identified from NCBI using CD-search. The motif search tool (http://www.genome.jp/tools/motif/) was also used to obtain functional motifs in the protein sequence.

3. Results and discussion

3.1 Retrieval of sequences

The lipase protein sequences of 11 different spp. of *Pseudomonas* (viz. *P. aeruginosa, P. fluorescences, P. alcaligenes, P. stutzeri, P. pseudoalcaligenes, P. putida, P. tolaasii, P. mandelii, P. lini, p. chlororaphis, and P. syringae*) and 84 strains of *P. aeruginosa* were retrieved from NCBI. The no. of amino acid residues were found variable from 290 to 353. Authentic databases like UniProtKB, NCBI are the hub of

raw materials for various kinds of Bioinformatics analyses (Verma et al., 2016; Pramanik et al., 2017a, 2017b, 2017c; Rani and Pooja, 2017; Udenigwe et al., 2013).

Table 1. Physicochemical characterization of selected 11 species of *Pseudomonas*

Sr. No.	Bacterial strain	AAR	MW(kDa)	pI	EC	II	AI	GRAVY
1.	*P. aeruginosa*	311	32.72	6.37	27515	23.75	92.22	-0.008
2.	*P. alcaligens*	313	32.84	5.50	29005	22.07	95.02	0.015
3.	*P. syringae*	339	36.53	7.88	43890	34.88	89.53	-0.091
4.	*P. chlororaphis*	296	32.62	9.29	44015	39.52	90.91	-0.201
5.	*P. fluorescens*	337	36.00	7.11	40910	28.96	92.11	0.012
6.	*P. lini*	293	31.48	9.15	22920	22.40	96.31	-0.100
7.	*P. mandelii*	290	31.70	6.08	34505	28.96	94.21	-0.104
8.	*P. pseudoalcaligenes*	339	36.37	6.72	40910	24.16	86.14	-0.113
9.	*P. tolaasii*	353	38.10	8.84	41620	45.65	82.97	-0.0194
10.	*P. putida*	339	36.33	8.89	42400	28.38	91.27	-.0.040
11.	*P. stutzeri*	308	32.45	5.45	27515	20.64	94.71	0.006

Table 2. Physicochemical characterization of 84 selected strains of *P. aeruginosa*

Sr. No.	Bacterial strain	Acc. No.	AAR	MW(kDa)	pI	EC	II	AI	GRAVY
1.	*P. aeruginosa* BL21	ERY19766.1	315	34.83	6.09	22920	43.86	104.67	-0.029
2.	*P. aeruginosa* BL20	ERY29272.1	315	34.83	6.09	22920	43.86	104.67	-0.029
3.	*P. aeruginosa* BL13	ERY35441.1	315	34.8	6.09	22920	43.86	104.06	-0.037
4.	*P. aeruginosa* BL12	ERY41611.1	315	34.83	6.09	22920	43.86	104.67	-0.029
5.	*P. aeruginosa* BL05	ERY49212.1	315	34.83	6.09	22920	43.86	104.67	-0.029
6.	*P. aeruginosa* BL06	ERY53882.1	315	34.86	6.09	22920	43.35	104.35	-0.037
7.	*P. aeruginosa* BL02	ERY55264.1	315	34.79	6.09	22920	44.25	103.75	-0.043
8.	*P. aeruginosa* BL03	ERY68759.1	315	34.83	6.09	22920	43.86	104.67	-0.029
9.	*P. aeruginosa* BL01	ERY71218.1	315	38.8	6.09	22920	43.86	104.06	-0.037
10.	*P. aeruginosa* BWHPSA016	ERY75684.1	315	34.83	6.09	22920	43.86	104.67	-0.029
11.	*P. aeruginosa* BWHPSA015	ERY84539.1	315	34.83	6.09	22920	43.86	104.67	-0.029
12.	*P. aeruginosa* BWHPSA008	ERY89724.1	315	34.86	6.09	22920	43.59	105.27	-0.021
13.	*P. aeruginosa* BWHPSA010	ERY90886.1	315	34.86	6.09	22920	43.59	105.27	-0.021
14.	*P. aeruginosa* BWHPSA007	ERZ04079.1	315	34.86	6.09	22920	43.35	104.35	-0.037
15.	*P. aeruginosa* S54485	ERZ12911.1	315	34.83	6.09	22920	43.86	104.67	-0.029
16.	*P. aeruginosa* JJ692	ERZ14698.1	315	34.83	6.09	22920	43.86	104.67	-0.029
17.	*P. aeruginosa* CF5	ERZ28743.1	315	34.86	6.09	22920	44.25	104.35	-0.037

18.	*P. aeruginosa* CF27	ERZ34986.1	315	34.82	6.09	22920	43.38	104.35	-0.03
19.	*P. aeruginosa* MSH10	ERZ41795.1	315	34.88	6.09	22920	43.35	104.67	-0.038
20.	*P. aeruginosa* PS50	ETU79227.1	315	34.83	6.09	22920	43.86	104.67	-0.029
21.	*P. aeruginosa* Z61	ETU81753.1	315	34.86	6.09	22920	43.86	104.35	-0.037
22.	*P. aeruginosa* BWHPSA048	ETU87856.1	315	34.79	6.09	22920	44.25	103.75	-0.043
23.	*P. aeruginosa* PS42	ETU96490.1	315	34.88	6.09	22920	43.35	104.67	-0.038
24.	*P. aeruginosa* BWHPSA046	ETV01190.1	315	34.85	6.09	22920	44.2	104.98	-0.028
25.	*P. aeruginosa* BWHPSA047	ETV02261.1	315	34.86	6.09	22920	43.35	104.35	-0.037
26.	*P. aeruginosa* BWHPSA045	ETV10583.1	315	34.86	6.09	22920	43.35	104.35	-0.037
27.	*P. aeruginosa* BWHPSA044	ETV19325.1	315	34.8	6.09	22920	43.86	104.06	-0.037
28.	*P. aeruginosa* BWHPSA042	ETV27751.1	315	34.86	6.09	22920	43.35	104.35	-0.037
29.	*P. aeruginosa* BWHPSA043	ETV29595.1	315	34.86	6.09	22920	44.25	104.35	-0.037
30.	*P. aeruginosa* BWHPSA041	ETV40984.1	315	34.86	6.09	22920	43.35	104.35	-0.037
31.	*P. aeruginosa* BWHPSA040	ETV43174.1	315	34.83	6.09	22920	43.86	104.67	-0.029
32.	*P. aeruginosa* BWHPSA039	ETV49980.1	315	34.83	6.09	22920	43.86	104.67	-0.029
33.	*P. aeruginosa* BWHPSA038	ETV57867.1	315	34.83	6.09	22920	43.86	104.67	-0.029
34.	*P. aeruginosa* BWHPSA037	ETV60618.1	315	34.86	6.09	22920	43.86	104.35	-0.037
35.	*P. aeruginosa* BWH033	EZN57465.1	315	34.86	6.09	22920	43.68	104.35	-0.037
36.	*P. aeruginosa* BWH035	EZN59824.1	315	34.89	6.09	22920	43.08	104.03	-0.045
37.	*P. aeruginosa* BWH032	EZN66495.1	315	34.88	6.09	22920	43.35	104.67	-0.038
38.	*P. aeruginosa* BWH031	EZN76279.1	315	34.79	6.09	22920	44.25	103.75	-0.043
39.	*P. aeruginosa* BWH029	EZN83273.1	315	34.86	6.09	22920	43.35	104.35	-0.037
40.	*P. aeruginosa* 3580	EZN94892.1	315	34.86	6.09	22920	43.35	104.35	-0.037
41.	*P. aeruginosa* 3581	EZN96632.1	315	34.86	6.09	22920	43.35	104.35	-0.037
42.	*P. aeruginosa* 3579	EZO06026.1	315	34.8	6.09	22920	42.98	104.67	-0.028
43.	P. aeruginosa 3577	EZO10580.1	315	34.83	6.09	22920	43.86	104.67	-0.029
44.	*P. aeruginosa* 3578	EZO14911.1	315	34.8	6.09	22920	42.98	104.67	-0.028
45.	*P. aeruginosa* 3576	EZO23631.1	315	34.85	6.09	22920	44.2	104.98	-0.028
46.	*P. aeruginosa* 3575	EZO26778.1	315	34.83	6.09	22920	43.86	104.67	-0.029
47.	*P. aeruginosa* PA01=GFP	EZO32237.1	315	34.83	6.09	22920	43.86	104.67	-0.029
48.	*P. aeruginosa* 3574	EZO37122.1	315	34.83	6.09	22920	43.86	104.67	-0.029
49.	*P. aeruginosa* BWH060	EZO47240.1	315	34.87	6.09	22920	43.47	103.43	-0.034
50.	*P. aeruginosa* 3573	EZO50352.1	315	34.88	6.09	22920	42.18	105.59	-0.02
51.	*P. aeruginosa* PS75	EZO53956.1	315	34.83	6.09	22920	43.86	104.67	-0.029
52.	*P. aeruginosa* BWH058	EZO64195.1	315	34.8	6.09	22920	43.68	104.06	-0.037
53.	*P. aeruginosa* BWH059	EZO71624.1	315	34.86	6.09	22920	43.35	104.35	-0.037

54.	*P. aeruginosa* BWH057	EZO77130.1	315	34.86	6.09	22920	43.35	104.35	-0.037
55.	*P. aeruginosa* BWH056	EZO84208.1	315	34.86	6.09	22920	43.35	104.35	-0.037
56.	*P. aeruginosa* BWH055	EZO87740.1	315	34.83	6.09	22920	43.86	104.67	-0.029
57.	*P. aeruginosa* BWH053	EZP01467.1	315	34.87	6.09	22920	43.47	103.43	-0.034
58.	*P. aeruginosa* BWH052	EZP03395.1	315	34.85	6.09	22920	44.2	104.98	-0.028
59.	*P. aeruginosa* BWH054	EZP05232.1	315	34.83	6.09	22920	43.86	104.67	-0.029
60.	*P. aeruginosa* BWH051	EZP15417.1	315	34.79	6.09	22920	44.25	103.73	-0.043
61.	*P. aeruginosa* BWH050	EZP20201.1	315	34.83	6.09	22920	43.86	104.67	-0.029
62.	*P. aeruginosa* BWH049	EZP23465.1	315	34.83	6.09	22920	43.86	104.67	-0.029
63.	*P. aeruginosa* VRFPA01	KFF35060.1	321	34.74	4.72	38765	48.31	96.45	-0.071
64.	*P. aeruginosa* PGPR2	KFB21463.1	321	34.65	4.8	38765	46.25	96.76	-0.046
65.	*P. aeruginosa* C0324C	KEA30031.1	321	34.76	4.8	44265	47.96	95.23	-0.078
66.	*P. aeruginosa* C2773C	KEA15800.1	321	33.88	5.37	35910	57.83	96.04	0.072
67.	*P. aeruginosa* C2159M	KEA11265.1	321	34.69	4.8	38765	48.05	96.45	-0.064
68.	*P. aeruginosa* C1913C	KEA09836.1	321	34.69	4.8	38765	48.05	96.45	-0.064
69.	*P. aeruginosa* ID4365	KAJ08836.1	321	34.72	4.84	38765	46.61	96.45	-0.069
70.	*P. aeruginosa* MTB-1	AHB55874.1	321	34.68	4.79	38765	47.45	96.45	-0.061
71.	*P. aeruginosa* SG17M	EWH24834.1	321	34.69	4.8	38765	48.05	96.45	-0.064
72.	*P. aeruginosa* ATCC 700888	EKA45986.1	315	34.83	6.09	22920	43.86	104.67	-0.029
73.	*P. aeruginosa* CI27	EKA45078.1	315	34.8	6.09	22920	43.86	104.06	-0.037
74.	*P. aeruginosa* CIG1	EJY61612.1	315	34.83	6.09	22920	43.86	104.06	-0.029
75.	*P. aeruginosa* DSM50071	AKO86690.1	321	34.69	4.8	38765	48.05	96.45	-0.064
76.	*P. aeruginosa* 0C2E	KUI88943.1	311	32.72	6.37	27515	25.37	92.22	-0.008
77.	*P. aeruginosa* AES-1R	ALT75138.1	311	32.72	6.37	27515	25.37	92.22	-0.008
78.	*P. aeruginosa* PA1	AHA16663.1	311	32.72	6.37	27515	23.75	92.22	-0.008
79.	*P. aeruginosa* PA01	NP-251639.1	315	34.83	6.07	22920	43.86	104.67	-0.029
80.	*P. aeruginosa* VRFPA05	ESR73027.1	321	34.68	4.79	38390	47.45	96.45	-0.061
81.	*P. aeruginosa* DHS01	AMA36701.1	311	32.72	6.37	27515	25.37	92.22	-0.008
82.	*P. aeruginosa* VRFPA08	ETD41872.1	321	34.68	4.79	38765	47.45	96.45	-0.061
83.	*P. aeruginosa* VRFPA04	AID85074.1	321	34.68	4.79	38765	47.45	96.45	-0.061
84.	*P. aeruginosa* SJTD-1	ANI11211.1	311	32.72	6.37	27515	23.76	92.22	-0.008

AAR = Amino acid residues, MW = Molecular weight, pI = Isoelectric point, EC = Extinction coefficient, II = Instability Indiex, AI = Aliphatic Index, GRAVY = Grand average of hydropathicities.

