Objective 1: Isolation of Endophytic bacteria from different tissues of some medicinal plants like *Glycosmis pentaphylla*, *Hygrophila auriculata*, *Phlogacanthus thyrsiformis* etc.

3.1.1 Collection and Authetication of the sample plant

Plant samples were collected from different locations like *G. pentaphylla* (6.444751°N, 90.287123°E, Altitude: 54.8m), *H. auriculata* (26.473437°N, 90.29795°E), *P. thyrsiformis* (26.469239°N, 90.296309°E, Altitude: 27m) of Kokrajhar district, Assam. Identification of Plants were done at the Bodoland University Botanical Herbarium, Department of Botany, Bodoland University and voucher specimen of three plants were also deposited for the accession number (Accession no. of *G. pentaphylla* is BUBH-0000143, *H. auriculata* is BUBH0000865 and for *P. thyrsiformis* is BUBH0000874) (Plate 1).

3.1.2 Culture media preparation

Nutrient agar (NA) and Luria Bertani (LB) Agar, Nutrient broth (NB) and Luria Bertani (LB) (procured from Himedia) media preparation was done by following Standard protocol of Himedia.

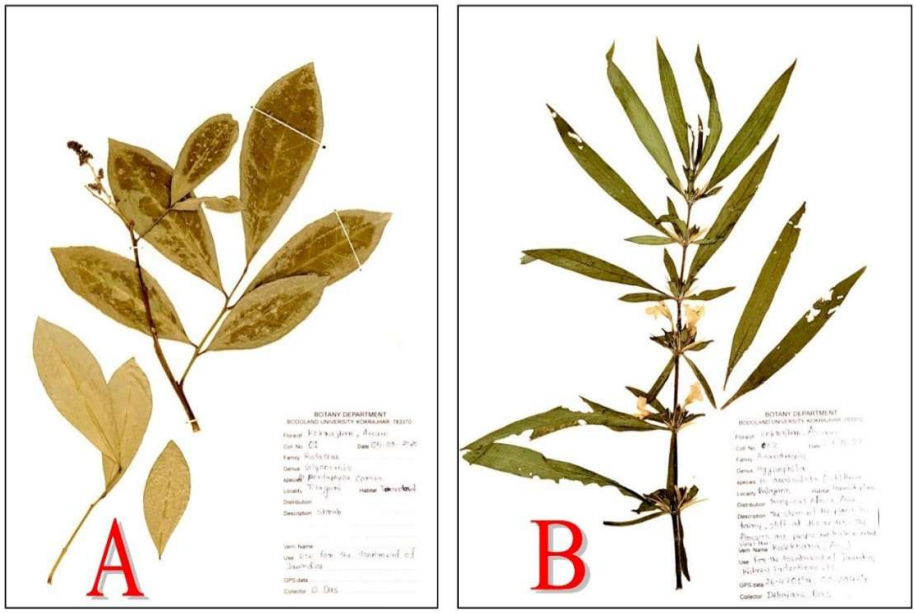
3.1.3 Isolation of Endophytic bacteria

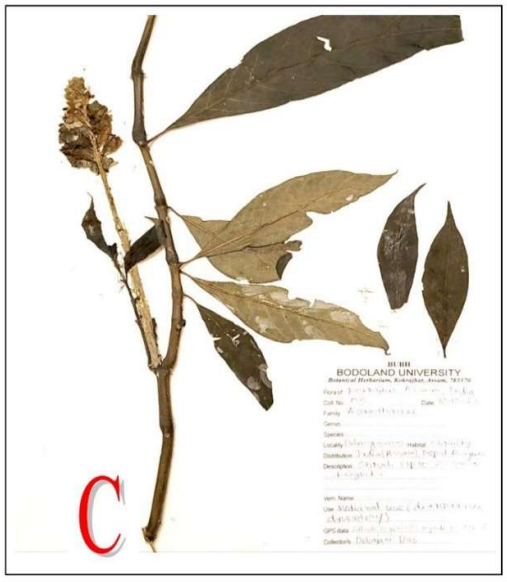
Healthy, visibly disease-free leaves, stems and roots (Fig 5) were collected from three medicinal plants. Surface sterilisation of samples was done by following the method of (Duhan et al., 2020) with some modifications. After collection, samples were wash with mild detergent and running tap water to remove soil or dust attached to it.

