Chapter-5

Discussion

Microorganisms control many of the vital processes on which the very maintenance and survival of an ecosystem depend. Endophytes that obtain nutrients from internal plant tissues produce amylase, cellulase, and protease for their sustainability (Sunitha *et al*., 2013). The endophytic bacteria grow inside the plant by taking essential metabolites from their host. These metabolites may be the active compound representing the medicinal value of the host plant. With this regard, the endophytic bacterial isolates may secrete the compound as secondary metabolites resembling their host. These active metabolites of biological interest could be isolated from bacteria through in vitro techniques. Thus, the current research aimed to isolate such endophytic bacteria that carry valuable compounds representing the host plant bioactivity. In the future, these endophytes could replace the plant when thought to be endangered due to their constant use in the pharmaceutical area.

For the present study of endophytic bacteria and their beneficial aspects, two medicinal plants, *M. citrifolia* and *M. pubescens* were selected from different locations of the Thrissur district of Kerala, India. A total of 15 different strains were isolated, among which 9 different strains were isolated from the surface-sterilized leaves of *M. citrifolia* and 6 from *M. pubescens*. Though there was an information on the isolation of endophytic bacteria from *M. citrifolia,* as mentioned in chapter 2, none of the strains obtained in this work were previously studied. And also, so far, no endophytic bacteria prevailing in *M. pubescens* have been reported. Thus, all the isolated six bacterial strains of *M. pubescens* were selected for the beneficial study.

5.1 Endophytic bacterial strain identification

The isolates were identified according to their morphology, physiology, and biochemical characters. The present study documented the distinctive colony morphology, cellular characteristics, and other biochemical and physiological properties of the isolates. All the isolates are gram-positive bacteria, except strain with sample code NMC15, is gram-negative. Also, all the isolates are pigmented with varying colony colors of light yellow, yellow, lemon yellow, orange, dark orange, and reddish-orange, indicating carotenoids' presence.

The physiological parameters (temperature and pH) for the growth of bacteria vary with isolates. The optimum growth temperature was 28º C for NMC07, NMC09, NMC11, NMT03, NMT04, NMT09 and 37º C for NMC01, NMC02, NMC03, NMC08, NMC10, NMC15, NMT06, NMT07, and NMT08. The optimum growth pH was 6 for NMC02, NMC08, NMT03, NMT06, NMT08, NMT09, and pH 7 for NMC01, NMC03, NMC07, NMC09, NMC10, NMC11, NMC15, NMT04, and NMT07. The biochemical tests shows a positive result for catalase and a negative for H_2S production, indole, and phenylalanine deaminase tests for all the isolates. In addition, the biochemical test results differ with the isolates giving a vague idea of bacteria at the genus level. Further identification of the isolates was made genotypically by the 16S rRNA gene sequencing method.

Owing to the characterization of bacteria, both phenotypic and genotypic identification gives different isolates. To sum up with the isolates characters, the NMC01 isolate was gram-positive non-spore-forming bacilli, with an orange colony, motile, circular shape, butyrous texture, entire margin, and raised elevation. In addition, the NMC01 isolate showed 94.71% similarity to *Exiguobacterium*

aurantiacum AN2. NMC03 isolate was gram-positive, non-spore-forming bacilli, with the orange colony, motile, circular shape, mucus texture, entire, and raised elevation. NMC03 isolate showed 97.64% similarity to *Exiguobacterium* sp. T230. NMC10 isolate was gram-positive, non-spore-forming bacilli, light orange colony, motile, circular, mucus texture, entire margin, and raised elevation. NMC10 isolate showed 100% similarity to *Exiguobacterium alkaliphilum* S13. Moreover, the construction of the phylogenetic tree for NMC01 isolates made to become an outgroup for NMC03 and NMC10 and other bacteria with the highest similarity score.