3.2 Phylogenetic analysis

Four phylogenetic trees were constructed to compare the evolutionary relationship among *Pseudomonas* spp. and among 84 strains of *P. aeruginosa*. The comparison based on the amino acid sequences of lipase protein showed *P. fluorescens* clustered with *P. syringae* with 100% similarity whereas *P. aeruginosa* clustered with *P. alcaligenes* and *P. stutzeri* with the same similarity percentage (Fig. 1). In addition, the phylogenetic tree based on cDNA (reverse translated) of lipase protein also showed that the *P. fluorescens* clustered with *P. syringae*. But *P. aeruginosa* cluster with *P. stutzeri* and those two again cluster with *P. alcaligenes* (Fig. 2).

Moreover, 84 strains of *P. aeruginosa* were also compared on the basis of lipase protein and cDNA (reverse translated). The comparison based on protein showed that the strain *P. aeruginosa* PA1 and *P. aeruginosa* SJTD-1 clustered together with 90% similarity. Those two again cluster with the strains *P. aeruginosa* 0C2E, *P. aeruginosa* AES-1R and *P. aeruginosa* DHS01 with 99% similarity (Fig. 3). The phylogenetic tree based on cDNA (reverse translated) of lipase protein of those 84 strains showed similar kind of cluster as found in the comparison of protein (Fig. 4). Similar phylogenetic analysis was previously worked out by a no. of authors (Verma et al., 2016; Pramanik et al., 2017a, 2017b, 2017c; Rani and Pooja, 2017; Udenigwe et al., 2013) to find if there was any correlation among the taxa in terms of their protein sequences compared with respective cDNA.

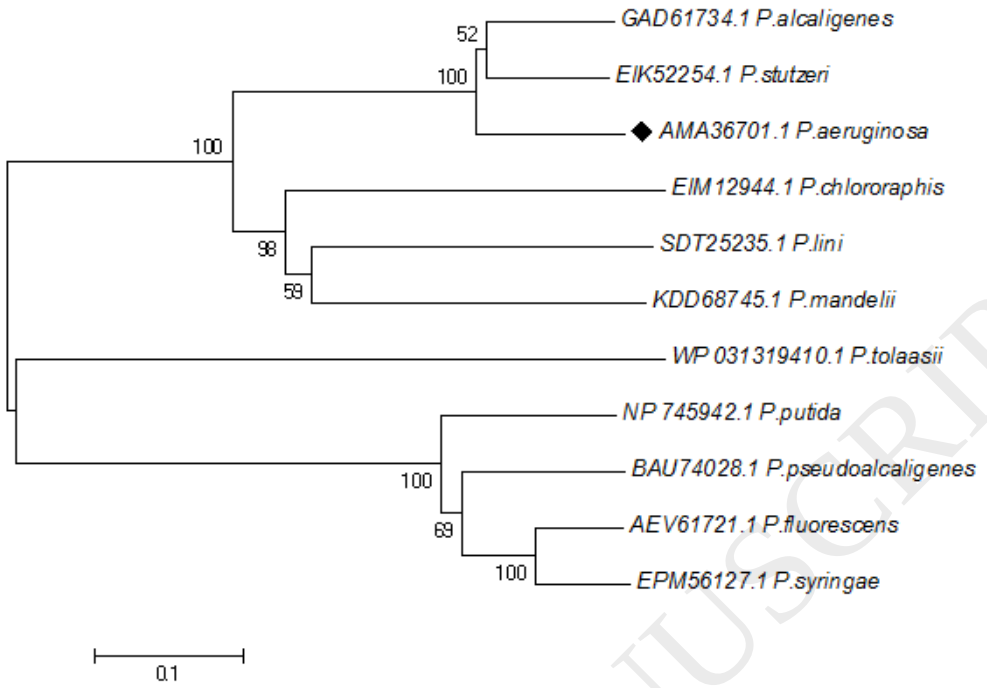


Fig. 1. Phylogenetic tree of lipase protein among different species of *Pseudomonas*.

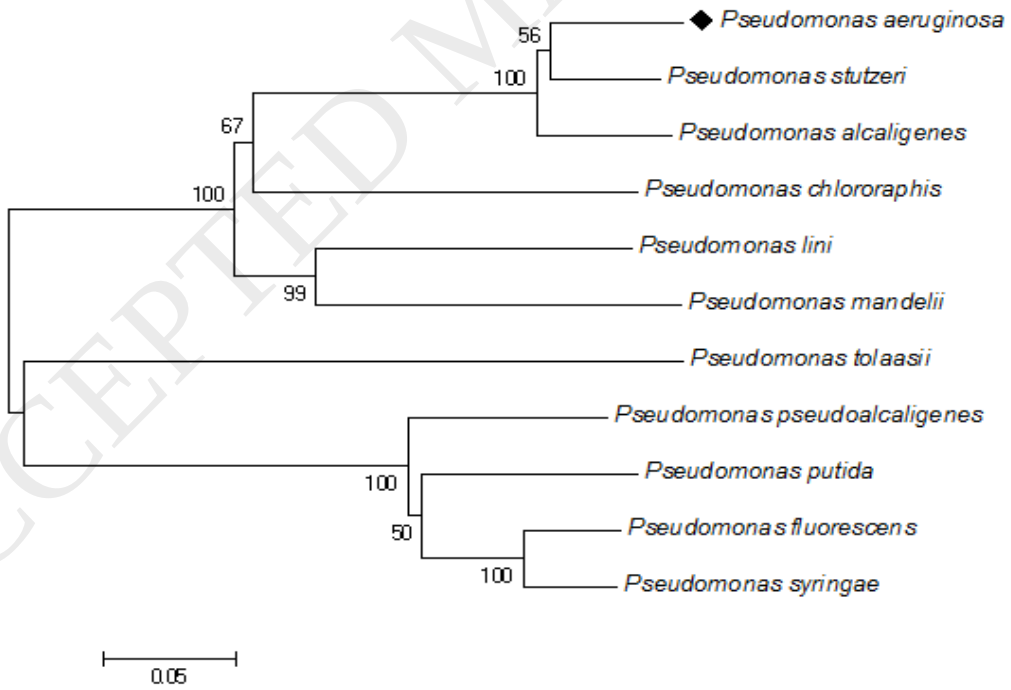


Fig. 2 Phylogenetic tree of cDNA of lipase protein of different species of *Pseudomonas*.

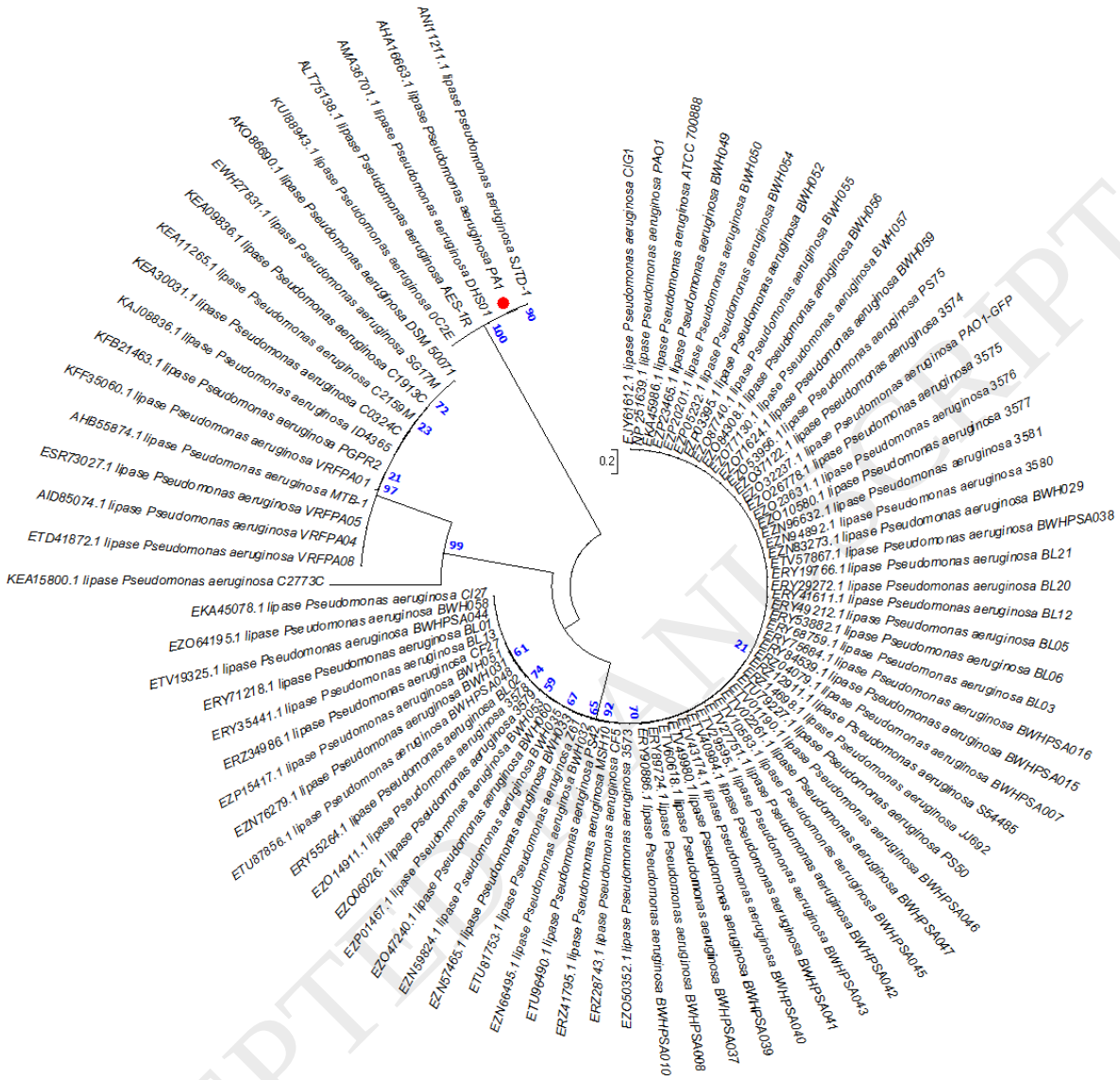


Fig. 3. Phylogenetic tree of lipase protein among different strains of *Pseudomonas aeruginosa*.

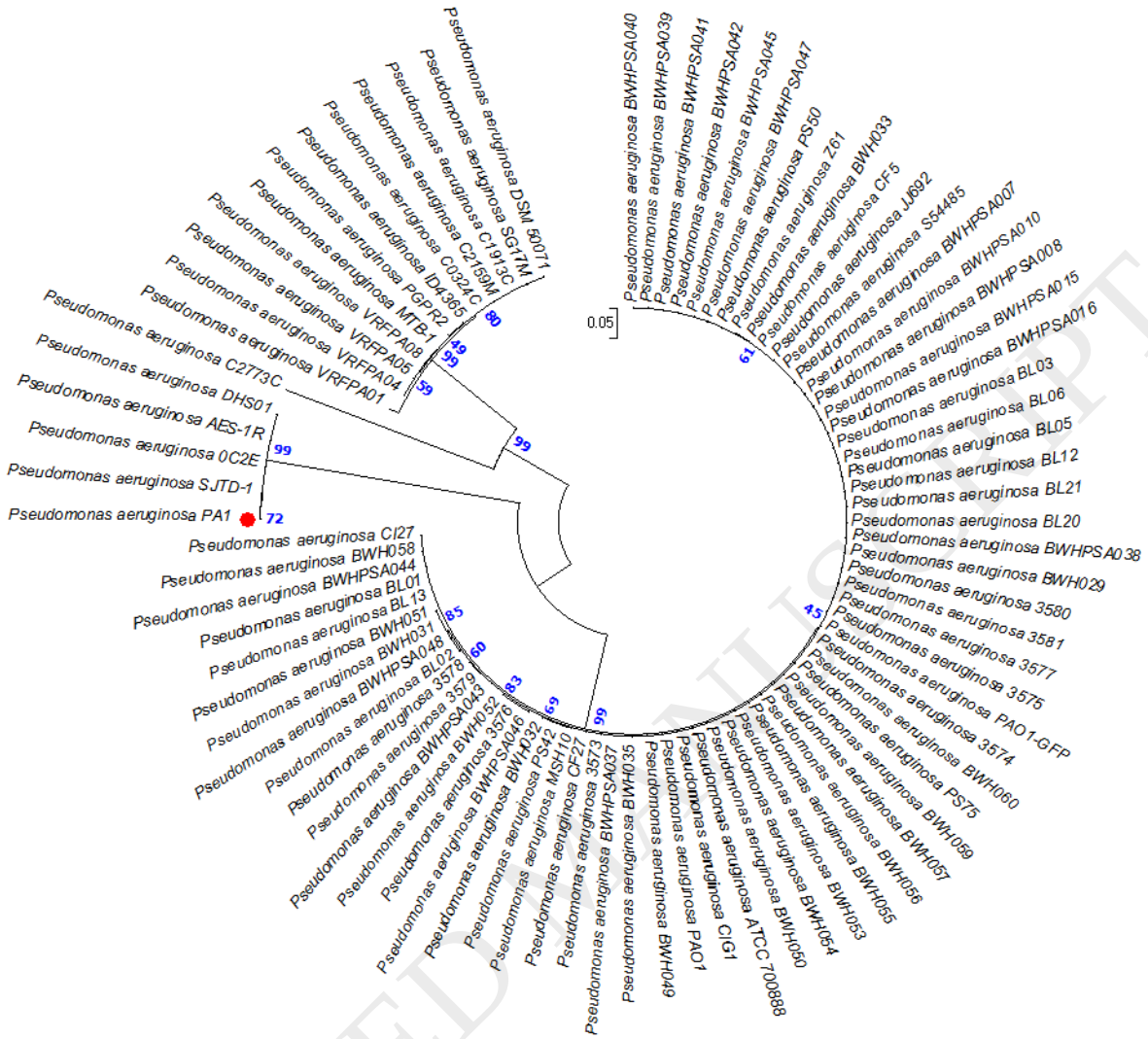


Fig. 4. Phylogenetic tree of cDNA among different strains of *Pseudomonas aeruginosa*.