Fig 5: Healthy tissues of *G. pentaphylla*, (A) Leaves; (B) Stems (C) Roots

PLATE: 1

Photograph of submitted herbarium sheets of sampled medicinal plants : (A) *G. pentaphylla* (BUBH0000143), (B) *H. auriculata* (BUBH0000865), (C) *P. thyrsiformis* (BUBH0000874)





Isolation from the tissues of *G. pentaphylla*: Leaf, stem and root samples were then taken to laminar flow and dip in 70% ethanol for 3 min and then treated 2% sodium hypochlorite 7-10 mins for leaves, for stems 10-12 mins and for roots 12-14 mins. After that samples were washed 3 to 5 times in sterilized distilled water. In order to prevent the growth of endophytic fungi, samples were then dipped in 10% sodium bicarbonate for ten mins and then samples were dried using sterilised Whatman filter paper and cut into small pieces using sterilised blade and placed in NA and LB agar containing plate. Small pieces of the samples were also transferred to NB and LB broth. All the plates and test tubes containing samples were incubated at 30℃ for 72 to 96 hours for the maximum recovery of endophytic bacteria.

Isolation from *H. auriculata*: Tissue samples were then taken to laminar flow and dip in 70% ethanol for 1 minute and then leaves samples treated 0.9% sodium hypochlorite 8-10 mins, stem samples were treated with 1% sodium hypochlorite for 12-14 mins, root samples were treated with 2% sodium hypochlorite for 10 to 12 mins. After that samples were washed 3 to 5 times in sterilised distilled water. In order to prevent the growth of endophytic fungi, samples were then submerged in 10% sodium bicarbonate for ten minutes and then samples were dried using sterilised Whatman filter paper and cut into small pieces using sterilised blade and placed in NA and LB agar containing plate. Small pieces of the samples were also transferred to NB and LB broth. All the plates and test tubes containing samples were incubated at 30℃ for 72 to 96 hours for the maximum recovery of endophytic bacteria.

Isolation from *P. thyrsiformis*: Tissue samples were taken to laminar flow after washing with mild detergent and running tab water and dip in 70% ethanol for 1 min and then treated 1% sodium hypochlorite 7-10 mins for leaves, 1.5% sodium hypochlorite for 10-12 mins for stems and 2% sodium hypochlorite for 10-12 mins for roots. For root samples high concentration of sodium hypochlorite was used as it was quite hard to sterilised the root samples. After that samples were washed 3 to 5 times in sterilised distilled water. Again, in order to prevent the growth of endophytic fungi, samples were then then treated with 10% sodium bicarbonate for ten minutes and then samples were dried using sterilized Whatman filter paper and cut into small pieces using sterilised blade and placed in NA and LB agar containing plate. Small pieces of the samples were also transferred to NB

and LB broth. All the plates and test tubes containing samples were incubated at 30℃ for 72 to 96 hours for the maximum recovery of endophytic bacteria.

3.1.4 Sterility test: A sterility check was conducted on each sample in order to evaluate the efficacy of the surface sterilisation method. Sterility test done by inoculating last sample washed water in both NA and LB agar media as well as sterilized tissue without cut was also inoculated to NB and LB broth and incubated at 30℃ for 10 days. No microbial growth was observed which means success of sterilisation process.

3.1.5 Sub-culture and Pure-culture

Bacterial growth was observed from leaves, stems and roots of all the three plant samples. Once bacterial growth was observed in the leaves, stems and root samples, sub culture was done for multiple times until pure culture was obtained. After obtaining pure culture all of the isolates were then kept in storage at 4°C for performing other experiments. During sub-culturing, it was observed that LB agar showed better results than NA media. For the rest of the experiment, LB agar media was used in place of NA media.

Objective 2: Morphological and Biochemical characterization of the isolates:

3.2.1 Morphological characterisation

After incubation for 48 to 96 hours of inoculation, the following characteristics of the bacterial isolate colonies were assessed: colour, shape, appearance etc.

3.2.2 Gram stain

Gram stain of the isolates were done by following standard protocol of Himedia (for each isolate one slide was used): 1. On a dry, clear glass slide, smear of the isolate was prepared, 2. The slides were then fixed by gentle heat, 3. Then, the slides were treated with Gram's Crystal Violet for 1 minute, 4. After that the slides were washed with tap water, 5. The smear was again treated with Gram's Iodine for 1 minute, 6. After that Gram's Decolourizer was used for 30 seconds to decolorized the smear, 7. Immediately the smears were washed with tap water, 8. Then the slides were counterstain for 20

seconds with 0.5% w/v Safranin and then washed with tap water, 9. After air drying, the slides were examined using an oil immersion objective.

3.2.3 Scanning Electron Microscopy (SEM) analysis

The characteristics of the endophytic isolates were further ascertained through characterisation using scanning electron microscopy (SEM). Methods outlined by Mahlangu & Tai, 2022 were used to prepare the cultures, with minor modifications. The isolates were cultured for 24 hours at 30℃ on LB agar medium. Over the cover slip, a bacterial smear was made and fixed for 24 hours with 2.5% glutaraldehyde and the smears were kept at 4℃. Following a 24-hour period, the cover slips containing the bacterial smears were washed with sterile distilled water and then dehydrated for 10 minutes each using ethanol at concentrations of 30%, 50%, 70%, 90%, 95%, and 100%. Coverslips were then crushed, adhered to aluminium stubs using hexamethyldisilazane (HMDS), and then placed on stubs that had been coated with carbon glue (Mahlangu & Tai, 2022) Bacterial samples were then coated with gold, and the shape of the bacterial cells was examined with a Scanning Electron Microscope (Zeiss Evo-10), and then the images were taken.