NMC02 isolate was gram-positive, spore-forming bacilli, light orange colony, motile, slightly irregular, viscid texture, entire margin, and raised elevation. NMC02 isolate showed 96.47% similarity to *Bacillus* sp. SCSSS02. NMC08 isolate was gram-positive, spore-forming bacilli, light yellow, motile, slightly irregular, viscid, entire margin, and elevation raised. NMC08 isolate showed 100% similarity to *Bacillus marisflavi* sp. CP31. NMT07 isolate was gram-positive, spore-forming bacilli, dark orange colony, motile, circular, mucus texture, entire margin, and raised elevation. NMT07 isolate showed 94.70% similarity to *Bacillus vietnamensis* KST183. NMT08 isolate was gram-positive, spore-forming bacilli, light yellow colony, motile, slightly irregular, viscid texture, entire margin, and raised elevation. NMT08 isolate showed 99.52% similarity to *Bacillus marisflavi* P/W8 AM. The phylogenetic tree construction for NMC02 isolates becomes an outgroup for NMC08, NMT07, NMT08, and other related bacteria with the highest similarity score.

NMC07 isolate was gram-positive, non-spore-forming cocci, yellow colony, non-motile, circular, butyrous texture, entire margin, and raised elevation. NMC07 isolate showed 100% similarity to *Micrococcus* sp. M14. NMC09 isolate was gram-positive,

non-spore-forming cocci, yellow colony, non-motile, circular, butyrous texture, entire margin, and raised elevation. NMC09 isolate showed 95.72% similarity to *Micrococcus* sp. FJFR62. NMC11 isolate was gram-positive, non-spore-forming cocci, bright yellow colony, motile, circular, butyrous texture, entire margin, and raised elevation. NMC11 isolate showed 100% similarity to *Micrococcus yunnanensis* 8G. NMT04 isolate was gram-positive, non-spore-forming cocci, yellow colony, non-motile, circular, butyrous texture, entire margin, and raised elevation. NMT04 isolate showed 99.91% similarity to *Micrococcus yunnanensis* 994. Based on phylogenetic analysis, NMC09 serves as an outgroup for the isolates NMC07, NMC11, NMT04, and other related isolates.

NMC15 isolate was gram-negative, non-spore-forming bacilli, reddish-orange colony, non-motile, circular, mucus texture, entire margin, and raised elevation. The isolate showed 99.92% similarity to *Brevundimonas vesicularis* C5-8. Also, it becomes an outgroup for other *Brevundimonas* sp. with maximum identity score. The NMT03 isolate was gram-positive, non-spore-forming bacilli, bright orange colony, non-motile, circular, mucus texture, entire margin, and raised elevation. NMT03 isolate showed 99.36% similarity to *Microbacterium kitamiense* AS66. NMT09 isolate was gram-positive, non-spore-forming bacilli, lemon yellow colony, motile, circular, mucus texture, entire margin, and raised elevation. In addition, the NMT09 isolate showed 99.51% similarity to *Microbacterium paraoxydans* CF 36. Also, both the isolates NMT03 and NMT09, vice versa, serve as outgroups derived from the same genus *Microbacterium*. NMT06 isolate was gram-positive, non-spore-forming bacilli, orange colony, non-motile, circular, mucus texture, entire margin, and raised elevation. NMT06 isolate showed 99.60% similarity to *Brevibacterium* sp. O1. An

outgroup for NMT06 and related species was *Brevibacterium* sp. SGAir0440 belongs to the same genus.

5.2 Metagenomic analysis of bacterial community

The metagenomic investigation of *M. pubescens* leaves reports the vast diversity of endophytic bacterial communities. The findings unveil for the first time the presence of the bacterial community that prevails in this medicinal plant using the Illumina Hiseq sequencing protocol. High-throughput sequencing was accurately performed using the appropriate primer pair to amplify a stretch of the V3-V4 region of the 16S rRNA gene. The correct choice of adopting various bioinformatics tools was equally important and challenging to reduce the errors hosted by the host DNA. The de-novo chimera removal method VSEARCH implemented by UCHIME was used to improve the overall quality of the reads. The utility of the sequencing platform for its high-resolution microbiota profiling (N90% at genus level) of endophytic bacterial communities shows that species were detected at similar abundances by MG-RAST and QIIME pipeline. The majority of them belong to the four most abundant phyla, Proteobacteria, Bacteroides, Firmicutes, and Actinobacteria. The study also elucidates the genus such as *Brevundimonas*, *Bacteroides*, *Serratia*, *Propionibacterium* by OTU distribution. The result shows that NGS technology uses the shotgun 16S rRNA gene sequencing, which discloses microbial communities' overall richness and diversity in plant tissues. The α-diversity analysis indicates that the species richness is more in Actinobacteria, while the species evenness is more in Proteobacteria. Also, the Simpson diversity index and Shannon index varies within the most prominent phyla.