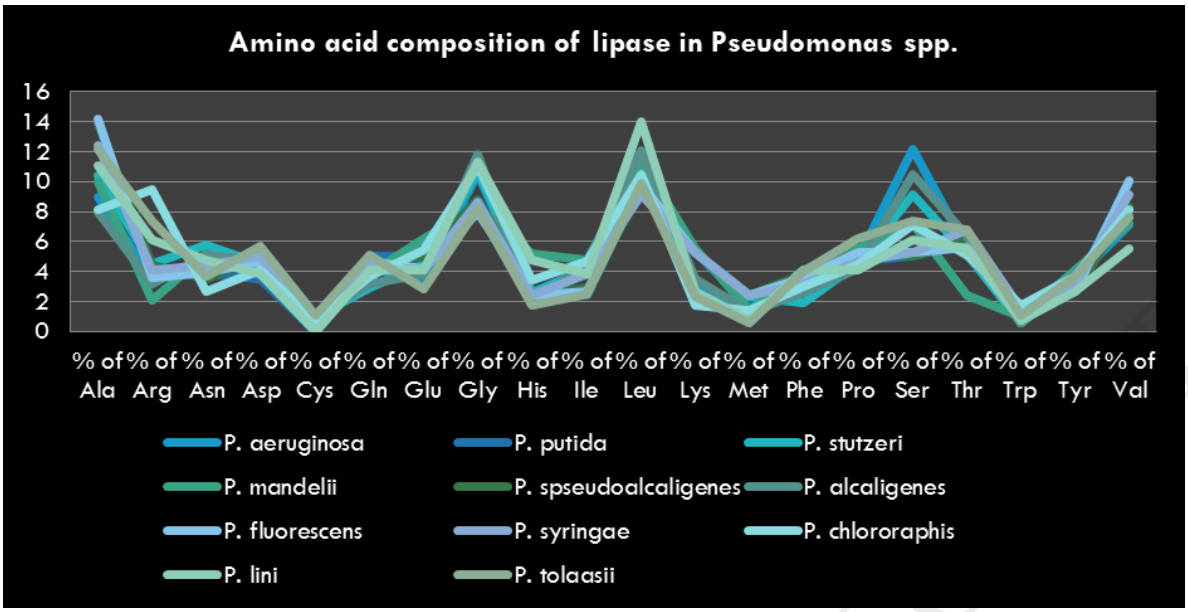
3.3Physicochemical characterization

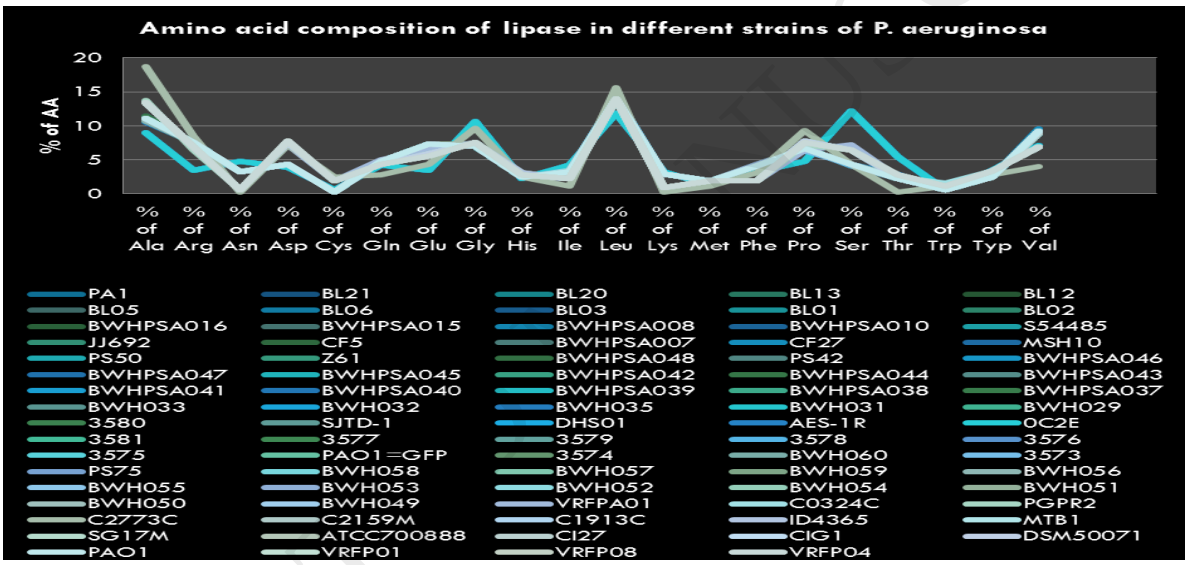
On the basis of physicochemical parameters 11 species of Pseudomonas and 84 strains of *P. aeruginosa* were characterized (Table 1, Table 2). The parameter includes no. of amino acid residues, molecular weight, theoretical pI, extinction coefficient, instability index, aliphatic index, GRAVY (Table 1, Table 2). The difference in amino acid composition graphically represented in Fig. 5 among different spp. of *Pseudomonas* as well as within different strains of *P. aeruginosa*. The selected sequences contained amino acid residues ranging from 311 to 321. The molecular weight of the sequences was ranging from 32.72 to 34.89 kDa. The pI of the sequences indicated that the lipase protein in *P. aeruginosa* strains was acidic in nature, as it ranged in between 4.72 and 6.09. The extinction coefficient (EC) of the said proteins was within a range of 27515 $M^{-1}cm^{-1}$ to 44265 $M^{-1}cm^{-1}$. The EC indicates the amount of light absorbed by a protein in a certain wavelength. Here the EC were calculated at 280 nm measured in water assuming all pair of Cys residue from cystines. Most of the strains have unstable lipase protein as their instability index is greater than 40 except 5 strains viz. *P. aeruginosa* 0C2E, *P. aeruginosa* AES-1R, *P. aeruginosa* PA1, *P. aeruginosa* DHS01, *P. aeruginosa* SJTD-1. The aliphatic index which positively indicates the thermostability of a globular protein ranges from 92.22 to 105.59. The GRAVY (Grand Average of Hydropathicity) of lipase protein is negative in almost all strains except in *P. aeruginosa* C2773C. Our results were corroborated by the similar determination of physicochemical characteristics of many other microbial proteins by earlier workers (Verma et al., 2016; Pramanik et al., 2017a, 2017b, 2017c; Rani and Pooja, 2017).

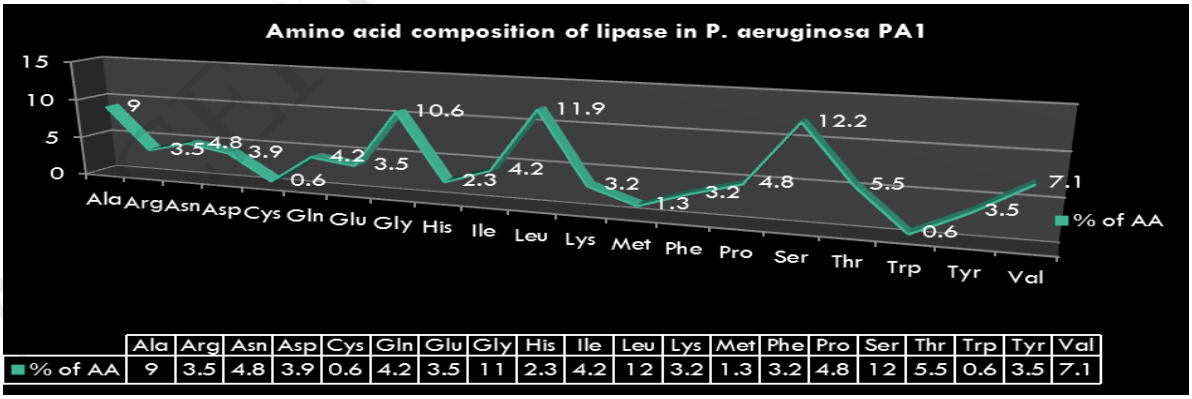
3.4 Secondary structural analysis

The secondary structure prediction result shows that the proteins has secondary arrangements of alpha helix 32.15%, extended stand 19.92%, beta turn 9.00% and random coil 38.91% (Fig. 6). Alpha helices are most stable element in protein folding and are the first element which formed during protein folding (Verma et al., 2016; Pramanik et al., 2017c; Rani and Pooja, 2017). The secondary elements i.e. alpha helix and extended stand involved in the stabilization of protein. Random coil indicates the absence of regular secondary structure. Here 38.91% of the protein remains unfolded as it exhibits random coils. This condition helps in interaction with

water. Generally the folded proteins are thermodynamically unstable. From the predicted result it can be stated that the protein is thermodynamically quiet stable.







	Ala	Arg	Asn	Asp	Cys	Gln	Glu	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Thr	Trp	Tyr	Val
% of AA	9	3.5	4.8	3.9	0.6	4.2	3.5	11	2.3	4.2	12	3.2	1.3	3.2	4.8	12	5.5	0.6	3.5	7.1

Fig. 5. Composition of amino acids within different species of *Pseudomonas* and within different strains of *P. aeruginosa*.

3.5 Homology modeling and evaluation

To obtain the 3D model (Fig. 7) of protein, the amino acid sequences of *P. aeruginosa* PA1 lipase (target sequence) was submitted to SWISS MODEL workspace. Here the target sequence is aligning to the template sequence (1ex.9.1.A) which has 100% sequence identities with the target sequence.

The built model result showed -0.32 QMEAN score. QMEAN is a composite scoring function to measure the global quality of entire model as well as for the local per residue analysis of different regions within a model (Benkert et al., 2011). The score close to 0 indicates the good quality of model. The QMEAN4 is a reliability score for the whole model which can be utilized to compare and rank alternative models of the same target. The quality estimated ranges between 0 and 1 with high values for batter models (Benkert et al., 2011). Here the normalized QMEAN score in comparison with non-redundant set of PDB structure is <1. In SAVES server, the ERRAT generated overall quality factors of the protein is 96.310 (Fig. 8).

The model was analyzed through Ramachandran plot (Fig. 8) and it was found that the no. of residues in favored region is 96.1%, in allowed region 3.9% and in outlier region is 0.0% and those results is very close to the expected result (Lovell et al., 2003). A good quality model would be expected to have over 90% in the most favored region (Yadav et al., 2013).

Fig. 6. Predicted secondary structural elements of *P. aeruginosa* PA1.

