Chapter-3

Materials and Methods

3.1 Materials

3.1.1 Collection of plant material

The leaves of *Morinda citrifolia* L. and *Morinda pubescens* J.E Smith were collected throughout the year from different locations of Thrissur district. The leaves collected for the study were kept in sterile plastic bags. In addition, a voucher specimen of *M. citrifolia* (15785) and *M. pubescens* (15090) were maintained in Kerala Forest Research Institute (KFRI), Peechi, Thrissur.

3.1.2 Plant description

Kingdom:	Plantae
Class:	Dicotyledonae
Order:	Gentianales
Family:	Rubiaceae
Genus:	*Morinda* L.

***Morinda citrifolia* L.**, commonly known as noni, an evergreen shrub belongs to the family Rubiaceae. The leaves are simple, entire, opposite, elliptic-lanceolate, glabrous, slightly acuminate at apex, pinnately nerved; long petioles with stipules broadly triangular. Globose head inflorescence with long peduncle; white, bisexual flowers; funnel-shaped corolla in which stamens inserted; bilobed stigma. Fruit ovoid seeded drupes, yellow-white (Orwa *et al*., 2009). In this study, fresh and healthy dark green leaves have been selected, as shown in fig. 3.1.

Fig. 3.1 Habit of *M. citrifolia*

***Morinda pubescens* J.E Smith**, commonly known as Indian mulberry, is a deciduous tree belongs to the family Rubiaceae. Leaves simple, entire, glabrous, opposite-decussate, apex acute to acuminate, long pubescent petiole, interpetiolar stipules, connate sheathing with bifid apex, elliptic-lanceolate or oblanceolate, intercostae reticulate. White, bisexual flowers, in the terminal, globose heads; long corolla tube; four stamens attached to the throat of corolla, inferior syncarp ovary, globose fruit with two-lobed style; with seeds not winged (Gamble, 1956). In the present work, the leaves of *M. pubescens* were taken for the study (Fig. 3.2).

Fig. 3.2 Habit of *M. pubescens*

3.1.3 Experimental animals

Male Swiss Albino mice weighing 26 ±5.2 g (Mean ±SD) used in this study were purchased from the Small Animal Breeding Station, Kerala Veterinary and Agricultural Sciences University, Mannuthy, Kerala, India. Animals were kept in polypropylene cages for quarantine for seven days with access to drinking water and standard rat feed (Lipton, India) ad libitum. The animals were maintained with a 12 h light: 12 h dark cycle at a controlled temperature of 26-28º C with 60-70% relative humidity. All the animal experiments were conducted during the present study with prior permission from the Institutional Animal Ethics Committee (ACRC/IAEC/20(1)-P13) (IAEC approved), which follows the regulations of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The animal experiments were conducted humanely in Amala Cancer Research Centre (ACRC), Kerala, India.

3.1.4 Cell lines

Breast cancer cell lines MCF-7 was obtained from National Centre for Cell Science (Pune, India). Cells were maintained in RPMI 1640 (Cell Grow, USA) medium with 10% fetal bovine serum (FBS), 100 mg/mL streptomycin and 100 units/mL penicillin, at 37° C, 5% CO_2 atmosphere. The experiments were performed on cell lines after 14 to 17 passages beyond the passage received.

3.1.5 Test organisms

The phytopathogenic fungal culture *Aspergillus* sp. and *Sclerotium rolfsii* were collected and identified by the microbiologist, Agricultural microbiology, Kerala Agricultural University, Vellanikara, Thrissur. The test organisms of bacterial strains such as *Escherichia coli* (ATCC 10799), *Listeria* sp. (ATCC 19111), *Klebsiella* sp. (ATCC 9621), *Shigella* sp. (ATCC 12022), and *Streptococcus* sp. (ATCC 11454) were obtained from global bioresource centre, ATCC (American Type Culture Collection), Washinhton D.C.

3.1.6 Chemicals and reagents

Ethylenediaminetetraacetic acid (EDTA), Folin-Ciocalteu reagent, Iodine, Nicotinamide adenine dinucleotide phosphate (NADPH), Nitroblue tetrazolium (NBT), Trichloroacetic acid (TCA), Tris-HCL, Chloroform, Isoamyl alcohol, Ethyl alcohol, Citric acid, Dimethylsulphoxide (DMSO), Di-sodium hydrogen phosphate, Ethyl acetate, Glycerol, Hydrochloric acid, Sulphuric acid, Barium chloride, Methanol, Potassium permanganate, Phosphate buffered saline (PBS), Sodium carbonate, Sodium chloride, Sodium dihydrogen phosphate, Sodium hydroxide, Ferric chloride, Ferrous sulfate, Thiobarbituric acid (TBA), Formaldehyde solution,

Hydrogen peroxide (H_2O_2), Picric acid, Ethidium bromide, Trypan blue, 3-(4,5-dimethythiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT), Cyclophosphamide, diclofenac, Congo red, Eriochrome Black T, Thioglycolate, Bromothymol blue, Indole reagent, Riboflavin, Sodium cyanide, 2,4,6-Tripyridyl-S-triazine (TPTZ) reagent, Lead acetate, Cadmium chloride, Chromium nitrate, Potassium chloride, Fetal Bovine Serum (FBS), Trysin, Acetate buffer, and Sodium acetate (SOD) buffer.

3.1.7 Culture Media

Nutrient agar:	HiMedia Laboratories, India.
Mueller-Hinton agar:	HiMedia Laboratories, India.
Bacteriological agar:	HiMedia Laboratories, India.
Simmons citrate agar:	Sisco Research Laboratories, India.
Kligler iron agar:	HiMedia Laboratories, India.
Christensen's Urea Agar:	HiMedia Laboratories, India.
Luria Bertani broth:	HiMedia Laboratories, India.
Potato dextrose agar:	HiMedia Laboratories, India.