5.3 Chemo-profiling of selected eight endophytic bacterial strains

Natural products remain to give exceptional chemical diversity with a broad bio-activitiy spectrum. Consequently, so far they have been the most promising sources for drug discovery and development. The selected isolates to study the biological activity were all pigmented. Literature establishes that the natural colorants produced from microbes were applied in food and pharmaceutical products. Apart from this, bacterial pigments have several pharmacological activities like antimicrobial, anticancer, antioxidant, anti-inflammatory, antiprotozoal, anti-allergic properties, and much more with enormous economic potential (Venil *et al*., 2020). In connection with these characteristics of the bioactive compounds of bacteria, Solieve *et al*., (2011) have earlier reported the highest bioactivity were shown by red, orange, yellow, and green pigmented bacteria. In this work, the isolated bacteria were different shades of orange and yellow pigmented which were chosen to study the biological property of medicinal value.

Cyclo(leucylopropyl), a dipeptide, is the primary compound identified from the *Exiguobacterium aurantiacum* NMC1 (NMC01) extract found using GC-MS profiling. Rhee, 2003, isolated this compound from *Streptomyces* sp. which shows antibacterial activity and is antagonistic to Vancomycin-resistant *Enterococci* (VRE) strains. The primary compound identified from *Brevundimonas vesicularis JAP* (NMC15) and *Bacillus vietnamensis* SMC (NMT07) extract is Dicarpyl phthalate. Studies reveal that this compound has antitumor properties (Gao and Wen, 2016).

Usually, phthalates are plasticizers, classified as environmental pollutants with some risk for humans. However, in recent years this compound has been produced by plants, fungi, and bacteria. Many aerobic bacteria degrade phthalates and forming key intermediates such as dicarpyl phthalic acid and monoester phthalate in anaerobic mineralization of phthalate esters (Ortiz and Sansinenea, 2018). In the present study, the isolated endophyte, *Brevundimonas vesicularis* and *Bacillus vietnamensis* SMC, are an aerobic bacterium that produces phthalate compounds. Therefore, it may be capable of degrading phthalates. In addition, reports on the isolation of two aromatic phthalate esters showing broad-spectrum antibacterial and cytotoxic activity from the bacterium *Brevibacterium mcbrellneri* of the genus, *Brevibacterium* have been identified (Rajamanikyam *et al*., 2017). Also, supporting evidences regarding dibutyl phthalate degradation by *Bacillus* sp. were provided in 2011 by Navacharoen, and Vangnai, and in 2019 Lotfy *et al*., documented enhanced production of phthalate by another sp. of *Bacillus* in their research study.

The main compound identified from the ethyl alcohol extract of *Microbacterium kitamiense* REGI is 1-Ethylsilatrane. In 1979, Voronkov has surveyed the biological activity of silatranes which have wide application in medical and agricultural fields. In *Micrococcus yunnanensis* STC, the primary compound identified was 2,4-ditert-butylphenol, which have been reported with antioxidant and antifungal activity by Varsha *et al*., 2015. 1,2-Benzenedicarboxylic acid is the main compound present in *Brevibacterium* sp. CVB, *Bacillus marisflavi* MENA, and *Microbacterium paraoxydans* REKA. This compound were earlier extracted from marine bacterium found to have cytotoxic activity (Krishnan *et al*., 2014).

5.4 Quantitative estimation of Total Phenolic Content

Total Phenolic content (TPC) of the isolates were estimated in the ethyl alcohol extract of the sample. From the eight samples taken under study *Bacillus Vietnamensis* SMC (NMT07) has more phenolic content with estimated value of 226.04 ±0.04 µg/mg of the extract calculated as gallic acid equivalent. The percentage yield of NMT07 was calculated to be 22.6%. Studies supporting TPC of *Bacillus* sp. shows that on fermentation with the *Bacillus* species, an increase in total phenolic compounds have been found soyabean plant (Juan and Chou, 2010).