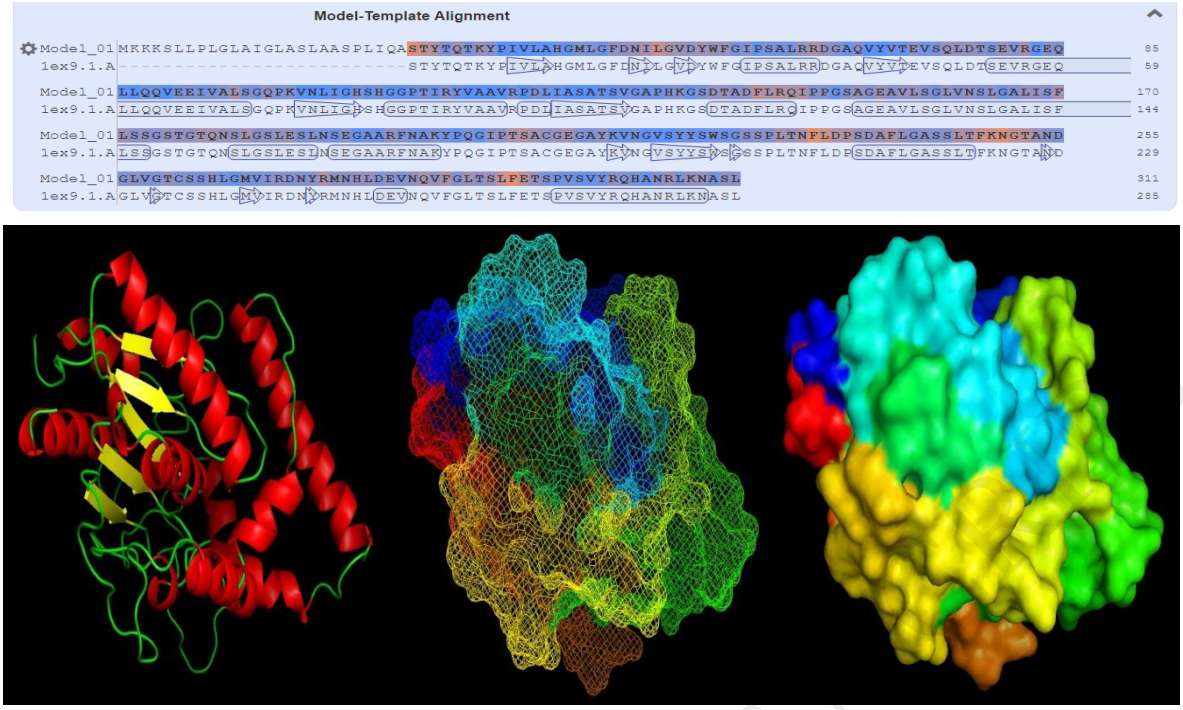
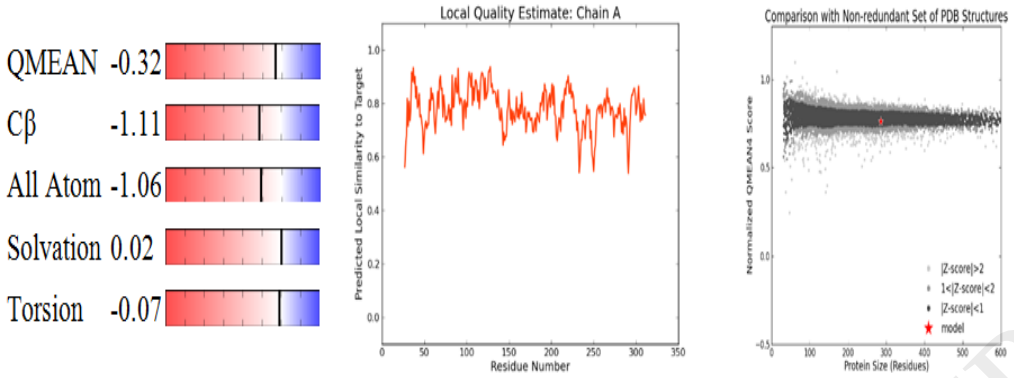


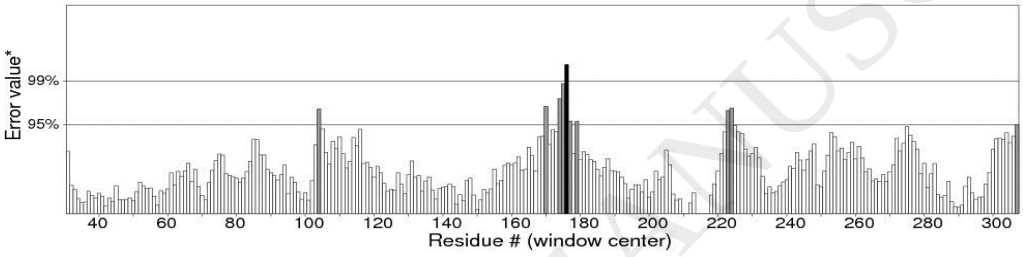
Fig. 7. Target-template alignment result and predicted 3D structures of P. aeruginosa PA1 with different views (cartoon, mesh and surface view)

3.6 Functional analysis

The STRING server detected (Fig. 9) a list of ten interacting proteins of lipase in *P. aeruginosa* cell viz. lipase chaperone (lipB) which involved in the folding of the extracellular lipase during the passage through the periplasm, diacylglycerol kinase (dgkA), two long chain fatty acid CoA ligases (fadD2 & fadD1), elastaseLasB (lisB, involved in the pathogenesis of P. aeruginosa), three hypothetical proteins (PA4921, PA3342 & PA1416), acyltransferase (PA0461) and 2'-5' RNA ligase (ligT). The *P. aeruginosa* PA1 lipase protein has putative conserved domains of Abhydrolase superfamily as detected by NCBI using CD search. There are five functional motifs in the protein viz. Abhydrolase_1, Abhydrolase_6, LCAT, DUF676 and Hydrolase_4 (Fig.10).

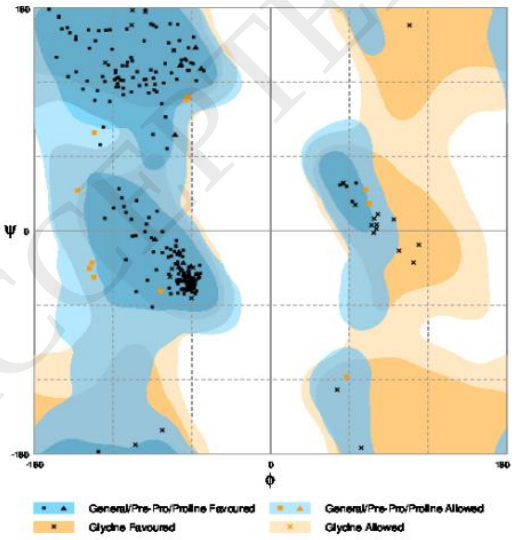


Program: ERRAT2
File: /var/www/SAVES/Jobs/74519413//errat.pdb
Chain#:1
Overall quality factor**: 96.310



*On the error axis, two lines are drawn to indicate the confidence with which it is possible to reject regions that exceed that error value.

**Expressed as the percentage of the protein for which the calculated error value falls below the 95% rejection limit. Good high resolution structures generally produce values around 95% or higher. For lower resolutions (2.5 to 3Å) the average overall quality factor is around 91%.



Evaluation of residues

Residue [A 55 :PHE] (-62.39, 107.84) in Allowed region
Residue [A 57 :ILE] (-83.74, -48.53) in Allowed region
Residue [A 96 :LEU] (-137.86, -30.29) in Allowed region
Residue [A 108 :SER] (58.13,-117.64) in Allowed region
Residue [A 180 :ASN] (-134.19, 79.14) in Allowed region
Residue [A 209 :CYS] (-146.92, 32.38) in Allowed region
Residue [A 231 :THR] (-134.55, -37.41) in Allowed region
Residue [A 250 :ASN] (72.72, 33.21) in Allowed region
Residue [A 252 :THR] (-64.49, 105.82) in Allowed region
Residue [A 257 :LEU] (-136.00, -25.80) in Allowed region
Residue [A 310 :SER] (75.26, 21.88) in Allowed region
Number of residues in favoured region (~98.0% expected) : 272 (96.1%)
Number of residues in allowed region (~2.0% expected) : 11 (3.9%)
Number of residues in outlier region : 0 (0.0%)

Fig. 8. Evaluation of the built model for *P. aeruginosa* PA1 showing overall quality factor and Ramachandran plot.

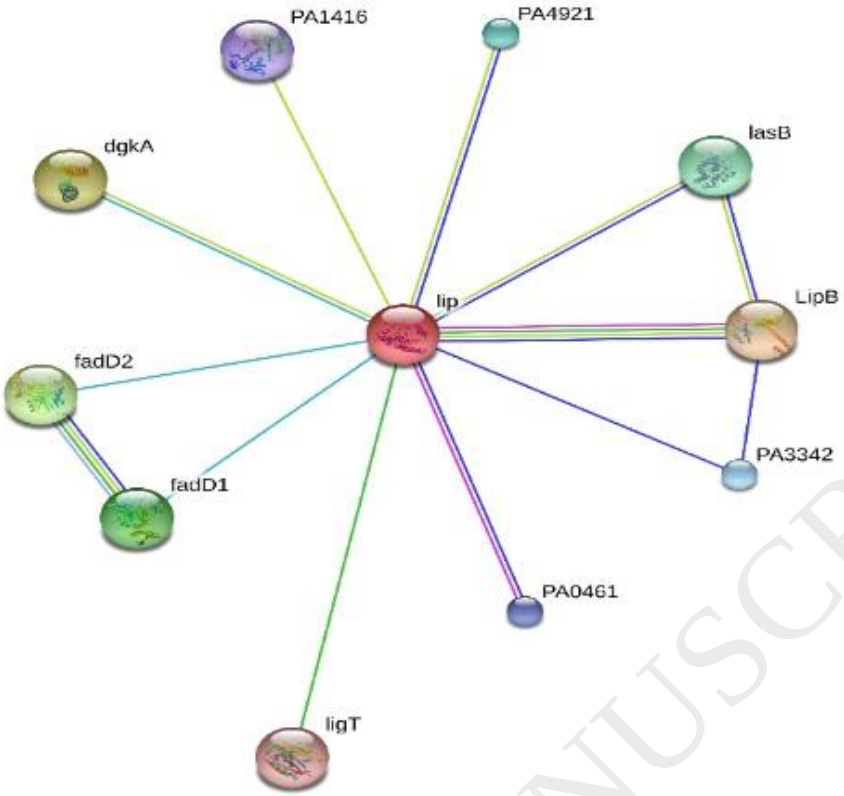


Fig. 9. STRING analysis result showing interacting proteins.

Pfam (5 motifs)

Pfam	Position(Independent E-value)	Description
Abhydrolase_1	35..280(7.4e-26)	PF00561, alpha/beta hydrolase fold
Abhydrolase_6	36..148(0.00095)	PF12697, Alpha/beta hydrolase family
LCAT	85..138(0.015)	PF02450, Lecithin:cholesterol acyltransferase
DUF676	80..119(0.025)	PF05057, Putative serine esterase (DUF676)
Hydrolase_4	88..137(0.099)	PF12146, Serine aminopeptidase, S33

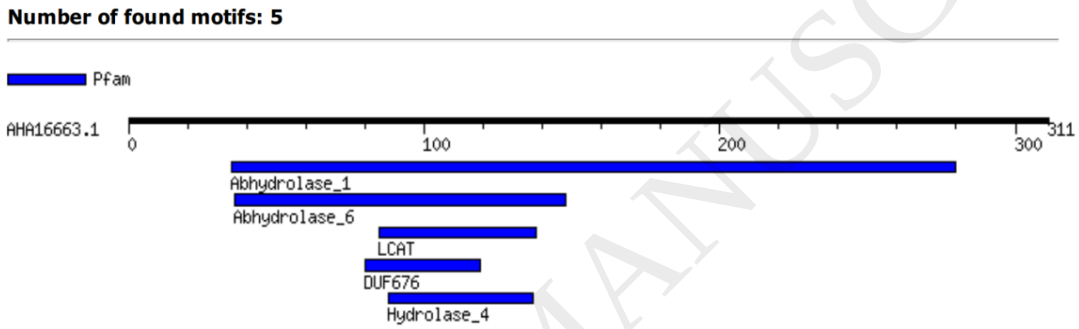


Fig. 10. Motif serach result showing functional motifs and their position.

Conclusions

Lipases are enzyme that hydrolyse long chain triacylglycerol at an oil-water interface and can also synthesize ester in non-aqueous condition. Lipases are variable in their property depending on their sources and gained great demands in various fields. In this study it was found that the phylogenetic tree obtained from amino acid sequences of the lipase protein and cDNA of *P. aeruginosa* clustered with *P. stutzeri*. The phylogenetic study of amino acid sequence of lipase protein and cDNA among different strains of *P. aeruginosa* indicated that *P. aeruginosa* PA1 clustered with *P. aeruginosa* SJTD-1. The lipase of *P. aeruginosa* PA1 was found monomeric, acidic and thermostable protein having molecular weight ranging in between 32.72 to 34.89 kDa. Random coils and alpha helices were abundant in its secondary structure. Moreover, tertiary model showed 96.310 score as overall quality factor. Hence, this *in silico* study is very important in terms of gathering useful information about the lipase protein without performing any crystal structure assessment by X-ray Crystallography or NMR study. So, it might be helpful for isolation and characterization of this enzyme in wet lab.

Conflict of interest

Authors declare no conflict of interest.

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L. Conferences:

1. Oral presentation: “Inhibitory effects of Luteolin, a flavonoid on Salmonella Typhi and on its Dihydrofolate Reductase protein” at **32nd West Bengal State Science and Technology Congress, 2025**.
2. Oral presentation: “Inhibitory effects of Luteolin, a flavonoid on Salmonella Typhi and on its Dihydrofolate Reductase protein” at **7th Regional Sciences and Technology Congress, 2025**.
3. Oral presentation: “The antityphoid efficacy of 2-Benzoxazolinone (BOA) identified from active fractions of root extract of Scoparia dulcis” at **AMR Conferences, 2024**.
4. Poster presentation: “Fighting Antimicrobial Resistance Together” at **World AMR Awareness Week, 2023**.
5. Poster Presentation: “In silico screening and docking of phytochemical constituents from selected fractions of Scoparia dulcis root extract with antibacterial activity against Salmonella typhi” at **91st Annual Meeting of the Society of Biological Chemists (India), 2022**.
6. Poster presentation: “In vitro screening of Scoparia dulcis root extract for its anti-Typhoid activity with in silico docking studies of its selected phytochemical constituents” at **17th Asian Conference on Diarrhoeal Disease and Nutrition, 2022.**

Inhibitory effects of Luteolin, a flavonoid on *Salmonella* Typhi and on its Dihydrofolate Reductase protein

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Luteolin is a naturally occurring flavonoid found in many fruits and vegetables and reported to have health benefits. It's an allelochemical produced by plants as a defence against pathogens. In the previous report, we identified its presence in *Scoparia dulcis* root extract, a well-known herb. In this report, we evaluated luteolin's activity against multidrug-resistant clinical isolates of *Salmonella* Typhi. *Salmonella* Typhi is a common food contaminant and a causal organism for human-restricted typhoid. WHO estimates as of 2019, there are 9 million cases of typhoid annually, resulting in about 110,000 deaths per year. The emergence of drug-resistant mutants of *Salmonella* Typhi has become a growing concern which demands alternatives to traditional antibiotics. Luteolin in *in vitro* test had MIC (Minimum Inhibitory Concentration) value ranging from $\leq$ 0.5 mg/ml to >0.01 mg/ml against tested *Salmonella* Typhi isolates and its effect can cause a decrease in growth rate. It also had a negative effect on biofilm formation and disruption and can restrict the *Salmonella* Typhi cells from invading mammalian macrophage cells. Luteolin, which in the computational screen showed druglike properties and was predicted to fall under toxicity class 4, had shown great binding affinity to the Dihydrofolate Reductase protein of *Salmonella* Typhi in virtual docking. And in *in vitro* setup, it showed a negative effect on the protein's activity.