3.2.4 Biochemical characterisation

For the biochemical characterisation, catalase, oxidase, indole, citrate, methyl red, voges proskauer test were performed.

Catalase test was performed adding a drop of 3% H_2O_2 a dry and clear glass slide and mix with bacterial colony. Formation of bubbles was considered as positive result (Shah et al., 2022). The *Staphylococcus aureus* was used as a positive control.

Endophytic bacterial isolates were inoculated onto Simmons citrate agar slants and then incubated for 24 hours at 30°C for the citrate test. The change in the media's colour from green to blue indicates positive result (Salo & Novero, 2020). *Bacillus subtilis* was used as positive control.

Indole test was done by following the method of MacWilliams, 2009 with minor modifications. The endophytic bacterial isolates were inoculated in a medium containing peptone (10g), sodium chloride (5g) and distilled water 1L. Then, the inoculants were

incubated at 30℃ for 2 to 3 days. After the incubation, 5 drops Kovacs reagent (procured from Himedia) was added to each tube. Formation of pink/red color on the top of the medium indicates positive result whereas yellow color indicates negative result. *E. coli* was used as a positive control.

For oxidase test, oxidase discs were procured (Himedia) and tests were performed by inoculating the smear of the bacterial culture onto the surface of the oxidase disc using inoculation loop. If the disc turns deep blue or purple, indicating the presence of cytochrome c oxidase enzyme. And if no color change or a delayed faint color change occurs after 30 seconds indicates negative result. *P. aeruginosa* used as positive control.

For, methyl red (MR) test, organisms were inoculated in MR-VP broth containing glucose and incubated for 48 hours at 30℃. After incubation, methyl red indicator was added. A red color indicates a positive result whereas a yellow or orange color represents a negative result. For voges-proskauer (VP) test, organisms are inoculated in the same MR-VP broth and incubated for 48 hours. After incubation, α-naphthol (VP reagent A) and potassium hydroxide (VP reagent B) were added. The tube was shaken to ensure oxygenation, which facilitates the reaction. A red or pink color after 10–20 minutes indicates a positive result, whereas no color change indicates a negative result (Barry & Feeney, 1967).

3.2.5 Antibiotic sensitivity test

Using a sterile cotton swab, all 24-hour old endophytic bacterial isolates growing LB broth were spreaded on Muller Hinton agar (MHA) plates. Then the standard antibiotic disc (Himedia): ampicillin (10 µg/disc), gentamicin (10 µg/disc), penicillin (10 µg/disc), ciprofloxacin (5 µg/disc), cefotaxime (5 µg/disc) were place on MHA agar plate and incubate for 24 hours at 30℃. The inhibition zone was measured following incubation to ascertain the pattern of antibiotic susceptibility. The organisms were classified as sensitive, intermediate, and resistant in accordance with the DIFCO Manual 10th edition (1984) based on the zone of inhibition's diameter, measured to the closest millimetre (mm) (Sharma & Mallubhotla, 2022).

Objective 3: Molecular identification of the isolates

3.3.1 DNA extraction from endophytic bacteria

Genomic DNA of endophytic bacteria was extracted using CTAB method of (Wilson, 2001) with some modifications.

- **Prepare all reagents:**

1. CTAB Buffer: 2% CTAB, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA, 1.4 M NaCl
2. Proteinase K: Prepare at 10 mg/mL in sterile water.
3. Chloroform: Isoamyl Alcohol: Mix in a 24:1 ratio.
4. Isopropanol: For DNA precipitation.

- **Steps of Genomic DNA extraction:**

1. Bacterial colony was inoculated into 1.5 mL of sterile LB broth and incubate at 30°C with shaking for 16–18 hours to obtain an overnight culture.
2. Centrifuge 1 mL of the culture at 10,000 × g for 5 minutes to pellet cells.
3. Discard the supernatant and resuspend the cell pellet in 500 µL of CTAB buffer.
4. Add 10 µL of Proteinase K solution, mix gently, and incubate at 65°C for 30 minutes.
5. Add an equal volume (500 µL) of chloroform:isoamyl alcohol (24:1).
6. Mix thoroughly by inversion for 5 minutes.
7. Centrifuge at 10,000 × g for 10 minutes. Carefully transfer the aqueous (upper) phase to a new tube.
8. Add 0.6 volumes of chilled isopropanol to the aqueous phase.
9. Mix gently and incubate at -20°C for 30 minutes to precipitate DNA.
10. Centrifuge at 10,000 × g for 10 minutes. Discard the supernatant and wash the DNA pellet with 70% ethanol.
11. Air-dry the DNA pellet for 1 to 2 hours.
12. Resuspend the DNA in 50 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

3.3.2 Gel electrophoresis: Genomic DNA was checked in 0.8% agarose gel.

3.3.3 Quantification: The Qubit 4 Fluorometer (Invitrogen) was used to quantify DNA in accordance with the manufacturer's instructions.