5.5 Biogenic synthesis of iron oxide nanoparticles

Bacterial mediated iron oxide nanoparticles synthesis is an eco-friendly approach. Extracellular microbial enzymes and secondary metabolites secreted by endophytic bacteria play a central role in synthesizing metal nanoparticles. Iron oxide nanoparticles were synthesized from the *Exiguobacterium aurantiacum* NMC1 strain using the extracellular methodology. The UV-Vis spectrophotometry showed a surface plasmon resonance (SPR) peak with maximum absorbance (λ-max) at 293.50 nm. The FTIR analysis showed that functional groups corresponding to the peak (3421.72 cm^{-1}) –OH stretching, (3742.72 cm^{-1}) –OH with hydrogen bonding, (2362.80 cm^{-1}) C-H stretching, (1627.92 cm^{-1}) –NH bending, (1546.91 and 1519.91 cm^{-1}) alkane groups, and (1022.27 cm^{-1}) P-O stretching, are associated with iron oxide nanoparticles. In contrast, peaks 669.30 and 524.64 cm^{-1} lies in the fingerprint region. The SEM images showed nanoparticles are spherical. The EDAX confirms the presence of a Fe and oxygen element, assuring the purity of synthesized nanoparticles. TEM analysis showed the particle's size range with a diameter between 9.05 nm and 51.21 nm. XRD peaks observed at 27.8, 32.0, 45.0, 54.0, 56.7 and 66.6 º corresponds

to 220, 311, 400, 422, 511 and 440 crystal planes of gamma Fe_2O_3. The absence of a peak at around 38.0 º corresponding to 222 planes clearly indicates the absence of the Fe_3O_4 phase. Thereby, the finding reveals that the synthesized iron oxide nanoparticle from the endophytic bacteria (NMC01) is γ-Fe_2O_3. In addition, different forms (α and γ) of Fe_2O_3 can be synthesized by annealing the sample at different temperatures.

5.5.1 Application of synthesized nanoparticles

The heavy metals, such as Pb (II), Cd(II), and Cr(III), in aqueous solutions, are toxic even at trace levels and have caused adverse health impacts on human beings. Hence, removing these heavy metals from the aqueous environment is essential to protect biodiversity, hydrosphere ecosystems, and human beings. Adsorption is an often-used method for removing heavy metal ions as this process possesses high efficiency, is easy to handle, and is cost-effective. Iron oxide-based nanomaterial was more attractive for removing heavy metals from the aqueous solution because of their size, high surface area, and magnetic property (Lingamdinne *et al*., 2019). Therefore in this research work, a preliminary screening of iron oxide nanoparticles to remove heavy metals from treated aqueous solutions was conducted. The result indicates that removal of lead from treated water was more efficient when compared to cadmium and chromium. Moreover, literature study reveals that the species of *Exiguobacterium* can remove heavy metals from wastewater. *Exiguobacterium* strains have wide application in bioremediation due to their unique characteristics, such as neutralizing highly alkaline wastewater, as reported by Kumar *et al*., 2006. In connection, other *Exiguobacterium* strains could bring about the removal of Cr (VI) over a broad range of pH, temperature, and salt concentrations (Okeke, 2008). Supporting documents on chromium removal were also provided by Batool *et al*., 2014 in their studies in free

and immobilized forms of *Exiguobacterium* sp. So, conclusively the present work mention that the probability of metal removal by synthesized maghemite nanoparticles may be due to their capping agents, which are functional groups derived from *Exiguobacterium aurantiacum*, NMC1 extracellular components.

Synthetic dyes are most widely used in the food and textile industries. Azo dyes are the most versatile and the largest class of dyes and account for more than 50% of the dyes produced worldwide (Puvaneswari *et al*., 2006). Azo dyes have vivid colors and are structurally diverse organic compounds having aromatic amines as their essential precursors. Therefore, azo dyes pose lethal effects, genotoxicity, mutagenicity, and carcinogenicity to humans and animals. Also, these dyes are not readily removed from wastewater by conventional wastewater treatment methods.