3.1.8 Diagnostic reagents and kits

Impregnated oxidase test disc:	HiMedia Laboratories, India.
Antibiotic dics:	HiMedia Laboratories, India.
PCR master mix:	HiMedia Laboratories, India.
Gel extraction kit QIAquick:	Qiagen, Mississauga, Canada.
BigDye™ Terminator v3.1 Cycle sequencing kit:	Thermo Fisher Scientific, USA.
Column-based purification kit:	Invitrogen, USA.
DNeasy Plant Minikit:	Qiagen, Mississauga, Canada.
Taq Polymerase master-mix kit:	Qiagen, Mississauga, Canada

3.1.9 Instruments

Rotavapor:	Buchi, India.
Lyophilizer:	Thermo Fisher Scientific, USA.
UV-Vis Spectrophotometer:	Shimadzu, Japan.
Atomic absorption spectroscopy:	Shimadzu, Japan.
Electronic weighing balance:	Shimadzu, Japan.
Compound microscope:	Magnus, India.
Inverted microscope:	Magnus, India.
Phase-contrast microscope:	Magnus, India.
Scanning electron microscope:	Jeol, Japan.
Transmission electron microscope:	Jeol, Japan.
Energy dispersive X-ray spectroscopy:	Jeol, Japan.
Fourier Transform infrared:	Shimadzu, Japan.
X-ray Diffraction:	Shimadzu, Japan.
Cooling centrifuge:	Eppendorf, Germany.
Qubit ^{R} 2.0 fluorometer:	Thermo Fisher Scientific, USA.
Nanodrop:	Thermo Fisher Scientific, USA.
Bacteriological Incubator:	Kemi, India.
Hot air oven:	Kemi, India.
Vortex mixer:	Kemi, India.
Orbital Shaker:	Kemi, India.
ELISA reader:	Biotek, India

3.1.10 Glassware and consumables

Glasswares and consumables were obtained from Borosil (India) and Merck (Germany).

3.2 Methods

The leaves of selected plants were washed thoroughly with tap water, and those with superficial injury visible to the naked eye were excluded. The isolation procedure was performed in triplicates, and each trial was composed of approximately 2 g of fresh leaves. The disinfection protocol was followed according to Araujo *et al*., (2002) with slight modifications. Briefly, the leaves were disinfected superficially through the following protocol: 70% ethyl alcohol for 1 min, sodium hypochlorite (2.5% W/V) for 3 min, ethyl alcohol for 30 sec, and finally rinse in sterile distilled water thrice for 10 minutes. The validity of the surface sterilization procedure was checked by plating the last rinsed water of the surface-sterilized tissues onto the agar plates to ensure no bacterial growth on plates after an incubation (37° C) period of 24 h. If any bacterial growth was found, the plates were discarded.

After validating the effective surface sterilization, the leaf tissues were macerated in 7 mL of an aqueous solution of 0.9% NaCl with sterile mortar and pestle. The tissue extract was allowed to stand for 3 hours at room temperature for the complete release of endophytic microorganisms from the host plant tissue. For endophytic bacteria isolation, the tissue extract was diluted in sterile distilled water and plated in nutrient agar for each serial dilution (10^{-1} to 10^{-7}) with spread plate method and incubated for seven days at 28 ±2° C. Subculturing of the isolates was performed several times to get a single pure colony, which was well-maintained and preserved in 50% glycerol stock in a deep freezer at -20° C (Fahy and Persley, 1983).

The bacterial isolates were selected for the study based on the traditional criteria of classification like colony morphology (color, texture, margin, and elevation), presence and absence of diffusible pigment production (Shirling and Gottlieb, 1966), viable subculturing, and pure culture maintenance for an extended period. In addition, morphologically distinct isolates were grouped and specified with code names for ease of identification.

3.2.1 Identification of endophytic isolates

3.2.1.1 Morphological and physiological identification

The selected isolates were morphological identified based on the above-described colony characters. In addition, a detailed study was done after Gram's staining to determine whether the isolates were gram-positive or negative. Also the cell shape (bacilli, cocci, vibrio, spirilla), presence and absence of flagella, endospore formation, and motility were noted. The physiological parameters such as temperature and pH desirable for the growth of isolates were monitored by growing them in various temperatures (20°, 28°, 37°, and 40° C) and pH (4, 5, 6, 7, and 10) conditions.

3.2.1.2 Biochemical identification

The isolated bacteria were then subjected to biochemical tests for carbohydrate fermentation, indole formation, H_2S production, urease test, nitrate reductase test, Phenylalanine deaminase test, citrate, catalase, and oxidase formation. The indole formation test was performed by inoculating the bacteria in a liquid tryptic soy broth medium with 10% NaCl and then incubated for 24 hours at 37° C, and also the Kovacs' indole reagent was subsequently added. The positive test result is marked with a red color formation. Kligler's Iron Agar (KIA) was conducted to differentiate organisms by H_2S production and fermentation of dextrose and lactose. The positive test for H_2S production is characterized by the formation of black color in the medium

and the positive result for carbohydrate fermentation is yellow color in slant and butt reaction, indicating dextrose and lactose fermentation. Also, the positive gas formation is characterized by bubbles in the medium. Phenylalanine deaminase (PAD) test was conducted to check the ability of bacteria to produce enzyme deaminase. The positive result is characterized by the formation of visible green color. Urease test was performed by inoculating isolate in Christensen's Urea Agar. The positive result is marked by pink color when kept for a week. Nitrate reduction test was done by inoculating bacterial suspension in nitrate broth and incubating it for 24 hours at 37° C. Then add 6 drops of nitrite reagent A followed by 6 drops of nitrite reagent B into it. The positive test is noted by the formation of red color. The citrate utilization test was performed by inoculating the isolates in Simmons citrate agar containing bromothymol blue indicator. After 24 hours of incubation at 37° C, a change in color of the culture medium from green to blue is noted. Methyl red and Voges Proskauer test was performed by inoculating pure culture of organism in MR-VP broth. The positive result for Methyl Red test is change in color from yellow to red after adding methyl red. Similarly, Voges Proskauer test result in cherry red color by adding α-naphthol and potassium hydroxide in inoculated MR-VP broth (Cappuccino and Sherman, 2002).

For the catalase test, drops of H_2O_2 was placed on a sterile slide, and then the bacterial isolate was aseptically seeded. Positive results are marked by the appearance of air or gas bubbles. For the oxidase test, bacterial isolates were applied to the surface of the oxidase discs that result in purple color on the discs after 5 seconds (Prescott *et al*., 2005).

3.2.1.3 Genotypic identification of bacterial isolates

The most frequently used molecular markers for identifying bacteria are 16S rRNA genes, which are highly conserved. Researchers have exploited these conserved

regions for phylogenetic analysis of bacteria and explored the possibility of 16S forward and reverse primers for amplification.