Government of West Bengal
Department of Science & Technology and Biotechnology

32nd West Bengal State Science & Technology Congress

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Inhibitory Effects of Luteolin, A Flavonoid on Salmonella Typhi and on its Dihydrofolate Reductase Protein
in the 32nd West Bengal State Science & Technology Congress held from 28th February to 2nd March, 2025
at Biswa Bangla Convention Centre, Kolkata.

The Co-authors were/was
Shanta Dutta, Utpal Mohan, Devendra Kumar Dhaked and Ravichandiran Velayutham

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The antityphoid efficacy of 2-Benzoxazolinone (BOA) identified from active fractions of root extract of Scoparia dulcis

Sunayana Saren[1]*, Paulami Dutta*[1]*, Utpal Mohan*[2]*, Devendra Kumar Dhaked*[2]*, Sohini Sikdar*[1]*, Ravichandiran Velayutham*[2]*, Shanta Dutta*[1]***

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Background: Scoparia dulcis is a well-known herb traditionally used for its anti-inflammatory, antioxidant, antimicrobial, anti-diabetic, and wound-healing properties. But there are very few works have been done to evaluate the antibacterial activity of *Scoparia dulcis* root extract especially against Salmonella Typhi. In our research work, we identified the presence of the active compound 2-Benzoxazolinone (BOA) in active fractions of root extract of Scoparia dulcis. BOA is a phytochemical that acts as a defense mechanism in plants against pathogens and herbivores.

Objective: The objective of our study was to evaluate the antibacterial activity of BOA against Salmonella Typhi.

Material and Methods: The physicochemical properties were computationally screened to detect drug-likeness and to predict possible toxicity. We performed microbroth dilution to detect MIC and MBC, time-kill assay, and antibiofilm assay against Salmonella Typhi strains. The computational docking was performed with two of the Salmonella proteins murA and folA. The in vitro enzyme assay was performed to test the inhibition of folA activity in presence of BOA. The therapeutic activity was studied on BALB/c mice which were challenged with a clinical isolate of Salmonella Typhimurium.

Results: The computational screen of physicochemical properties showed that BOA had zero violation in Lipinski's rule of five in Druglikeness and its fall under acute toxicity class IV with predicted LD50 of 890 mg/kg (in mice). MIC and MBC of BOA ranged from $\leq$ 1.87 mg/ml to $\geq$ 0.46 mg/ml and $\leq$ 3.75 mg/ml to $\geq$ 0.93 mg/ml respectively. The *Salmonella* Typhi strains showed a decrease in growth rate in the presence of BOA and had mild antibiofilm activity. In docking, BOA showed more affinity towards folA protein with a docking score of -6.01. The enzyme assay showed the inhibition of the activity of folA in the presence of BOA. In In vivo, test groups treated with BOA showed 100 percent survival and less colonization of Salmonella Typhimurium in the liver and intestine compared to the untreated group with 33 percent survival.

Conclusions: BOA a naturally occurring active compound which was identified from fractions of Scoparia dulcis root extracts in our research work, has antibacterial activity against *Salmonella* Typhi and has the potential to be used as therapeutics to treat typhoid.

AMR CONFERENCE 2024

Theme: ""Exploring the Bioresources of India to fight against Antimicrobial Resistance (AMR)"

Organized by

Institute of Bioresources and Sustainable Development (IBSD)
Takyelpat, Imphal, Manipur, India

ICMR-National Institute of Cholera and Enteric Diseases (ICMR-NICED)
CIT Road, Beliaghata, Kolkata, India

In Association with

Society for Ethnopharmacology (SFE)
Jadavpur, Kolkata, India

April 04-05, 2024

Venue: Science City Auditorium, J.B.S. Haldane Avenue Kolkata-700 046, India

Certificate for Poster/ Oral Presentation

This to certify that

~~Mr.~~/Ms./~~Prof.~~/~~Dr.~~ .Sunayana Sahen..... participated & presented a paper in the ~~Poster~~/ Oral presentation session in in the National Conference on Antimicrobial Resistance (AMRC 2024) at Science City, Kolkata, India during April 04-05, 2024.

S. Dutta

Dr. Shanta Dutta
Chairman, AMRC 2024 &
Director, ICMR-NICED

Dr. S. Indira Devi
Organizing Secretary, AMRC 2024 &
Scientist, IBSD

Sushmita Bhattacharya

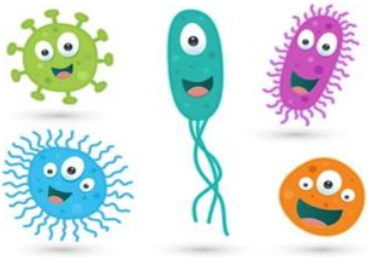
Dr. Sushmita Bhattacharya
Organizing Secretary, AMRC 2024 &
Scientist, ICMR-NICED

Fighting Antimicrobial Resistance Together

What is Antibiotic

Antibiotics are medicines that treat or prevent bacterial infections in humans and animals. They work by killing bacteria or making it difficult for them to grow and multiply

Antibiotic



Microorganisms

What is the problem?

Superbugs are strains of bacteria, viruses, parasites and fungi that are resistant to most of the antibiotics and other medications commonly used to treat the infections they cause

Microorganisms are Getting Stronger

Antimicrobial resistance (AMR) is when microbes, like bacteria, viruses, fungi, and parasites, develop the ability to resist the effects of antimicrobials.

What we can do?

WASH YOUR HANDS RELIGIOUSLY

Only take antibiotics prescribed by a qualified doctor

FIVE DAYS MEANS FIVE DAYS; NO CHEAT DAY!

Always complete full course of antibiotics

Don't purchase/ sell medicine without a doctor's prescription if it shows a red line on the strip

Presenter:
Sunayana Saren,
Bacteriology

ICMR INDIAN COUNCIL OF MEDICAL RESEARCH | NICED NATIONAL INSTITUTE OF CHOLERA AND ENTERIC DISEASES

ICMR-National Institute of Cholera and Enteric Diseases
Indian Council of Medical Research

WORLD AMR AWARENESS WEEK
18th - 24th November 2023

Certificate of Participation

This is to certify that ~~Mr.~~ / Mrs. Sunayana Saren has participated in the poster making competition on the topic "Fighting Antimicrobial Resistance Together".

S. Dutta

Dr. Shanta Dutta
Director
ICMR-NICED

In silico screening and docking of phytochemical constituents from selected fractions of *Scoparia dulcis* root extract with antibacterial activity against *Salmonella* Typhi

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Abstract: Typhoid fever, caused by *Salmonella* Typhi progresses to life-threatening conditions if not treated with appropriate antibiotics due to the emergence of multidrug-resistant bacteria. This emergence of multidrug-resistant *S.* Typhi hindered effective treatment of the cases, leading to the need for the development of alternatives to traditional antibiotics. In this study, we evaluated the antibacterial activity of *Scoparia dulcis* root extract against *S.* Typhi using one standard strain and two clinical isolates. The TLC-bioautography indicated the growth inhibition activity by the extract. The minimum bactericidal concentrations (MBCs) of the extract against test strains were ranged from $\leq$ 15 mg/ml to > 3.75 mg/ml. The time-kill assay indicated the activity was time- as well as concentration-dependent. The crude extract was fractionated into 13 fractions by column chromatography of which, five fractions with improved minimum inhibitory concentrations (MICs) and MBC were selected. Through LC-MS a total of 43 compounds were identified from those five fractions and were computationally screened to state their drug-likeness using the SwissADME web tool. Out of those, 35 compounds showed drug-likeness and were computationally docked with the Dihydrofolate reductase (folA) protein of *S.* Typhi using Schrodinger's Maestro Software. Compound (2R)-7-methoxy-2H-1,4-benzoxazin-3(4H)-one 2-O-β-galactopyranoside showed the highest docking score of -10.9. The stability of the compound-protein was observed by studying the molecular dynamic (MD) using Schrodinger's Desmond Software. The average MMGBSA Energy value of the complex was -56.53 ± 10.59 at 100 nanoseconds simulation. Further *in vitro* and *in vivo* studies are necessary to comment on the effectiveness of identified compounds against S. Typhi.

In silico screening and docking of phytochemicals constituents from selected fractions of *Scoparia dulcis* root extract with antibacterial activity against *Salmonella typhi*

Sunayana Saren[1], Devendra Kumar Dhaked[2], Utpal Mohan[2], Ravichandiran Velayutham[2], **Shanta Dutta**[1*]

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BACKGROUND

- Typhoid or enteric fever is a bacterial infection caused by *Salmonella enterica subspecies enterica* serovar Typhi (*S.* Typhi), a Gram-negative, rod-shaped flagellated bacterium restricted to humans only.
- Typhoid often progresses to life-threatening conditions if not treated with appropriate antibiotics.
- Over the time *S.* Typhi disappeared from many high-income countries (HICs) while in many low- to middle-income countries (LMICs) increasingly resistant variants remain as endemic strains.
- The International Vaccine Institute estimated 11.9 million cases and 129,000 deaths in LMICs in 2010.

Extensively drug-resistant (XDR) H58 clade is the most worrisome event in the evolutionary history of *S.* Typhi.
Azithromycin is currently the last effective and affordable antibiotic but azithromycin-resistant strains have been already reported.

→ This emergence of antimicrobial-resistant *S.* Typhi hindered effective treatment of the cases leading to the need for the development of alternative medicines in addition to the traditional antibiotics.

→ Plants can be a good source of effective antimicrobial compounds with reduced toxicity and less side effects which are the major shortcomings of synthetic drugs.
The bioactive compounds from plant sources may be combined with commercial drugs to have synergistic effects.

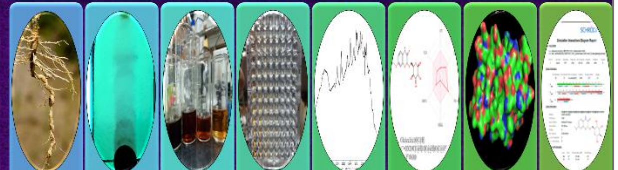
AIM

- Evaluate the antibacterial effect of ***Scoparia dulcis* root extract** against *Salmonella* Typhi to validate its **anti-typhoid activity**.

Scoparia dulcis L. (Plantaginaceae)

- **Identify the phytochemical constituents** from most active fractions of *Scoparia dulcis* root extract.
- ***In silico* screening and docking study** of identified phytochemical constituents.

METHODOLOGY



1. The *Scoparia dulcis* root samples were extracted in methanol by Maceration technique.
2. The crude extract was tested for its antibacterial effect against *S.* Typhi by performing TLC-Bioautography, Microbroth dilution, and Time-kill assay using one standard strain (MTCC734), and two clinical isolates (KOL557 and KOL558)
3. Crude extract was fractionated by Column Chromatography using solvents of different polarity viz. Petroleum Ether-Ethyl acetate (1:0, 4:1, 3:2, 2:3, 1:4, 0:1), Methanol, Water.
4. Each collected fraction was tested for its antibacterial effect against *S.* Typhi by performing Microbroth dilution.
5. The presences of phytochemical constituents were identified in most active fractions by LC-MS.
6. Identified phytochemicals were computationally screened to state their drug-likeness using the SwissADME web tool.
7. The phytochemicals or compounds were computationally docked with Dihydrofolate reductase (folA) protein of S. Typhi in Schrödinger's Maestro Software.
8. The molecular dynamic (MD) of the protein-compound complex with good docking score was studied in Schrödinger's Desmond Software.

RESULT and DISCUSSION

1. TLC-Bioautography

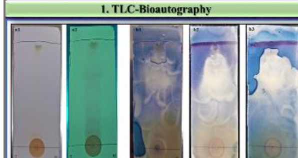


Fig.1: TLC plates; a1, a2: showing separated bands under visual light and UV light respectively; b1, b2, b3: showing blue area of bacterial growth (of *S.* Typhi MTCC734, *S.* Typhi KOL557, *S.* Typhi KOL558 respectively) surrounding the clear zone which indicates the growth inhibition activity.