Azo dyes such as Eriochrome Black T, Congo red, and Trypan blue, which are categorized as carcinogens, were chosen for the present work. Moreover, these dyes are inevitable in laboratories experiments by researchers. The study reports that decoloration of dye after adding synthesized γ-Fe_2O_3 nanoparticles was observed, and absorbance was recorded spectrophotometrically across with maximum absorbance wavelength of respective dyes. The results showed maximum percentage of dye decoloration in Congo red (88.2%), followed by Trypan blue (63.69%) and Eriochrome Black T (50%). Also, literature studies evidence that *Exiguobacterium aurantiacum* isolated from the textile industry is potent in azo dye decoloration (Kumar, 2016). Therefore, this evidence supports the preliminary study on azo dye decolorization by γ-Fe_2O_3 nanoparticles synthesized from *Exiguobacterium aurantiacum* NMC1. The functional active compound principles in the bacterium capping the nanoparticle enables the azo dye's decoloration.

5.6 Antimicrobial activity

The foremost human health issue is the infection caused by the multidrug-resistant (MDR) bacteria resistant to commercial antibiotics (Van Duin and Paterson, 2016). The pigmented bacteria exhibited maximum antimicrobial activity. On the other hand, the multi-drug resistant bacteria were inhibited by orange-pigmented bacteria (Ayuningrum *et al.*, 2017). In the present study, the antimicrobial capability of the endophytic strains has been evaluated by preliminary screening. The antibacterial activity of selected isolates was initially screened for using the cross-streak plate method. The study was conducted following standard microbial testing protocol by adjusting the turbidity of bacterial culture suspension to 0.5 McFarland reference standard. The result showed that out of eight strains, the pure colony of *Exiguobacterium aurantiacum* NMC1 bacterial culture showed inhibition against some human pathogenic bacteria, *Escherichia coli*, *Listeria* sp., *Klebsiella* sp., and *Streptococcus* sp. In the study, the major compound identified in exometabolites extract was Cyclo(leucylopropyl). There are reports on the bactericidal activity of *Exiguobacterium* species in food-borne pathogens (Mekala *et al*., 2020). Moreover, Putra and Karim (2020) isolated Cyclo(leucylopropyl) from the fungus *Fusarium* shows antibacterial activity against *Escherichia coli*. This supports the present study of *Exiguobacterium aurantiacum* NMC1 extracts showing antibacterial activity may be due to this significant compound. However, the study of the antibacterial activity of the endophytic bacterium *Exiguobacterium aurantiacum* has not been previously reported.

A preliminary study of the antifungal activity of the isolated endophytic bacteria was studied using the dual plate culture method against the plant pathogens,

Sclerotium rolfsii and *Aspergillus* sp. The study results that the isolates *Microbacterium kitamiense* REGI (NMT03), *Micrococcus yunnanensis* STC (NMT04), and *Microbacterium paraoxydans* REKA (NMT09) showed significant inhibition on the growth of *Sclerotium rolfsii* at 29.26, 47.04, and 45.55 %, respectively. The inhibition of *Aspergillus* sp. growth was noticed effectively at 35.93, 42.96, and 52.96 % by *Microbacterium kitamiense* REGI (NMT03), *Micrococcus yunnanensis* STC (NMT04), and *Brevibacterium* sp. CVB (NMT06) isolates. Moreover, these results could be justified by the work of de Boer *et al*., (2004), indicating that most bacteria secrete some extracellular enzymes such as chitinase, cellulase, glucanase, and protease, which digest the fungal mycelia.

5.7 Antioxidant activity

Antioxidants play a primary role to humans in protecting against various infections and degenerative diseases by inhibiting and scavenging free radicals. Unfortunately, many synthetic antioxidants are used to retain the oxidation process, which has potential health hazards, and researchers are focusing on screening alternative antioxidants from natural sources. Therefore, natural antioxidants derived from microbes are nowadays replacing the synthetic with their incredible effects. The present study on the antioxidant capacity of bacterial isolates results in getting appreciable isolates that suppress oxidants with low extract concentration. Furthermore, the scavenging of free radicals like DPPH and superoxide, and also lipid peroxidation inhibitory activity and ferric reducing power of the samples were analyzed in the study.