3.2.1.3.1 Genomic DNA isolation

Genomic DNA of endophytic bacterial strain was isolated from an overnight culture grown in Luria Bertani liquid media incubated at 37° C in an orbital shaker (180 rpm). Three milliliters of each strain's liquid culture were taken separately and centrifuged for 1 min at 10,000 rpm. The cell pellets thus obtained were washed with phosphate buffer (0.1 M, pH 7.2), and finally, cells were taken in 500 µL TE buffer (25 mM Tris-HCl of pH 8.0, 25 mM EDTA). For proper lyses, cells were treated repeatedly with lysozyme and 10% SDS. Using chloroform-isoamyl alcohol mixture (24:1), DNA was extracted from cell lysate, and the aqueous layer was separated carefully. Finally, genomic DNA was precipitated with isopropyl alcohol. Precipitated DNA was washed with 70% ice-cold ethanol and centrifuge at 14,000 rpm for 10 min. The pellet was allowed to dry to remove alcohol content and add 50 µL of nuclease-free water. The genomic DNA thus isolated can be directly used as a template for PCR amplification.

3.2.1.3.2 Amplification of 16S rRNA gene

The 16S rRNA gene of the bacterial isolates was amplified using primers 27F (5’ AGAGTTTGATCCTGGCTCAG 3’) and 1492R (5’ GGTTACCTTGTTACGACTT 3’) (Jose *et al*., 2011). The reactions were performed in a PCR machine (Thermo Fischer, USA) with 50 µL reaction mixture which contains 1 µL genomic DNA, 1 µL forward and reverse primer each, 38 µL sterile water, 1 µL Taq DNA polymerase (HiMedia), 5 µL 10X assay buffer and 3 µL 10 mM dNTP mix. Reaction parameters as follows: initial denaturation 94° C for 1 min followed by 30 cycles of denaturation

at temperature 94° C for 30 s, annealing 60° C for 30 s, primer extension temperature of 72° C for 60 s and a final extension of 72° C for 60 s was done. The PCR products were checked for appropriate size by agarose gel visualization using ethidium bromide dye. The DNA amplicon was purified using a commercial column-based purification kit (Invitrogen, USA). The purified amplicon's forward and reverse DNA sequencing reaction was carried out with the same primer using BigDye^{TM} Terminator v3.1 Cycle sequencing kit on ABI 3730 XL cycle Sequencer. Both the sequences, forward and reverse were assembled and trimmed low-quality bases to generate contig using Bioedit software.

3.2.1.3.3 Phylogenetic tree construction

Consensus sequences of 16s rRNA generated from forward and reverse sequence data of all the isolated endophytic bacteria were used to carry out BLAST with the NCBI GenBank non-redundant (nr) database. Based on maximum similarity, sequences were selected and aligned using the multiple sequence alignment tool ClustalW. Ribosomal database project (RDP) was used for distance matrixes. To assign isolates at the proper position, a phylogenetic tree was constructed using MEGA X (Kumar *et al*., 2018). The neighbor-Joining method (Saitou and Nei, 1987) was inferred to study the evolutionary history and the computation of evolutionary distances using the Kimura 2-parameter method (Kimura, 1980).

3.2.2 Metagenomic study using Next Generation Sequencing technology

3.2.2.1 Sampling, DNA extraction, and PCR amplification

The leaves of *Morinda pubescens* were collected from Athani, Thrissur (Lat. 10°37'06"N and Lon. 76°12'17"E), Kerala, and transferred into sterile biosafety bags. Surface sterilization of the sample was performed with 70% ethanol for 2 mins

followed by 2.5% (W/V) sodium hypochlorite solution for 10 mins and finally washed thrice with sterile distilled water for 10 mins. The tissues were gently disrupted with sterile mortar and pestle, and genomic DNA of the endophytic bacterial isolates were extracted using Qiagen DNeasy Plant Minikit (Cat no.69104) and lysozyme. First, 0.1g of the sample is grinded using sterile mortar and pestle and then add 400 µL AP1 buffer and 4 µL RNase into the homogenate, then vortex and incubate the mixture for 10 min at 65° C. Centrifuge the lysate for 5 min at 14,000 rpm, pipette the lysate into a QIAshredder spin column, and centrifuge for 2 min. Add 1.5 volumes of Buffer AW1 and mix well by pipetting, then transfer 650 µL of it into Mini spin column kept in a collection tube of 2 mL. Centrifuge for 1 min at ≥8000 rpm. Add 500 µL Buffer AW2, and centrifuge at 20,000 x g for 1 min and repeat this process after discarding the flow-through and then add 100 µL of elution buffer AE Incubate for 5 min room temperature (www.qiagen.com/handbooks). The extracted DNA was quantified, and purity was checked (∼1.8) using Qubit ^{R} 2.0 fluorometer and Nanodrop (Thermo Fisher Scientific, USA) at absorbance 260/280 nm and stored at −20° C.

3.2.2.2 Illumina Library preparation

The genomic DNA of bacterial isolates from leaf tissue samples was standardized to concentration 10 ng/µL. PCR amplification of V3–V4 conserved regions of 16S rRNA gene sequences was carried out using the forward and reverse primers (341F: 5’ CCTACGGGAGGCAGCAG 3’ and 806R: 5' GGACTACHVGGGTWTCTAAT 3’). The PCR library preparation was carried out using Qiagen TaqPCR Master-mix Kit (Cat. No.201900). Concisely, PCR reaction was assembled into 50 µL volume containing 4 µL of genomic DNA template, 25 µL Taq DNA polymerase, Qiagen

PCR buffer, PCR- grade dNTPs mix (10mM), 2.0 µL each forward and reverse primer and nuclease-free sterile water. PCR reactions were started initially with denaturation step for 2 min at 94° C followed by 30 cycles at 94° C denaturations for 30 s, 55° C primer annealing for 45 s, and 72° C for 1 min extension and ended with a final extension step at 72° C for 5 min. The PCR products were then analyzed via 2% agarose gel electrophoresis, and purified DNA was recovered using gel extraction kit QIAquick (Qiagen, Mississauga, Ontario, Canada). Illumina HiSeq sequencer were used to sequence samples by loading the sample DNA onto the paired-end flow cell of the system, which performs dual index sequencing with automated cluster generation (Illumina guide 2013).