2. Microbroth Dilution

- The **crude extract was most effective against** the standard strain, **MTCC734** with The Minimum Bactericidal Concentration (**MBC**) **ranging from ≤7.5 mg/mL to >3.75 mg/mL**.
- In both the clinical isolates **(KOL557 and KOL558)** the **MBCs ranged from ≤15 mg/mL to >7.5 mg/mL**.

3. Time-Kill Assay

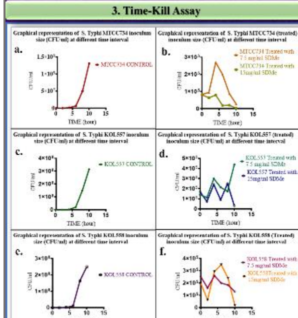


Fig.2: The graphical representation of the time-kill curve of S. Typhi strains (Left: control; Right: treated) at 10 hours of time span (a, b: time-kill curve of MTCC734; c, d: time-kill curve of KOL557; e, f: time-kill curve of KOL558; CFU/mL: Colony Forming Unit per milliliter; SDMe: methanolic crude extract of *S. dulcis* root).

- **The growth patterns and growth rates indicated time-dependent as well as concentration-dependent activity**

4. Column Chromatography

- **The crude extract was separated into 13 fractions.**
- Five fractions (SDFRC1, SDFRC2, SDFRC3, SDFRC4, and SDFRC5) were collected in pet. ether-ethyl acetate mixture (4:1, 3:2, 2:3, 1:4, 0:1 respectively), in 100% methanol five fractions (SDFRC6 to SDFRC10) and in water three fractions (SDFRC11, SDFRC12 and SDFRC13) were collected.

5. Microbroth dilution of collated fractions

Table 1: The MIC and MBC of isolated fractions against *Salmonella* Typhi strains

Fractions	MTCC 734		KOL557		KOL558	
	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)
SDFRC1	≤3.75 (>1.8)	≤15 (>7.5)	≤7.5 (>3.75)	≤15 (>7.5)	≤7.5 (>3.75)	≤15 (>7.5)
SDFRC2	≤3.75 (>1.8)	≤3.75 (>1.8)	≤3.75 (>1.8)	≤7.5 (>3.75)	≤3.75 (>1.8)	≤7.5 (>3.75)
SDFRC3	≤3.75 (>1.8)	≤7.5 (>3.75)	≤7.5 (>3.75)	≤7.5 (>3.75)	≤7.5 (>3.75)	≤7.5 (>3.75)
SDFRC4	≤3.75 (>1.8)	≤3.75 (>1.8)	≤7.5 (>3.75)	≤7.5 (>3.75)	≤7.5 (>3.75)	≤7.5 (>3.75)
SDFRC5	≤7.5 (>3.75)	≤7.5 (>3.75)	≤7.5 (>3.75)	≤7.5 (>3.75)	≤7.5 (>3.75)	≤15 (>7.5)
SDFRC6	≤7.5 (>3.75)	≤15 (>7.5)	≤15 (>7.5)	>15	≤15 (>7.5)	≤15 (>7.5)
SDFRC7	≤15 (>7.5)	≤15 (>7.5)	≤15 (>7.5)	≤15 (>7.5)	≤15 (>7.5)	≤15 (>7.5)
SDFRC8	≤15 (>7.5)	>15	≤15 (>7.5)	>15	≤15 (>7.5)	>15
SDFRC9	>15	>15	>15	>15	>15	>15
SDFRC10	≤15 (>7.5)	≤15 (>7.5)	>15	>15	≤15 (>7.5)	≤15 (>7.5)
SDFRC11	≤15 (>7.5)	≤15 (>7.5)	≤15 (>7.5)	≤15 (>7.5)	≤15 (>7.5)	≤15 (>7.5)
SDFRC12	≤15 (>7.5)	≤15 (>7.5)	≤15 (>7.5)	>15	≤15 (>7.5)	≤15 (>7.5)
SDFRC13	≤15 (>7.5)	≤15 (>7.5)	≤15 (>7.5)	≤15 (>7.5)	≤15 (>7.5)	≤15 (>7.5)

6. LC-MS

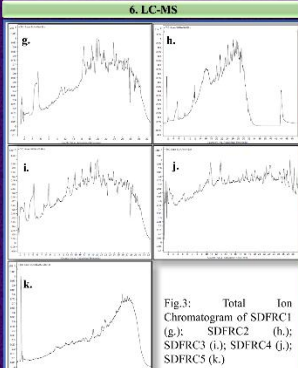


Fig.3: Total Ion Chromatogram of SDFRC1 (g.); SDFRC2 (h.); SDFRC3 (i.); SDFRC4 (j.); SDFRC5 (k.)

Table 2: List of 43 Identified compounds in the selected five fractions

Serial No.	Name of the compound	Molecular formula	M/Z	Mass (g/mol)	Fractions
1	(2R)-7-methoxy-2H-1,4-benzoxazin-3(4H)-one 2-O-β-galactopyranoside	$C_{15}H_{19}NO_9$	380.096	357.1074	SDFRC3
2	3-hydroxy-6-methoxy-2-benzoxazolinone	$C_8H_7NO_4$	182.045	181.0385	SDFRC1
3	4-epi-scopadulcic acid B	$C_{27}H_{34}O_5$	439.25	438.2394	SDFRC4
4	5,7,4'-trihydroxy-3'-methoxyisoflavone	$C_{16}H_{12}O_6$	301.071	300.0635	SDFRC1, SDFRC2, SDFRC3
5	6-methoxybenzoxazolinone	$C_8H_7NO_3$	166.05	165.0428	SDFRC1, SDFRC2, SDFRC3, SDFRC4, SDFRC5
6	Acacetin	$C_{16}H_{12}O_5$	285.075	284.0683	SDFRC2, SDFRC3
7	Apigenin	$C_{15}H_{10}O_5$	271.06	270.0531	SDFRC2, SDFRC3, SDFRC4, SDFRC5
8	Apigenin 6-C-pentosyl-8-C-hexoside	$C_{26}H_{28}O_{14}$	586.132	563.1356	SDFRC2
9	Apigenin 7-O-R-L-3-O-acetylrhamnopyranosyl-(1→6)-β-D glucopyranoside	$C_{29}H_{32}O_{15}$	621.181	620.1744	SDFRC5
10	Benzoxazine	C_8H_7NO	134.06	133.0835	SDFRC2
11	Benzoxazolinone	$C_7H_5NO_2$	136.039	135.0331	SDFRC1, SDFRC2, SDFRC3
12	Betulinic acid	$C_{30}H_{48}O_3$	457.368	456.3588	SDFRC1, SDFRC2, SDFRC3, SDFRC4, SDFRC5
13	Cirsiliol	$C_{17}H_{14}O_7$	353.067	330.0759	SDFRC2
14	Cirsimaritin	$C_{17}H_{14}O_6$	315.086	314.0816	SDFRC1, SDFRC3, SDFRC4, SDFRC5
15	Coixol	$C_8H_7NO_3$	178.05	177.0436	SDFRC2, SDFRC3, SDFRC4, SDFRC5
16	Dulcidiol	[illegible]	427.281	426.2743	SDFRC2
17	Ferruginoside C	[illegible]	821.295	798.2939	SDFRC4
18	Gentisic acid	$C_7H_6O_4$	155.034	154.0264	SDFRC1, SDFRC3
19	Ginsenoside F1	$C_{36}H_{62}O_9$	661.428	638.4403	SDFRC3
20	Glutinol	$C_{30}H_{50}O$	427.393	426.3855	SDFRC2
21	Gonzalitosin I	$C_{18}H_{16}O_6$	329.102	328.0951	SDFRC3, SDFRC4
22	Hexacosanol	$C_{26}H_{54}O$	419.373	396.3833	SDFRC3
23	Hydroxy-tetramethoxyflavone	$C_{19}H_{18}O_7$	359.113	358.1054	SDFRC1
24	Hymenoxin	$C_{19}H_{18}O_8$	375.107	374.1001	SDFRC4
25	Ifflaionic acid	$C_{30}H_{46}O_3$	455.353	454.3452	SDFRC1
26	Isorhoifolin	$C_{27}H_{30}O_{14}$	579.171	578.1667	SDFRC1, SDFRC3
27	Linarin	$C_{28}H_{32}O_{14}$	593.187	592.1793	SDFRC4
28	Luteolin	$C_{15}H_{10}O_6$	287.055	286.0589	SDFRC2, SDFRC4
29	Nevadensin	$C_{18}H_{16}O_7$	345.096	344.0905	SDFRC1, SDFRC2
30	Nicotiflorin	$C_{27}H_{30}O_{15}$	595.168	594.1619	SDFRC2, SDFRC3, SDFRC4
31	Nicotinic acid	$C_6H_5NO_2$	124.039	123.0673	SDFRC3
32	Palmitic amide	$C_{16}H_{33}NO$	256.263	255.2599	SDFRC2, SDFRC3
33	P-coumaric acid	$C_9H_8O_3$	165.055	164.0468	SDFRC1, SDFRC3
34	Scopadulcic acid A	$C_{27}H_{34}O_6$	477.231	454.242	SDFRC1, SDFRC3
35	Scopanolal	$C_{27}H_{36}O_5$	447.25	424.2558	SDFRC1, SDFRC3, SDFRC4, SDFRC5
36	Scoparic acid A	$C_{27}H_{36}O_5$	463.171	440.1864	SDFRC2, SDFRC3, SDFRC4
37	Scoparic acid B	$C_{27}H_{32}O_5$	413.232	412.2252	SDFRC2, SDFRC3
38	Scoparic acid C	$C_{26}H_{32}O_5$	447.213	424.2238	SDFRC2, SDFRC4, SDFRC5
39	Scoparic acid D	$C_{19}H_{28}O_4$	305.175	292.1785	SDFRC1
40	Stigmasterol	$C_{29}H_{48}O$	413.378	412.3712	SDFRC4, SDFRC5
41	Trans Acteoside	$C_{29}H_{36}O_{15}$	625.21	624.2035	SDFRC4, SDFRC5
42	Trihydroxyoctadecadienoic acid	$C_{18}H_{32}O_5$	351.214	328.2244	SDFRC2, SDFRC3, SDFRC4, SDFRC5
43	Tuberonic acid glucoside	$C_{18}H_{28}O_9$	411.165	388.1757	SDFRC1

7. Drug-likeness

Out of 43, **eight violated** (Nicotiflorin, trans Acteoside, Apigenin 6-C-pentosyl-8-C-hexoside, Apigenin 7-O-α-L-3-O-acetylrhamnopyranosyl-(1→6)-β-D glucopyranoside, Ferruginoside C, Ginsenoside F1, Isorhoifolin, and Linarin) **Lipinski's rule of five for drug-likeness**.

8. Docking

Table.3: Docking score of compounds with folA protein

COMPOUNDS	Score (XP)	Interacting amino acids
(2R)-7-methoxy-2H-1,4-benzoxazin-3(4H)-one 2-O-β-galactopyranoside	-10.9	ALA7, ASN18, PHE31(PI-PI)
trihydroxyoctadecadienoic acid	-10.58	ASN18, ALA19, TRP22, LEU24
Cirsiliol	-10.1	ALA7, ASN18, SER49
5,7,4' trihydroxy 3' methoxyisoflavone	-8.79	ILE14, ASP27, SER49
co-folA	-8.42	ARG52, ARG57, ASP122
luteolin	-8.35	ALA7, ASN18
Tuberonic acid glucoside	-8.09	MET20, ASP27, MET20, SER49, ARG52
Cirsimaritin	-7.79	ALA7, ASN18
3-hydroxy-6-methoxy-2-benzoxazolinone	-7.71	ALA7, TYR100
stigmasterol	-7.6	
Nicotinic_acid	-7.55	ALA7, THR46, ILE94
Gonzalitosin_I	-7.27	ILE5, PHE31(Pi-Pi), ARG52
coixol	-7.1	ALA7
apigenin	-7.07	ALA7, ASN18
acacetin	-6.91	ILE5, ASP27, PHE31(Pi-Pi)
nevadensin	-6.8	ARG52, ILE94
Hymenoxin	-6.78	PHE31(Pi-Pi), ARG52, ILE94
gentisic_acid	-6.77	ALA7, ILE14, ILE94
Benzoxazine	-6.74	ILE94
Scoparic_acid_C	-6.37	ARG52
Hydroxy-tetramethoxyflavone	-6.16	ARG52
2-Benzoxazolinone	-6.01	ALA7
6-Methoxy-2-benzoxazolinone	-5.75	ALA7
p-coumaric_acid	-5.56	ASP27, ILE94
Scoparic acid B	-4.74	MET20, PHE31(PI-PI), SER49, ARG52
Scoparic acid A	-4.46	ASN23, PHE31(PI-PI), SER49
Scopanolal	-4.33	MER20, SER49
Palmitic amide	3.37	ASN18, THR46
Ifflaionic acid	2.35	
Glutinol	1.84	
4-epi-scopadulcic_acid_B	-0.89	

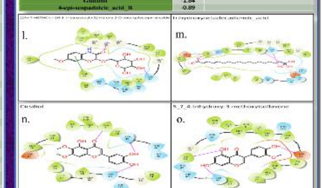


Fig.4: 2D diagram of (2R)-7-methoxy-2H-1,4-benzoxazin-3(4H)-one 2-O-β-galactopyranoside (l.), Trihydroxyoctadecadienoic acid (m.), Cirsiliol (n.), and 5,7,4'-trihydroxy-3'-methoxyisoflavone (o.) interacting with amino acids of folA protein.