DPPH, a relatively stable organic free radical, has been used widely to determine the antioxidant activities of various biological compounds. The discoloration of the

deep purple color of DPPH radical on contact with the sample extract points out the scavenging property of the isolates. All the samples were found to scavenge the radical effectively in a concentration-dependent manner. Moreover, out of eight samples, *Microbacterium kitamiense* REGI (NMT03) showed maximum radical scavenging efficacy of 83.2% with an IC_{50} value of 49.13 ±0.76 µg/mL, followed by *Microbacterium paraoxydans* REKA (NMT09) of 82% with an IC_{50} value of 74.13 ±0.76 µg/mL in comparison with ascorbic acid (19.02 ±1.11 µg/mL) used as a standard drug. Both the *Microbacterium* species elevated their scavenging potentiality among the eight samples selected for the study. This may be due to the presence of fatty acid constituents like nonadecane (1.73%), tetracosane (2.08%), tetradecane (2.36%), eicosanol (2.41%), and methyltetracosane (1.59%) identified in GC-MS analysis of NMT03 and methyl-7-hexadecenoate (1.96%) in NMT09.

Superoxide radical scavenging activity (SRSA) for all the selected samples was determined by the NBT reduction method. All the samples showed superoxide radical scavenging properties. The isolate *Brevundimonas vesicularis* JAP (NMC15) exhibited significant scavenging activity of 79.97% with an IC_{50} value of 170.26 ±0.36 µg/mL and could serve as a potential source of natural antioxidants. Also, *Brevibacterium* sp. CVB (NMT06) extract shows effective scavenging with a percentage of 75.81% with an IC_{50} value of 63.71 ±0.84 µg/mL, and the standard drug (ascorbic acid) has an IC_{50} value of 23.02 ±1.02 µg/mL. The GC-MS profiling of NMC15 and NMT06 reveals phthalate derivatives. Studies by Zhong-Ji *et al*., 2012 supports the present findings on the explanation that phthalate has antioxidant property.

Lipid peroxidation inhibition study of the isolates extract was carried out in rat liver homogenate. All the samples taken for the analysis significantly inhibited lipid peroxidation except the *Brevundimonas vesicularis* JAP (NMC15). NMT06 showed the highest inhibition activity of 70.17% with an IC_{50} value of 27.3 ±0.65 µg/mL followed by NMT03 with an IC_{50} value of 32.96 ±0.37 µg/mL having 68.93% inhibition capacity.

In the FRAP assay, the reducing power of each compound was estimated by a color change from yellow to different shades of green. In the present research, all the samples exhibited this assay has reducing potential. Among which *Bacillus vietnamensis* SMC (NMT07) has a maximum reducing power of 76.27% with an IC_{50} value of 33.55 ±0.69 µg/mL, and ascorbic acid has 4.45 ±1.27 µg/mL. The study on the antioxidant potential using FRAP assay was investigated by Patkar *et al*., in 2021 from the pigments of *Bacillus* sp. supports the present study.

5.8 Cytotoxicity study

Cytotoxicity is one of the chemotherapeutic targets of antitumor activity. The majority of the clinically tested antitumor agents possess significant cytotoxic activity in cell culture systems. Therefore, in vitro cytotoxic study of selected eight bacterial isolates was conducted. An ethyl alcohol extract of the isolates was evaporated to remove alcohol residue and reconstituted in DMSO solvent, i.e., 10 mg of extract in 1 mL of solvent. Through Trypan blue exclusion method, the short-term cytotoxic effect of the sample extract was studied. The findings show that *Brevundimonas vesicularis* JAP (NMC15) and *Bacillus vietnamensis* SMC (NMT07) have an IC_{50} value of 47.51 ±0.183 and 44.12 ±0.311 µg/mL, respectively; found to be below 50 µg/mL was selected for in vivo studies. The higher cytotoxic activity of sample

extract against DLA cell lines explains its increased antitumor activity against ascites and solid tumors compared to other extracts.