3.2.2.3 Sequence data processing

The paired-end sequence thus formed contains the V3-V4 region and some portion of the conserved region. The quality parameters of the sequence obtained from the sequencer are checked by the FastQ tool (Fastq quality-check). The Phred score recorded greater than 30 ensures an excellent quality sequence. As a preliminary step, the conserved region was removed from paired-end reads to study V3-V4 metagenomics alone. Using the Trimmomatic tool, unwanted sequences were trimmed from original paired-end data, and a consensus V3-V4 region sequence is constructed using the FLASH program to improve genome assemblies. Chimeras and singletons are removed using the de-novo chimera removal method UCHIME implemented in the open-source metagenomics tool VSEARCH. Finally, the raw data containing forward and reverse reads were merged using QIIME and MG-RAST pipeline alignment methods to evaluate their accuracy in assigning taxonomic units.

3.2.2.4 Characterization of bacterial community composition

The pre-processed consensus V3-V4 sequences were clustered in operational taxonomic units (OTUs) based on sequence similarity using an algorithm implemented in the Uclust program having a dissimilarity threshold of 3% and similarity cut off of 97%. QIIME program, a quantitative insight in microbial ecology, was used to perform downstream analysis such as microbial community analysis (Caporaso *et al*., 2010). Using the PyNAST program, the representative sequence was identified for each OTU. aligned against the Greengenes database (DeSantis *et al*., 2006). Also, the SILVA OTUs database raised by the RDP classifier was used to quantify the microbial diversity, richness, composition, and taxonomy classification of inferred OTUs.

3.2.3 Extraction of compounds and qualitative GC-MS analysis

The pure colony of selected endophytic isolates was inoculated into the LB broth separately. The culture broth was incubated at 37° C for ten days in an orbital shaker. After incubation, the liquid culture was centrifuged at 3000 rpm for 20 min to obtain an extracellular component containing supernatant. The obtained culture filtrate of 500 mL was extracted twice with ethyl acetate as solvent. The volume of solvent taken with the filtrate for extraction is in the ratio of 1:1 (v/v). After shaking vigorously for 20 minutes, the ethyl acetate phase was separated from the aqueous phase of the culture supernatant using the separating funnel. The ethyl acetate layer was concentrated using a lyophilizer, and the residue was dissolved in an ethyl alcohol solvent for further studies (Ahmed 2007).

GC-MS profiling of the samples was carried out in a combined gas chromatography system (Shimadzu QP2010S) and mass spectrophotometer fitted with ELITE-5MS fused silica column (5% phenyl methyl siloxane 30.0 m × 250 μm, film thickness 0.25 μm). Helium gas act as a carrier and the flow velocity of the column was adjusted to 1.0 mL/min. Other GC-MS parameters are ion-source temperature, 200° C; interface temperature, 280° C; pressure, 61.5 kPa and 1μL injection mode splitless with injection temperature of 260° C. The column temperature started at 70° C for 2min and changed to 200° C at the rate of 10° C/min. The oven temperature was elevated to 280° C at 5° C/min and held for 15 min. The total flow was 54 mL/min. By comparing the average peak to the total area of each component, the relative percent amount can be calculated. GCMS solution software provided by the supplier uses a Real-Time Analysis module for system control and data acquisition. Volatile Organic Compounds (VOCs) were identified based on the m/z peaks representing mass to charge ratio. Recorded chromatogram and mass spectra of the compounds obtained were analyzed following the standard mass spectrum of the NIST library and WILEY 8 library of the corresponding organic compound.

3.2.4 Total Phenolic content

The total phenolic compounds in the extracts were estimated using the Folin-Ciocalteu method Nabavi *et al*., (2008). The stock solutions of 10 mg/mL of the samples extract were made. From it, 50 μL of the ethanol extract of the sample (0.5 mg) was pipetted into a 50 mL Erlenmeyer flask containing 2.5 mL of one-tenth dilution of Folin-Ciocalteu's reagent, 2 mL of sodium carbonate solution (7.5% W/V), and the volume was made up to 10 mL with distilled water. The reaction mixture was then incubated for 15 mins at 45º C. During that time, oxidation of phenolic

compounds occurs due to the reduction of phosphomolybdic acid and phosphotungstic acid present in the Folin-Ciocalteu's reagent to blue-colored molybdenum and tungsten oxides. The absorbance of blue coloration of the test sample was measured at 765 nm against a blank sample. The standard curve of gallic acid solutions at different concentrations was prepared. The total phenolic content was measured as Gallic Acid Equivalent (GAE, mg/g of extract). The experiment was conducted in triplicates.

3.2.5 Iron oxide nanoparticles production efficacy of isolates

The selected bacterial isolate was screened for its iron oxide nanoparticle production. The iron oxide NPs were synthesized by the method proposed by Chomchoey *et al*., 2009 with certain modifications. First, inoculate bacterial isolates in 100 mL LB broth and incubate at room temperature for ten days in a rotary shaker (200 rpm). After incubation, the inoculated broth was centrifuged at 10,000 rpm for 15 mins, and the supernatant was collected. Iron oxide nanoparticles were prepared by adding 2 mM freshly prepared $FeCl_3$ solution to the supernatant in a 1:1 volume ratio. were harvested by separating bacterial biomassimmediately obtained with the reduction process by a change in color. The reaction composite was stirred for 60 mins and then allowed to stand at room temperature for another 30 mins. The obtained colloidal suspensions of bacterial iron oxide nanoparticles were then centrifuged and washed in deionised water several times and were dried in an oven at 80° C for 5 h in air. Thereafter, the dried black powders were then calcined in air at 200° C. The final product was stored in glass vials with airtight lid and submitted for characterization and laboratory examinations.

3.2.5.1 Characterization of nanoparticles

3.2.5.1.1 UV-Vis spectral analysis

UV-Visible spectroscopy is the most extensively used technique to investigate the optical properties of the particles. This analysis specifies the time point of maximum production of Fe nanoparticles by taking absorption spectra at a range of 200 nm to 400 nm wavelength range. The reduction of ferric chloride into FeNPs was monitored by measuring the UV-Vis spectra of the reaction medium after diluting small aliquots of the sample into deionized water. It has been reported that maximum absorbance was observed at 200-324 nm regions to form iron nanoparticles (Balamurugan *et al.,* 2014).