9. Molecular Dynamic

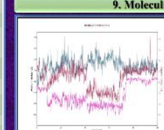


Fig.5: Protein-Ligand Root Mean Square Deviation (RMSD)

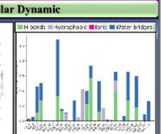


Fig.6: Protein-Ligand Contacts

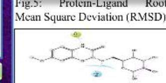


Fig.7: A schematic of detailed ligand atom interactions with the protein residues. Interactions that occur more than 30% of the simulation time.

- **The average** Molecular mechanics with generalized Born and surface area solvation **(MMGBSA) Energy value was -**

CONCLUSION AND FUTURE WORK

- **The *Scoparia dulcis* root extract showed antibacterial activity against *Salmonella* Typhi test stains** at certain concentrations and The antibacterial activity could be due to its possession of active phytochemicals.
- The identified phytochemicals from the most active fraction of the crude extract can lead us to a potential anti-typhoid drug.
- The phytochemicals with good docking scores to S. Typhi proteins could be a potential inhibitor to target those proteins.
- The **(2R)-7-methoxy-2H-1,4-benzoxazin-3(4H)-one 2-O-β-galactopyranoside**, which had a good docking score with folA protein and showed overall stability in the molecular dynamic study and good average MMGBSA energy **could be developed into an antityphoid drug to target folA protein.**
- Further *in vitro* and *in vivo* studies with (2R)-7-methoxy-2H-1,4-benzoxazin-3(4H)-one 2-O-β-galactopyranoside and other identified phytochemicals are necessary.

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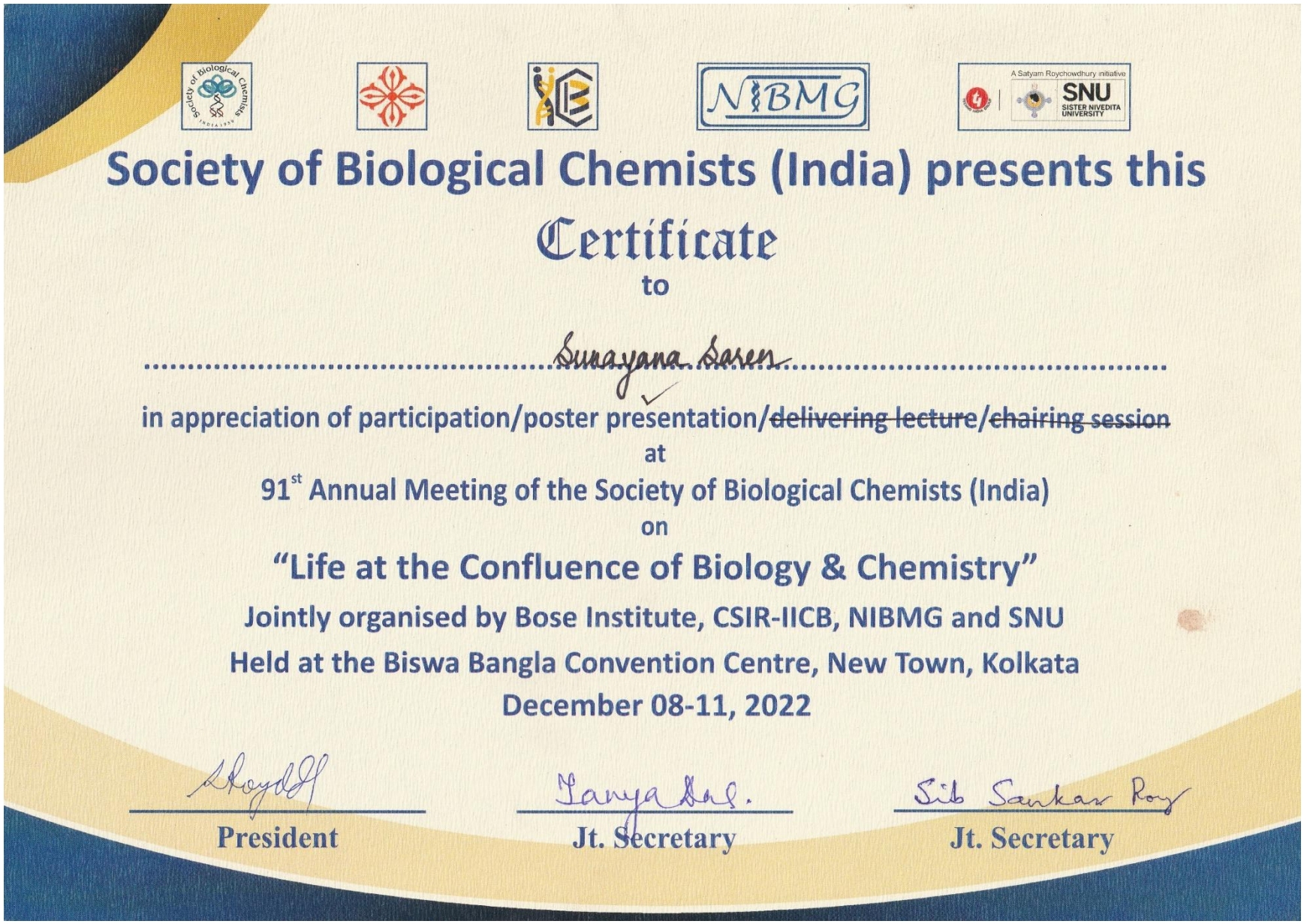
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In vitro screening of *Scoparia dulcis* root extract for its anti-Typhoid activity with *in silico* docking studies of its selected phytochemical constituents

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Background: The increasing antimicrobial resistances in *Salmonella* Typhi strains, predominantly in low- to middle-income countries is a growing concern among physicians, which leads to need for development of alternatives. The traditionally used plants, a source of potential bioactive compounds could show antibacterial activity against *S.* Typhi with reduce toxicity and less side effects. Attempts need to be made for development of ethnomedicine against typhoid fever.

Methods: The *Scoparia dulcis* roots were extracted in methanol and fractionated by column chromatography. The minimum bactericidal concentrations (MBCs) of crude extract and collected fractions were determined using one standard referral strain (MTCC734) and two clinical isolates (KOL557 and KOL558) of *S.* Typhi. The compounds were identified from the most active fraction by LC-MS and computationally screened to state their drug-likeness and docked with UDP-N-acetylglucosamine 1-carboxyvinyltransferase (murA) and Dihydrofolate reductase (folA) protein of *S.* Typhi followed by molecular dynamic (MD) study of selected compound in Schrödinger Software.

Result: The MBC of crude extract against test strains were ranged from ≤ 15 mg/ml to > 3.75 mg/ml. The crude extract was fractionated in 13 fractions, of which SDFRC2 showed improved activity in comparisons to other fractions with MBC range of ≤7.5 mg/ml to >1.8 mg/ml. A total of 20 compounds were identified from SDFRC2 by LC-MS; of these two compounds violated the Lipinski's rule of five for drug-likeness. In docking study, the compound trihydroxyoctadecadienoic acid (ligand) showed best score of -10.58 to folA protein by interacting with ASN18, ALA19, TRP22, LEU24 amino acids. The MD study of folA-trihydroxyoctadecadienoic acid showed that in 100 nanoseconds stimulation time the Root Mean Square Deviation (RMSD) of protein and ligand were within the accepted range of stability. The average free energy for binding of trihydroxyoctadecadienoic acid to folA protein, calculated by molecular mechanics-generalized Born surface area (MMGBSA) was -51.55 ± 5.31. The ligand had high interaction with ASN18 amino acid of folA protein in form of H-bond, ionic bond and water bridge.

Conclusion: The *S. dulcis* root extract can cause cell death of *S.* Typhi strains at certain concentration and can be a source of bioactive compounds having anti-typhoid activity. The compounds with good docking score could be the potential candidate for developing of anti-typhoid drug.

Keywords: *Salmonella* Typhi; *Scoparia dulcis*; Antityphoid; *in silico*; Protein-drug docking

In vitro screening of *Scoparia dulcis* root extract for its anti-Typhoid activity with *in silico* docking studies of its selected phytochemical constituents

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BACKGROUND

- Typhoid or enteric fever is a bacterial infection caused by *Salmonella enterica subspecies enterica* serovar Typhi (*S.* Typhi), a Gram-negative, rod-shaped flagellated bacterium restricted to humans only.
- Typhoid often progresses to life-threatening conditions if not treated with appropriate antibiotics.
- Over the time *S.* Typhi disappeared from many high-income countries (HICs) while in many low- to middle-income countries (LMICs) increasingly resistant variants remain as endemic strains.
- The International Vaccine Institute estimated 11.9 million cases and 129,000 deaths in LMICs in 2010.

Extensively drug-resistant (XDR) H58 clade is the most worrisome event in the evolutionary history of *S.* Typhi. Azithromycin is currently the last effective and affordable antibiotic but azithromycin-resistant strains have been already reported.

→ This emergence of antimicrobial-resistant *S.* Typhi hindered effective treatment of the cases leading to the need for the development of alternative medicines in addition to the traditional antibiotics.

→ Plants can be a good source of effective antimicrobial compounds with reduced toxicity and less side effects which are the major shortcomings of synthetic drugs. The bioactive compounds from plant sources may be combined with commercial drugs to have synergistic effects.

AIM

- Evaluate the antibacterial effect of ***Scoparia dulcis* root extract** against *Salmonella* Typhi to validate its **anti-typhoid activity**.
- **Identify the phytochemical constituents** from most active fractions of *Scoparia dulcis* root extract.
- ***In silico* screening and docking study** of identified phytochemical constituents.

METHODOLOGY

The Scoparia dulcis root samples were extracted in methanol by Maceration technique.

→ The crude extract was tested for its antibacterial effect against S. Typhi by performing TLC-Bioautography, Microbroth dilution, and Time-kill assay using one standard strain (MTCC734), and two clinical isolates (KOL557 and KOL558)

→ Crude extract was fractionated by Column Chromatography using solvents of different polarity viz. Petroleum Ether-Ethyl acetate (1:0, 4:1, 3:2, 2:3, 1:4, 0:1), Methanol, Water.

→ Each collected fractions were tested for its antibacterial effect against S. Typhi by performing Microbroth dilution.

→ The presences of phytochemical constituents were identified in most active fractions by LC-MS.

→ Identified phytochemicals were computationally screened to state their drug-likeness using the SwissADME web tool.

→ The phytochemicals or compounds were computationally docked with UDP-N-acetylglucosamine 1-carboxyvinyltransferase (murA) and Dihydrofolate reductase (folA) protein of S. Typhi in Schrödinger's Maestro Software.

→ The molecular dynamic (MD) of the protein-compound complex with good docking score was studied in Schrödinger's Desmond Software.

RESULT and DISCUSSION

1. TLC-Bioautography

The **clear zone surrounded by the blue area** appeared on the TLC plates (Fig.1). **indicated that the extract** might possess some compounds which were able **to inhibit the growth of *S.* Typhi test strains.**

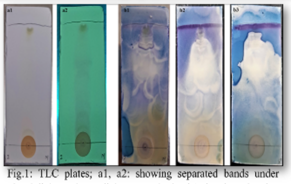

Fig.1: TLC plates; a1, a2: showing separated bands under visual light and UV light respectively; b1, b2, b3: showing blue area of bacterial growth (of S. Typhi MTCC734, S. Typhi KOL557, S. Typhi KOL558 respectively) surrounding the clear zone.

2. Microbroth Dilution

- The **crude extract was most effective against** the standard strain, **MTCC734** with The Minimum Bactericidal Concentration **(MBC) ranging from ≤7.5 mg/mL to >3.75 mg/mL**.
- In both the clinical isolates **(KOL557 and KOL558) the MBCs ranged from ≤15 mg/mL to >7.5 mg/mL**

3. Time-Kill Assay

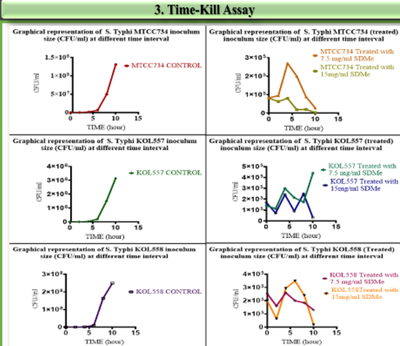

Fig.2: The graphical representation of the time-kill curve of S. Typhi strains (Left: control; Right: treated) at 10 hours of time span (a, b: time-kill curve of MTCC734; c, d: time-kill curve of KOL557; e, f: time-kill curve of KOL558; CFU/ml: Colony Forming Unit per milliliter; SDMe: methanolic crude extract of *S. dulcis* root).

- Both the concentrations of crude extract were bactericidal towards MTCC734 and KOL558 strains after 24-hour incubation; but only 15 mg/mL concentration of the extract was bactericidal to KOL557 strain after 24-hour incubation.
- **The growth patterns and growth rates indicated time-dependent as well as concentration-dependent activity**

4. Column Chromatography

- **The crude extract was separated into 13 fractions.**
- Five fractions (SDFRC1, SDFRC2, SDFRC3, SDFRC4, and SDFRC5) were collected in pet. ether-ethyl acetate mixture (4:1, 3:2, 2:3, 1:4, 0:1 respectively), in 100% methanol five fractions (SDFRC6 to SDFRC10) and in water three fractions (SDFRC11, SDFRC12 and SDFRC13) were collected.