The long-term cytotoxicity of isolate *Brevundimonas vesicularis* JAP (NMC15) and *Bacillus vietnamensis* SMC (NMT07) extract was determined by MTT assay. Approximately 1×10^6 of MCF-7 breast cancer cells were seeded in flat bottomed 96 well plates with different concentrations of extracts and incubated at 37° C for 24 h. After treatment, 20 µL of MTT (3-(4,5-dimethythiazol-2yl)-2,5-diphenyl tetrazolium bromide) was added to all the wells and incubated for 3 h. Formazan crystals developed were dissolved in DMSO; the absorbance of the colored product was measured at 570 nm, and the death rate of cells was calculated. The average absorbance of untreated negative controls was taken as 100% survival. By comparing the percentage of death of the treated cell population with the untreated control, cytotoxicity was determined. The extracts also showed a dose-dependent inhibition of MCF-7 cells in culture with an IC_{50} value of 79.36 ±1.91 µg/mL for NMC15 and 98.91 ±1.86 µg/mL for NMT07 sample extract.

5.9 In vivo study

5.9.1 Toxicity analysis

The drugs and chemicals exposure often induce toxicity to living organisms. The pharmacokinetics of the compound is the main factor determining the toxicity of the drug followed by the metabolism and the response of target organ. Safety dosage of drug can be analyzed in animal study, which helps to determine the ratio between the beneficial dose and the toxic dose of drug. Thus the dose level recommended for the treatment of disease are studied in the animal models. Also, the OECD guidelines enables the characterization of adverse effects of repeatedetly exposure to a test. In

the present study, the acute toxicity of drug applied as single dose (300 mg/Kg. b.wt.) was accessed in male Swiss albino mice. The drug was safe with no signs of toxicity and was able to fix the lower dose (25 mg/kg) and higher dose (50 mg/kg) for in vivo biological studies on the proportion of one-tenth and one-fifth of the drug dose taken for toxicity.

5.9.2 Anti-inflammatory activity

Inflammation is an immunological defense mechanism caused due to mechanical injuries (Mueller *et al*., 2010). The quest for naturally occurring anti-inflammatory agents with less toxic effects is currently a worldwide race. Pharmacological treatment for employing chronic inflammatory diseases is usually associated with severe side effects (Mckellar *et al*., 2007). Medicinal plants being an excellent source for non-toxic anti-inflammatory agents, the microbial candidates are now investing out numerous natural phlogistic agents. Lipoxygenases (LOXs) catalyze the biosynthesis of leukotrienes, initiators of inflammation. LOXs are sensitive to antioxidants because antioxidants inhibit lipid hydroperoxide formation, which acts as a substrate necessary for the catalytic cycle of LOX. Thus antioxidants can limit the availability of this substrate, thereby inhibiting LOXs (Rackova *et al*., 2007).

Carrageenan-induced acute inflammation in animals is one of the most suitable test procedures to screen anti-inflammatory agents. The cellular and molecular responses of carrageenan-induced inflammation are well marked. It has been stated that various mediators are released by carrageenan in the paw and are believed to show a biphasic reaction. (Williams and Morley, 1973; White, 1999). This accompanies leukocyte migration and then prostaglandin in the later phases (Castro *et al*., 1998). The inflammatory agent carrageenan determined the acute anti-inflammatory activity of

Propylene glycol extract of sample NMC15 and NMT07 in mice. When compared with the control, extract-treated animals showed a significant reduction in carrageenan-induced paw edema. NMC15 extract at 25 and 50 mg/kg doses exhibited activity with 32.71 and 34.57 % inhibition at the 3rd hour, respectively. NMT07 also effectively reduced acute inflammation induced by carrageenan with percentage inhibition of 17.75 and 37 % for 25 and 50 mg/kg b. wt., respectively. Administration of standard anti-inflammatory agent diclofenac at the 3rd hour also reduced carrageenan-induced inflammation with 45.79% inhibition.