3.2.5.1.2 Fourier Transform Infrared spectroscopy

Fourier transform infrared (IR) spectroscopy deals with the vibration of chemical bonds in a molecule at various frequencies depending on the elements and nature of bonds. It is the most widely used vibration technique to study the chemical composition of the material. After the absorption of electromagnetic radiation, the vibration frequency of a chemical bond increases leading to the transition between the ground state and various excited states. These absorption frequencies represent the vibrational excitations of chemical bonds and are specific to the type of bond and atoms groups involved in the vibration (Chandran *et al*., 2016). Nanoparticles characterization using FTIR determines the functional groups attached to NPs. FTIR analysis was performed out through the potassium bromide (KBr) pellet in a 1:100 ratio, and the spectrum was recorded FTIR Spectrophotometer (Shimadzu, Japan). The infrared absorption spectrum of a compound possibly represents its most unique physical property, and the spectrum is often called the fingerprint of a molecule. The

mid-infrared region 4000-500 cm^{-1} is the most used FTIR range for various applications (Mathlouthi *et al*., 1986).

3.2.5.1.3 Energy Dispersive Spectroscopy

Energy Dispersive X-ray (EDAX) or EDS is an analytical technique for analyzing elements or chemical characterization of a sample. It is one type of X-ray fluorescence spectroscopy based on investigating a sample through the interactions of electromagnetic radiation and matter, analyzing the X-rays emitted by matter in response to the impact of charged particles and identifiable elements. The characterization capabilities are primarily due to the fundamental principle that each element has a unique atomic structure that allows X-rays to be unique to the element's atomic structure, thus identifies each element. The emission of X-rays characteristic of a sample is excited when a high-energy electron beam is focused on the sample under examination. At rest, an atom within the sample contains ground-state (or unexcited) electrons at discrete energy levels or electron shells bound to the nucleus. The incident beam can excite an electron in an inner shell, ejecting it from the shell, creating a hole in the electron's place. An outer shell of an electron with higher energy fills the hole, and the energy difference between the upper energy shell and the lower energy shell can be released in the form of X-rays. EDAX has confirmed the elemental state of metal nanoparticles.

3.2.5.1.4 Scanning Electron Microscopy

Scanning electron microscopy (SEM) is an electron microscope that images a sample by scanning it with a high-energy beam of electrons. The morphological features such as the size of the synthesized iron nanoparticles from Hemigraphis plant extract were studied by SEM (JEOL-MODEL 639O) at an accelerating voltage of 20

kV. The biosynthesized metal nanoparticles were mounted on carbon stubs, and the images were examined using a scanning electron microscope (SEM-EDX JEOL Model L6390). The mean particle size and morphology of the iron oxide nanoparticles were determined by SEM analysis.

3.2.5.1.5 X-ray Diffraction analysis

X-ray diffraction is an important analytical technique that reveals comprehensive information about the crystallographic structure, chemical composition, and physical properties of particles. These techniques mainly focus on observing the diffuse intensity of an X-ray beam incident on a sample as a function of wavelength, polarization, and diffused angle. XRD patterns were recorded on Phillips PW-1710 automated diffractometer using a Copper tube operated at 40 kV and 35 mA. The instrument was fitted with a diffracted curved beam graphite monochromator and a proportional counter filled with Xe. When a monochromatic beam of lambda X-ray wavelength is projected onto a crystalline material with a theta angle, diffraction occurs only when the distance traveled by the rays reflected from successive planes differs by a total number of wavelengths. The incident rays that interact with the sample produce constructive interference (and a diffracted beam) when the conditions satisfy Bragg's law ($n\lambda=2d\sin\theta$). This law relates the wavelength (λ) of the electromagnetic radiation with the diffraction angle (θ) and the lattice spacing (d) in a crystalline sample (Borchert *et al*., 2005). The biosynthesized nanoparticles were released onto a glass substrate for analysis, and the powder X-ray diffraction analysis was performed on an X-ray diffractometer (Shimadzu, Japan). The pattern was recorded from the Cu K (alpha) radiation with λ of 1.54 A°. According to the Debye-

Scherrer equation, the average size of the metal and metal oxide nanoparticles was determined by the reflection width.

$$D = 0.9\ \lambda/\beta\ \cos\theta$$

Where β is the line extending to full width to half the maximum (FWHM) of the peak in radians, θ is the diffraction angle (Bragg angle), and λ is the X-ray wavelength.

3.2.5.1.6 Transmission Electron Microscopy

The transmission electron microscope (TEM) technique was used to visualize the morphology of the FeNps. This method helps to observe the particle size in nano-dimensions and study the crystal structure meticulously (Herrera-Becerra *et al*., 2008). In a high-resolution transmission electron microscope, a thin sample or specimen is irradiated with a sharp, high electron beam (usually in 100-200 kV). Selected area electron diffraction (SAED) studies show the crystal planes of elemental iron.

3.2.5.2 Application of iron oxide nanoparticles

3.2.5.2.1 Preliminary study on heavy metal removal

Heavy metals removal by synthesized nanoparticle was detected using Atomic Absorption Spectroscopy (AAS). This technique is used to determine the concentration of a particular element in the sample. AAS is based on the absorption of optical radiation or light by free metallic ion. The heavy metal such as lead (II) acetate, cadmium (II) chloride, and chromium (III) nitrate was taken for the study. The amount of lead in lead acetate, cadmium in cadmium chloride, and chromium in chromium nitrate were found to be 0.66, 0.61, and 0.21 g/mol, respectively in 1 g of each heavy metal taken. Under this optimum condition 0.5 g/L of synthesized iron

oxide nanoparticles were added into the 100 ppm heavy metal. The absorbance were recorded for control and test sample of heavy metal in Atomic absorption Spectroscopy (Dave and Chopda, 2014).