5. Microbroth dilution of collated fractions

- Out of 13, **SDFRC2 showed improved activity** in comparison to the crude and other fractions.
- The SDFRC2 had MIC and MBC ranges from ≤3.75 mg/mL to >1.8 mg/mL and from ≤7.5 mg/mL to >1.8 mg/mL respectively.
- SDFRC2 was selected for further study

Table 1: The MIC and MBC of isolated fractions against *Salmonella* Typhi strains

Fractions	MTCC 734		KOL557		KOL558	
	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)
SDFRC1	≤3.75 (>1.8)	≤15 (>7.5)	≤7.5 (>3.75)	≤15 (>7.5)	≤7.5 (>3.75)	≤15 (>7.5)
SDFRC2	≤3.75 (>1.8)	≤3.75 (>1.8)	≤3.75 (>1.8)	≤7.5 (>3.75)	≤3.75 (>1.8)	≤7.5 (>3.75)
SDFRC3	≤3.75 (>1.8)	≤7.5 (>3.75)	≤7.5 (>3.75)	≤7.5 (>3.75)	≤7.5 (>3.75)	≤7.5 (>3.75)
SDFRC4	≤3.75 (>1.8)	≤3.75 (>1.8)	≤7.5 (>3.75)	≤7.5 (>3.75)	≤7.5 (>3.75)	≤7.5 (>3.75)
SDFRC5	≤7.5 (>3.75)	≤7.5 (>3.75)	≤7.5 (>3.75)	≤7.5 (>3.75)	≤7.5 (>3.75)	≤15 (>7.5)
SDFRC6	≤7.5 (>3.75)	≤15 (>7.5)	≤15 (>7.5)	>15	≤15 (>7.5)	≤15 (>7.5)
SDFRC7	≤15 (>7.5)	≤15 (>7.5)	≤15 (>7.5)	≤15 (>7.5)	≤15 (>7.5)	≤15 (>7.5)
SDFRC8	≤15 (>7.5)	>15	≤15 (>7.5)	>15	≤15 (>7.5)	>15
SDFRC9	>15	>15	>15	>15	>15	>15
SDFRC10	≤15 (>7.5)	≤15 (>7.5)	>15	>15	≤15 (>7.5)	≤15 (>7.5)
SDFRC11	≤15 (>7.5)	≤15 (>7.5)	≤15 (>7.5)	≤15 (>7.5)	≤15 (>7.5)	≤15 (>7.5)
SDFRC12	≤15 (>7.5)	≤15 (>7.5)	≤15 (>7.5)	>15	≤15 (>7.5)	≤15 (>7.5)
SDFRC13	≤15 (>7.5)	≤15 (>7.5)	≤15 (>7.5)	≤15 (>7.5)	≤15 (>7.5)	≤15 (>7.5)

6. LC-MS

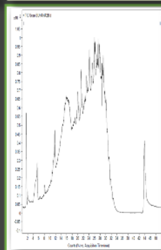

Fig.3: Total Ion Chromatogram of SDFRC2.

Table2: The phytochemical constituents of SDFRC2.

Formula	m/z	Mass	RT	Width	Height	Area	Name
C15 H10 O5	271.0604	270.0531	9.3	0.35	3868	62915	Apigenin
C15 H10 O6	287.0546	286.0589	19.9	7.77	1522	14080	Luteolin
C9 H7 N O3	178.0496	177.0426	3.28	0.17	35027	801356	Coixol
C27 H36 O5	463.1713	440.1864	14.67	0.1	4892	12405	Scoparic acid A
C16 H12 O5	285.0754	284.0682	22.33	0.63	3311	88194	acacetin
C25 H32 O5	413.2323	412.2252	13.15	0.15	51647	459789	Scoparic acid B
C30 H48 O3	457.3675	456.3588	26.52	0.23	25813	330544	Betulinic acid
C17 H14 O7	353.0667	330.0759	5.38	0.3	9132	189897	Cirsiliol
C18 H16 O7	345.0961	344.0905	13.33	0.58	3047	51428	Nevadensin
C16 H33 N O	256.2634	255.2559	29.1	0.15	146057	1620166	Palmitic amide
C27 H38 O4	427.281	426.2742	15.75	0.34	12230	116605	Dulcidiol
C30 H50 O	427.3925	426.3855	25.44	0.14	61972	414030	Glutinol
C16 H12 O6	301.0709	300.0635	9.79	0.57	7545	226049	5,7,4'-trihydroxy-3'-methoxyisoflavone
C7 H5 N O2	136.0394	135.0321	4.76	0.41	163163	2922726	Benzoxazolinone
C8 H7 N O3	166.0501	165.0428	5.38	0.84	5221801	245402962	6-methoxybenzoxazolinone
C8 H7 N O	134.0595	133.0835	4.11	1.37	3056	64478	Benzoxazine
C26 H27 O14	586.1315	563.1356	1.33	0.14	4406	32331	Apigenin 6-C-pentosyl-8-C-hexoside
C26 H32 O5	447.2132	424.2238	1.46	0.03	8788	15250	Scoparic acid C
C27 H30 O15	595.168	594.1619	1.93	0.07	4449	21340	Nicotiflorin
C18 H32 O5	351.214	328.2244	12.09	0.58	12957	428720	Trihydroxyoctadecadienoic acid

7. Drug-likeness

Out of 20, **two compounds** (Apigenin 6-C-pentosyl-8-C-hexoside and Nicotiflorin) **violated Lipinski's rule of five for drug-likeness**.

8. Docking

Table.3: Docking score of compounds with murA protein

Serial no.	Compounds	Docking score	Interacting amino acid
1	CO-murA	-11.95	ASN23, ARG91, ARG120, ASP123, LEU124, LYS160, SER162, VAL163, GLY164, ASP305, VAL327
2	Cirsiliol	-5.98	ARG91, ASP123, LYS160, PHE328(Pi-Pi)
3	5,7,4'-trihydroxy-3'-methoxyisoflavone	-5.9	ASP123, LYS160, SER162, GLU188
4	Luteolin	-5.53	ARG91, VAL161, ASP305, PHE328(Pi-Pi)
5	acacetin	-4.78	ASP123, LEU124, HIE125, LYS160, SER162, PHE328(Pi-Pi)
6	Apigenin	-4.58	ARG120(Pi-Cation), PRO121, SER162, GLY164, GLU188
7	Scoparic acid A	-4.34	ARG91, SER162, THR326
8	Nevadensin	-4.1	GLU188, ASP305, PHE328(Pi-Pi)
9	Scoparic acid B	-3.83	ARG91, ARG120(Pi-cation), PRO121, LYS160, SER162, GLY164
10	Coixol	-3.25	ARG91, PHE328(Pi-Pi)
11	6-methoxybenzoxazolinone	-3.02	ARG91, ARG120(Pi-cation), SER162, VAL164
12	Benzoxazine	-2.75	VAL161
13	Scoparic acid C	-2.51	ARG120(Pi-Cation), VAL161, SER162
14	Glutinol	-1.57	ARG91
15	Palmitic amide	-1.26	VAL161, SER162
16	Trihydroxyoctadecadienoic acid	-1.09	CYS115, ARG120, PRO121, ASP123, ARG397
17	Dulcidiol	-0.98	THR326
18	Betulinic acid		

Table.4: Docking score of compounds with folA protein

Serial no.	Compounds	Docking score	Interacting amino acid
1	Trihydroxyoctadecadienoic acid	-10.58	ASN18, ALA19, TRP22, LEU24
2	Cirsiliol	-10.1	ALA7, ASN18, SER49
3	5,7,4'-trihydroxy-3'-methoxyisoflavone	-8.79	ILE14, ASP27, SER49
4	CO-folA	-8.41	ARG52, ARG57, ASP112
5	Luteolin	-8.35	ALA7, ASN18
6	Coixol	-7.1	ALA7
7	Apigenin	-7.07	ALA7, ASN18
8	acacetin	-6.91	ILE5, ASP27, PHE31(Pi-Pi)
9	Scoparic acid A	-6.46	ASN23, PHE31(Pi-Pi), SER49
10	Nevadensin	-6.8	ARG52, ILE94
11	Scoparic acid B	-4.74	MET20, PHE31(Pi-Pi), SER49, ARG51
12	6-methoxybenzoxazolinone	-5.55	ALA7
13	Benzoxazine	-4.74	ILE94
14	Scoparic acid C	-4.37	ARG52
15	Glutinol	-1.84	
16	Palmitic amide	-3.37	ASN18, THR46
17	Dulcidiol		
18	Betulinic acid		

- In case of murA protein non of the compounds had better docking scores compared to its co-crystallizer.
- **In case of folA protein 3 compounds** viz. **Trihydroxyoctadecadienoic acid (-10.58), cirsiliol (-10.1), and 5,7,4'-trihydroxy-3'-methoxyisoflavone (-8.79) had better docking scores than its co-crystallizer.**

Fig.4: Trihydroxyoctadecadienoic acid interacting with amino acids of folA protein.

9. Molecular Dynamic

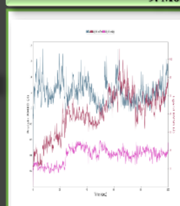

Fig.5: Protein-Ligand Root Mean Square Deviation (RMSD)

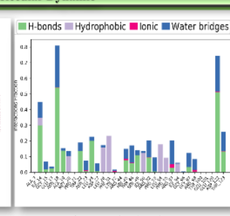

Fig.6: Protein-Ligand Contacts

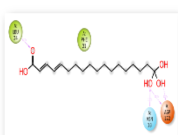

Fig.7: A schematic of detailed ligand atom interactions with the protein residues. Interactions that occur more than 15.0% of the simulation time.

- The molecular dynamic of folA-Trihydroxyoctadecadienoic acid was studied.
- **The RMSD indicated the stability**
- **The** average Molecular mechanics with generalized Born and surface area solvation **(MMGBSA) Energy value was -51.55 ± 5.31**

CONCLUTION AND FUTURE WORK

- **The *Scoparia dulcis* root extract showed antibacterial activity against *Salmonella* Typhi test stains** at certain concentrations and The antibacterial activity could be due to its possession of active phytochemicals.
- The identified phytochemicals from the most active fraction of the crude extract can lead us to a potential anti-typhoid drug.
- The phytochemicals with good docking scores to S. Typhi proteins could be a potential inhibitor to target those proteins.
- The **Trihydroxyoctadecadienoic acid**, which had a good docking score with folA protein and showed overall stability in the molecular dynamic study and good average MMGBSA energy **could be developed into an antityphoid drug to target folA protein.**
- Further *in vitro* and *in vivo* studies with Trihydroxyoctadecadienoic acid and other identified phytochemicals are necessary.
- The *in vitro* and *in vivo* experiments to evaluate the antibacterial activity of selected phytochemicals against S. Typhi were under consideration.

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Dr./Mr./Ms. Sunayana Saren

has participated in the 16th Asian Conference on Diarrhoeal Disease and Nutrition in Kolkata, India and presented ~~ORAL~~/ POSTER during the Conference.

T. Ramamurthy
Dr. T. Ramamurthy
Organizing Secretary
ASCODD 2022, Kolkata

S. Dutta
Dr. Shanta Dutta
Organizing Chairperson
ASCODD 2022, Kolkata

M. Workshops:

1. **Scientific Writing and Research Ethics for Medical and Clinical Science Community**, Organized by DBT/Wellcome Trust India Alliance, 2020.
2. **Ethics in Human Health Research and Good Clinical Practice**, 2019, organized by ICMR-National Institute for Research in Bacterial Infections (NIRBI, formerly NICED).

IndiaAlliance
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Certificate of Participation

This is to certify that

Sunayana

has attended the **Online Workshop on Scientific Writing** and **Research Ethics** for **Medical and Clinical Science community**, organized by DBT/Wellcome Trust India Alliance on 19 September 2020.

Shahid Jameel

Dr Shahid Jameel
CEO, DBT/Wellcome Trust India Alliance

Knowledge and training partner: Editage

Workshop

'Ethics in Human Health Research and Good Clinical Practice'

Theme

Current Regulatory and Ethical Requirements for Clinical Research in India

Sub Themes

Changing Regulatory Environment for Conduct of Clinical Trials in India
Challenges in the Conduct of Academic Trials in India
Conduct of Biomedical and Health Research in India and the National Ethical Guidelines
Good Clinical Practice: Concept and Principles
Ethics Committee – Review and Oversight of Clinical Research
Role of Individual Members of the Ethics Committee
Safety Reporting in Clinical Trials and Compensation Issues
Registration and Accreditation of Ethics Committees

Organized by:

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Date:

7th May, 2019.

Venue:

Seminar hall (4th Floor), ICMR-NICED Building II (ID Hospital Campus)

Certificate of Appreciation

Certified that

Dr./Mr./Mrs./Ms. Sunayana Saren *of* ICMR-NICED *attended the Workshop and actively participated in the deliberations. Her/his performance in the post-event assessment was satisfactory.*

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