Formalin-induced paw edema is considered one of the most suitable test procedures to screen chronic anti-inflammatory agents as it closely resembles human arthritis (Greenwald, 1991). Moreover, a nociceptive biphasic effect with an early neurogenic component followed by a later tissue-mediated response was detected on inducing formalin in mice (Tominaga *et al*., 1998). The samples NMC15 and NMT07 propylene glycol extracts were effective in reducing chronic inflammation induced by formalin. In the case of NMC15 treated animals, percentage inhibition for 25 and 50 mg/kg b. wt was 20.23 and 32.14 % and NMT07 extract administration also reduced formalin-induced inflammation with percentage inhibitions of 26.19 and 40.47 % for 25 and 50 mg/kg b.wt, respectively on the 7th day. Diclofenac-treated animals at 10 mg/kg b. wt showed 58.33% inhibition on the 7th day.

The study's finding suggests that extracts of *Bacillus vietnamensis* SMC and *Brevundimonas vesicularis* JAP showed noticeable anti-inflammatory activity by lowering the paw edema volume in mice. Treatment with a high dosage (50 mg/kg b.wt.) of bacterial extracts attenuated carrageenan and formalin-induced paw edema in mice. The mechanism behind the anti-inflammatory activity conducted by bacterial

extract could be explained by the efficient antioxidant activity of the extracts. Results indicate that these bacterial extracts are helpful in the treatment of inflammation.

5.9.3 Antitumor activity

Chemotherapy is still the conventional treatment method for the most dreadful cancer disease (Felisa *et al*., 2015). However, this treatment is cytotoxic to normal cells, affecting tumor development and worsens patients' recovery (Ravin *et al*., 2017). Hence, there is a demand to develop cheaper, safer natural products to treat cancer to challenge the dreadful human disease. Bacterial pigments seem to have massive potential as a source of anticancer compounds and deserve a comprehensive investigation.

Although many studies reported the screening of bacterial metabolites for anticancer properties, no works of literature were available on the screening of *Bacillus vietnamensis* metabolites for anticancer properties. This prompted an attempt to study the anticancer properties of *Bacillus vietnamensis* metabolites. The findings of the present investigation demonstrate the significant antitumor activity of propylene glycol extract of *Bacillus vietnamensis* SMC against both ascites and solid tumors.

In the ascites tumor model, the standard reference drug cyclophosphamide at a dose of 15 mg/kg. body wt., given orally for ten consecutive days, was toxic and lethal to animals. The lethality may be due to the combined effect of cyclophosphamide and cancer cell lines in the animal. Hence, a lower dose of cyclophosphamide (10 mg/kg body wt.) was selected, which was significantly effective in inhibiting ascites tumors.

The sample NMT07 could prevent 78.97% solid tumor growth at 50 mg/kg body weight when administered 24 hours after tumor implantation. The tumor volume was 0.878 ±0.456 cm^3 in propylene glycol extract of the treated group (50 mg/kg b.wt) at the end of the 5^{th} week was significantly lower ($P<0.01$) than the control group. Furthermore, the lower dose of extract treated groups (25 mg/kg b.wt) also significantly reduced tumor volume (2.95 ±0.410 cm^3) at the end of the 5^{th} week. Thus, the 50 mg/kg sample extract inhibited the tumor proliferation as effectively as the standard reference drug (10 mg/kg) cyclophosphamide. Finally towards the end of the study, tumor-bearing mice were sacrificed using a CO_2 chamber.

The hematological parameters such as hemoglobin content, RBC, WBC, neutrophils, lymphocytes, and platelets of all surviving animals were studied. The hemoglobin content for normal control, standard, and higher dose are 14.45 ±0.21, 13.35 ±0.21, and 13.05 ±0.21 g/dL, respectively found to be similar in comparison with tumor control (10.70 ±0.42 g/dL) and lower dose (11.30 ±0.42 g/dL). Likewise, RBC and WBC for normal control, standard, a lower dose and higher dose have no differences in addition to tumor control. Thus, the study indicates that standard and higher dose drugs have less or no difference statistically than normal control. However, tumor control and lower dose drug groups showed statistically significant differences ($P < 0.001$) with the normal control.

Histopathology analysis of tumor bearing control mice shows many necrotic cells and more infiltration, thus leading to the deterioration of muscle fibers. Also, the lower dose drug tumor tissues show angiogenesis due to inflammation, reflected in dispersed necrotic cells. But the standard group and higher dose drug of sample NMT07 confer less necrotic cells and infiltration in muscle fibers.