3.2.5.2.2 Preliminary study on dye decoloration

Eriochrome Black T (EB), Congo Red (CR), and Trypan Blue (TB) dyes were employed for this experiment. Add 0.5 mg of the dyestuff in 100 mL of deionized water, and each dye solution was mixed with 10 mg of the synthesized FeNps. The reaction mixture was kept under a UV lamp (KODAK, 40 W), constant stirring at 25° C. The maximum absorbance wavelength (λmax) for the dye taken was used for absorbance reading using a spectrophotometer (Bekhit *et al*., 2020). Deionized water was served as a reference. Absorbance spectra of the dye were recorded at 545, 497, and 540 nm wavelengths for EB, CR, and TB, respectively. The degradation efficiency was calculated using the formula,

$$\% \text{ Dye degradation} = (Abs_o - Abs_t) \times 100/Abs_o$$

where "Abs_o" is the absorbance of the solution before adding nanoparticles and "Abs_t" is the absorbance of the solution after adding nanoparticles.

3.2.6 Screening of antimicrobial activity

The in vitro antibiotic susceptibility test for the endophytic isolates was done following the Kirby–Bauer disc diffusion method on Mueller Hinton agar (HiMedia), as described by Hudzicki (2009). Six commercially available antibiotic discs (Himedia® OD 2/4) were used in the following concentrations: ampicillin (10 µg); penicillin (10 µg); streptomycin (10 µg); ciprofloxacin (5 µg); gentamicin (30 µg) and chloramphenicol (30 µg). Also, all the selected endophytic bacteria were screened for

primary antagonistic activity against human pathogenic bacteria and plant pathogenic fungus using appropriate techniques.

3.2.6.1 Antibacterial activity of isolates

The preliminary screening of selected endophytic bacteria was done by the perpendicular streak method against human pathogenic bacterial strains. The desired bacteria were suspended in liquid broth and kept overnight at 37° C in an orbital shaker. All the bacterial cell suspensions were adjusted to 0.5 McFarland turbidity standards to prepare 1×10^8 bacterial/ mL inoculum. Fifty microliters of the suspension were streaked as a single straight line through the center of the agar seeded in a Petri dish and incubated for 48 h at 37° C. After incubation, all five tested human pathogenic bacteria were streaked perpendicular to the antagonistic bacteria. The test pathogen includes *Escherichia coli*, *Klebsiella* sp., *Listeria* sp., *Streptococcus* sp., and *Shigella* sp. In addition to streaking, the streak's length was marked on the plate using an appropriate marker. The observations were made and recorded after 24 h of incubation. The presence of antimicrobial substances produced by the endophytic bacteria was expected to inhibit the growth of tested human pathogens (Nike *et al*., 2013).

3.2.6.2 Antifungal activity of isolates

The antifungal activity of isolates against phytopathogenic fungus was performed using a dual culture technique by growing the pathogen and test bacteria on Potato dextrose agar (PDA). The biocontrol efficacy of isolated endophytic bacteria may be due to diffusible compounds. The bacterial strains were grown in LB liquid medium overnight. The bacterial suspensions (50 µL) were streaked in a line on PDA in conventional Petri dishes 1 cm away from the plate end. Fungal plugs of diameter 5 mm were then kept on the agar 2.5 cm away from the bacterial streak immediately

after the bacteria were plated. Sterile distilled water streaked against fungal plug was kept as control, and these plates were incubated at 30° C. The fungal diameter was measured in both directions, and the average was recorded. The percent inhibition on the growth of the fungal pathogen was calculated using the formula given below by Rabindran and Vidyasekaran (1996).

$$\text{Percentage of growth inhibition} = [(R_1-R_2)/ R_1] \text{ X } 100$$

Where R_1 = Diameter of colony growth of the pathogen in the control plate

R_2 = Diameter of pathogen towards the test bacteria plate

3.2.7 Estimation of in vitro antioxidant activity

The in vitro antioxidant assay of the ethyl alcohol extract of selected isolates samples were studied by the following assays:

3.2.7.1 DPPH scavenging activity

The extract of the sample was tested for their scavenging activity against the stable free radical DPPH (2, 2-diphenyl-1-picryl hydrazyl) by the method of Aquino *et al*., (2001). The reaction mixture contained 1mL of freshly prepared DPPH (0.0025 g in 10 mL methanol) solution, different concentrations of sample extract (0, 25, 50, 75, 100, 125, 150, 175, 200, 225 and 250 µg/mL) and the volume was made up to 3 mL with methanol. After 20 mins of incubation at room temperature, the absorbance was measured at 515 nm. The effect of the sample in scavenging DPPH radical was determined by a decrease in color intensity compared to control. The percentage of DPPH scavenging was calculated using the formula-

$$\% \text{ of DPPH scavenging activity} = [(A_0-A_1)/ A_0 \times 100]$$

Where A_0 and A_1 is the absorbance of the control and the sample, respectively.

3.2.7.2 Superoxide radical scavenging activity

Superoxide radical scavenging activity was detected by the NBT reduction method introduced by Mc Cord and Fridovich (1969). It depends on the light-induced superoxide radical generation by riboflavin. Different concentration (0, 50, 100, 150, 200, 250, and 300 µg/mL) of the extract was added to the reaction mixture contained 0.12 mM riboflavin (50 µL), 1.5 mM NBT (100 µL), 0.003 mg NaCN in 0.1M EDTA (200 µL), and 0.6 M SOD buffer (pH 7.8) in to final volume of 3 mL. The tubes containing the reaction mixture were uniformly illuminated for 20 mins using an incandescent lamp (Philips, 40W), and the optical density was measured at 560 nm before and after illumination. The Superoxide radical thus formed reduces the NBT to a blue color formazan complex, and the effect of the sample to scavenge superoxide radical was evaluated by comparing the optical density of control and treated samples using the formula,

$$\% \text{ of Superoxide radical scavenging activity} = [(A_0 - A_1)/ A_0 \times 100],$$

Where A_0 represents control absorbance and A_1 the sample absorbance.

3.2.7.3 Lipid peroxidation inhibition activity

The lipid peroxide generation was measured by Thiobarbituric acid reaction substances (TBARS) formation Ohkawa *et al*., (1979). Different concentrations of the extract (0, 100, 200, 300, and 400 µg/mL) were incubated at 37º C for 1 hr with the reaction mixture contained 500 µL of rat liver homogenate (20% W/V) in Tris-HCl buffer (40 mM, pH 7.4) for the induction of lipid peroxide, 50 µL KCl (30 mM), 50 µL ferrous sulfate (15 mM), 50 µL of ascorbic acid. The reaction mixture was made up to 2 mL with distilled water. After incubation, add 1.5 mL of 20% acetic acid (pH 3.5) and centrifuge at 1500 rpm for 20 min. After vigorous shaking, 400 µL of the

supernatant was incubated with 200 µL SDS (8%), 1.5 mL of TBA, and 1.5 mL acetic acid (20%, pH 3.5) for 20 min at 100º C. A pink-colored product was formed by reaction of thiobarbituric acid (TBA) reagent with Malondialdehyde (MDA), produced during peroxidation. The optical density of control and sample was measured at an absorption maximum of 532 nm. The lipid peroxidation inhibition was calculated as follows,

$$\% \text{ of lipid peroxidation inhibition activity} = [(A_0 - A_1)/ A_0 \times 100],$$

Where A_0 represents the control absorbance and A_1 is the sample absorbance.

3.2.7.4 Ferric Reducing Antioxidant Power assay

The extract of the sample was analyzed for their antioxidant activity against the stable free radical FRAP reagent by the method Benzie *et al*., (1996). The intense blue coloration can visualize the reduction of ferric tripyridyl-s-triazine (Fe III TPTZ) complex to ferrous form at low pH. The reaction mixture contained 900 µL of FRAP reagent (75 µL ferric chloride (20 mM), 75 µL TPTZ (10 mM), 750 µL acetate buffer of pH 3.6). The sample extract of different concentrations (0, 20, 40, 60, 80, and 100 µg/mL) was made to 1 mL final volume using acetate buffer. Incubate the reaction mixture for 15 mins at 37º C, and optical density readings were monitored at 593 nm against 1 mL acetate buffer as blank. The change in absorbance is correlated to the total reducing power of the sample extract present in the reaction mixture. The value obtained from a $FeSO_4.7H_2O$ standard graph is expressed. The ferric reducing activity of drug concentration is equal to the reducing power $FeSO_4.7H_2O$ and is calculated by the following formula,

$$\% \text{ of FRAP assay} = [(A_1 - A_0)/ A_1 \times 100],$$

Where A_0 denotes the control absorbance, and A_1 is the sample absorbance.

3.2.8 In vitro cytotoxicity studies

3.2.8.1 Trypan Blue Exclusion test of cell viability

Cytotoxic activity of the endophytic bacterial extract was studied using the trypan blue dye exclusion technique by analyzing the percentage of viability of Daltons Lymphoma Ascites (DLA) cells. DLA cells were grown in the peritoneal cavity of mice weighing 25-30 g by injecting cell suspension ($1x10^6$ cells/mL). On the 15^{th} day, tumor cells were aspired from the peritoneal cavity of the mice and centrifuged for 15 min at 1500 rpm after washing with PBS (0.2 M, pH 7.4). The pellet was again dissolved in PBS, and the process was repeated thrice. At last, the cells were suspended in a known quantity of PBS, and the cell count was adjusted to $1x10^6$ cells/ mL. Next, cell suspension of 0.1 mL was dispensed in 0.8 mL of phosphate buffer and incubated with different concentrations (10-200 µg/mL) of samples for 3 h at 37º C. After 3 h, add 0.1 mL trypan blue dye (1%), and cells were examined in a compound microscope using a hemocytometer. The IC_{50} value was calculated (Moldeus *et al*., 1978).

3.2.8.2 MTT assay

The cytotoxic potential of sample extract against breast cancer cell line MCF-7 was tested using MTT (3-(4,5-dimethythiazol-2yl)-2,5-diphenyl tetrazolium bromide) assay (Mosmann,1983). The monolayer cell culture was trypsinized (0.25% Trypsin and 0.03% EDTA), and the cell count was adjusted to $1x10^6$ cells/mL in the Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal bovine serum (FBS). About $1x10^6$ cells were seeded in each well of the 96 well plates. When a partial monolayer was formed after 24 h, the supernatant was flicked off, and 100 µL of test extracts (12.5, 25, 50, 75, and 100 µg/mL) were added to the cells in microtiter plates

incubated at 37º C in an atmosphere of 5% CO_2 for 24 h. Cells incubated in a complete medium without test extract were kept as control. After incubation, the medium was discarded, and 20 µL MTT (5 mg/mL) was pipetted, and the cells were again incubated for 3 h. After the incubation, the MTT solution covering the cells was removed using a micropipette. 100 µl of dimethyl sulphoxide was added to the wells of the microtitre plate. The absorbance were noted in a microplate reader (ELISA Reader, Biotek) at 570 nm to determine cell viability. The experiment was repeated thrice. The percentage of cell viability and IC_{50} value of the test extract was calculated using the formula,

Percentage of cell viability = [Mean OD of experimental wells/ Mean OD of control wells] × 100

3.2.9 In vivo animal study

3.2.9.1 Toxicity analysis

Two groups of Swiss albino mice (6 mice/group) for the acute toxicity study were taken. Group, I was placed as control, and Group II was administrated orally with a single dose drug (300 mg/Kg b.wt.) as per OECD guidelines. The animals were monitored for visible changes in behavior, body weight, hair loss, food, and water intake for two weeks. Also, the mortality rate of the mice will be noted (Yamanaka *et al*., 1990).

3.2.9.2 Anti-inflammatory activity

The anti-inflammatory activity of ethyl alcohol extract of samples NMC15 and NMT07 were evaluated using acute (carrageenan) and chronic (formalin)

inflammation models in male Swiss albino mice. For in vivo study, extracts were dissolved in 1% propylene glycol, used as vehicle control.

3.2.9.2.1 Carrageenan-induced acute paw edema

The carrageenan-induced paw edema model evaluated the acute anti-inflammatory activity according to Winter *et al*., 1962. The experimental animal male Swiss albino mice were divided into five groups. Each group contains six animals, which is the minimum required to meet a statistical conclusion of acceptable standards (Erb, 1990). Each group was pre-treated for five consecutive days with the drug at different concentrations administrated orally. After one hour of the fifth dose, edema was induced in the right hind paw of mice by sub plantar injection of 0.02 mL freshly prepared suspension of 1% carrageenan in 0.1% carboxymethyl cellulose (CMC). The paw edema was measured before and after administering phlogistic agents using digital vernier calipers and recorded every hour up to the 6^{th} hour. The inhibition percentage of paw edema was calculated using the formula-

$$[(tCn-tC0) - (tTn-tT0)]/ (tCn-tC0) \times 100$$

Where tC0, tCn represents paw edema of control animal and tT0, tTn paw edema of treated animal at respective hours before and after injection of phlogistic agents.

Groups and dosages

Group 1: Control, injected with 1% carrageenan (20 µl)

Group 2: Vehicle control (1% Propylene glycol) + 1% carrageenan (20 µl)

Group 3: Administered with 10 mg/kg b.wt. diclofenac + 1% carrageenan (20 µl)

Group 4: Administered with 25 mg/kg b. wt. sample extract + 1% carrageenan (20 µl)

Group 5: Administered with 50 mg/kg b. wt. sample extract + 1% carrageenan (20 µl)

3.2.9.2.2 Formalin-induced chronic inflammation

The chronic anti-inflammatory activity in Swiss albino mice was evaluated by formalin-induced paw edema according to the method by Chau, 1989. The experimental animals were categorized into five groups containing six animals each. The drug was administrated orally for five consecutive days. On the fifth day, all animals induced chronic inflammation by sub-plantar injection of freshly prepared 0.02 mL of 2% formalin on the right hind paw. The inflammation was measured using digital vernier calipers before and after injection of formalin for six consecutive days. An increase in paw thickness as a measure of inflammatory edema was calculated using the formula of, P_t-P_o where P_o is the initial thickness before induction. The inhibition was calculated by the formula, P_c-P_t/ P_c×100, where P_t increases the thickness of the treated mice and P_c is the control.

Groups and dosages

Group 1: Control, injected with 2% formalin (20 µl)

Group 2: Vehicle control (1% Propylene glycol) + 2% formalin (20 µl)

Group 3: Administered with 10 mg/kg b.wt. diclofenac + 2% formalin (20 µl)

Group 4: Administered with 25 mg/kg b. wt. sample extract + 2% formalin (20 µl)

Group 5: Administered with 50 mg/kg b. wt. sample extract + 2% formalin (20 µl)

3.2.9.3 Antitumor study

3.2.9.3.1 EAC-induced ascites tumor model

The antitumor activity of the extract will be studied using the EAC-induced ascites tumor model (Kuttan *et al*., 1985). The experimental Male Swiss albino mice will be categorized into five groups of six animals each. EAC cells will be aspirated from the peritoneal cavity of the tumor-bearing mice on the first day, and the ascites tumor will

be induced by injecting 0.1 mL of the cell lines (1×10^6 cells/animal) intraperitoneally to the animals. The control group will be injected with ascites tumor only and the standard with cyclophosphamide drug, while the treated groups will be administered with low and high doses of NMT07 extract. The drug will be administered for ten consecutive days. The death pattern of animals due to tumor burden was recorded, and the percentage increase in life span was calculated using the formula-

$$\% \text{ ILS} = (\text{T-C/C}) \times 100$$

Where 'T' and 'C' are mean survival of treated and control mice, respectively.

Groups and dosages

Group 1: Control, injected with tumor alone (EAC)

Group 2: Vehicle control (1% Propylene glycol) + EAC cells (100 µL)

Group 3: Administered with 10 mg/kg Cyclophosphamide + EAC cells (100 µL)

Group 4: Administered with 25 mg/kg sample extract + EAC cells (100 µL)

Group 5: Administered with 50 mg/kg sample extract + EAC cells (100 µL)

3.2.9.3.2 DLA-induced solid tumor model

The antitumor activity of the extract will be studied using DLA induced solid tumor model (Chihara *et al*., 1970). The experimental male Swiss mice will be divided into four groups of six animals each. On the first day, DLA cells will be aspirated from the peritoneal cavity of the tumor-bearing mice. The solid tumor will be induced by injecting 0.1 mL of the cell lines (1×10^6 cells/animal) intramuscularly to the right-hand limb of the animals. The drug will be given to mice for ten consecutive days. The solid tumor development on the animal in each group was determined by measuring the diameter of the tumor in two perpendicular planes using

a vernier caliper. The readings were taken at a three days interval up to 30 days. The tumor volume was calculated according to the following formula, $V = 4/3\ \pi\ r_1^2\ r_2$, where r_1 is the minor radius, and r_2 is the major radius. The percentage inhibition of tumor growth was calculated using the formula-

$$\% \text{ inhibition} = [(C-T)/C] \times 100$$

C is the tumor volume of control animals on the 30^{th} day, and T is the tumor volume of treated animals on the 30^{th} day.

Groups and dosages

Group 1: Control, injected with tumor alone (DLA)

Group 2: Administered with 10 mg/kg Cyclophosphamide + DLA cells (100 µL)

Group 3: Administered with 25 mg/kg sample extract + DLA cells (100 µL)

Group 4: Administered with 50 mg/kg sample extract + DLA cells (100 µL)

3.2.9.3.3 Hematological parameters

Towards the end of the fifth week, the blood was removed from each group of Dalton Lymphocytes Ascites tumor-bearing mice by cardiac puncture to obtain the maximum possible blood for analysis. The mice blood was collected in tubes containing the anticoagulant BD Vacutainer™ K2EDTA. The assessment of hematological parameters such as hemoglobin (Hb) content, red blood cell (RBC) and total white blood cell (WBC) count, platelet count, neutrophils (%), and lymphocytes (%) using fully automated equipment Mindray BC 20s analyzer following the rules of good practice in clinical laboratory analysis.

3.2.9.3.4 Histopathology of tumor-induced mice

A portion of the tumor was cut and washed in PBS, fixed in 10% formaldehyde solution for 24 hrs. The tissue was embedded in paraffin wax. Sections of 5-6 microns in thickness were taken using a manual microtome with the disposable blade of thickness 4 mm at an angle of 45°. The slides were double-stained with hematoxylin and eosin dyes. The photograph of the stained tissues was taken in a phase-contrast microscope (Magnus, India) at 200 X magnification (Su *et al*., 2012).

3.2.10 Statistical analysis

All the invitro and in vivo experiments were repeated atleast thrice, and the values were expressed in Mean ±SE. The data obtained were statistically analyzed with significance difference of values ($P<0.001$, $P<0.01$and $P<0.05$) using one-way ANOVA followed by an appropriate post hoc test (Dunnett's multiple comparison test) using Prism 9.0 GraphPad software Inc. USA.