3.3.4 Polymerase Chain Reaction: 16S rDNA gene amplification was done using universal primer:

(i) 27F (AGAGTTTGATCCTGGCTCAG),
(ii) 1492R (CGGTTACCTTGTTACGACTT)

Reaction mixture contain final volume 25µl containing: 12.5 µl of 2X concentration of PCR master mix (Genei), Forward primer (10 pmol), Reverse primer (10pmol), template 90 ng concentration and rest of the volume was made up by adding nuclease free water.

PCR reaction was carried out in 2720 Thermal Cycler of applied biosystem by Thermo Fisher Scientific and below the table (Table 3) showing conditions which used during amplification.

Table 3: Conditions for PCR amplification of 16S rDNA gene

Steps	Temperature	Time	Cycle
Initial denaturation	94 ℃	4 minutes	1 cycle
Denaturation	94 ℃	1 minute	35 cycle
Annealing	55 ℃	1 minute	
Extension	72 ℃	2 minutes	
Final extension	72 ℃	10 minutes	1 cycle

The amplicons were checked at 1.5% agarose gel electrophoresis and the concentration was determined using a Qubit 4 Fluorometer (Invitrogen) following the successful amplification of the PCR result.

3.3.5 Sequencing

Using 27F and 1492R primers, the 16S rDNA gene was successfully amplified. The amplicons were then purified using Gel Extraction Kit (Genetix Brand). The forward and reverse DNA sequencing processes for the PCR amplicon were performed using the BDT v3.1 Cycle sequencing kit on an ABI 3730xl Genetic Analyser.

3.3.6 Phylogenetic analysis and identification of the isolates

Using aligner software and forward and reverse sequence data, consensus sequences of the 16S rDNA gene were produced. For 16S rDNA gene sequences, the NCBI GenBank database was used to perform the Basic Local Alignment Search Tool (BLAST). The first ten sequences were selected on the basis of the maximum identity score and aligned using Clustal W, a multiple alignment software application (Duhan et al., 2020). Based on the Kimura 2 parameter model, a maximum likelihood phylogenetic tree was produced using MEGA version 11. 1000 bootstrapping repetitions were used to determine the reliability of the branching and clustering patterns (Beiranvand et al., 2017). To obtain their accession numbers, the sequences of all the bacterial isolates were submitted to GenBank.

Objective 4: To study a number of plant growth promotion activities of the isolated endophytic bacteria

3.4.1 Phosphate solubilisation

Phosphate solubilisation test was done by following the method of Shah et al., 2022 with minor modifications. Isolates were inoculated on Pikovskaya's agar medium containing yeast extract: 0.5g; Dextrose: 10g; Calcium phosphate: 5g; Ammonium sulphate: 0.5g; Potassium chloride: 0.2g; Magnesium sulphate: 1g; Manganese sulphate: 0.0001; Ferrous sulphate: 0.0001; Agar: 15g; Distilled water: 1L. After inoculation plates incubated for 5 to 7 days at 28 °C to conduct the phosphate solubilization test. The formation of clear zone around the colonies indicated as positive result for phosphate solubilization. The zone formation can start after 24 hours of incubation. The measurements of clearing zone were done after 7 days of incubation. Phosphate

solubilization index (SI) was also calculated by using the formula, SI = (colony diameter + halo zone diameter) / colony diameter.

3.4.2 Ammonia production

Ammonia production ability of the isolated endophytic bacteria performed by following the method of (Alkahtani et al., 2020) with some minor modifications. The stored bacterial cultures were sub cultured on LB agar medium, and after 24 hours of incubation, isolates were inoculated in peptone water, which contains 10 g peptone, 5 g NaCl, and 1L distilled water. Then the isolates were incubated at 30℃ for 5 days. As a control, peptone water without isolates was also kept for incubation. After 5 days of incubation 1 ml of Nessler's reagent was added to all tubes containing bacterial isolates and also added to control tubes. If the colour of the media containing isolates changes to faint to deep yellow indicates positive results for ammonia production test. Whereas faint yellow indicates positive results and deep yellow indicates highly positive.

3.4.3 IAA production

The IAA production test was done following the method of (Singh et al., 2022) with minor modifications. The isolated endophytic bacteria were sub cultured in the LB agar medium, and then the 24-hour-old culture was inoculated in LB broth supplemented with 400 µg/ml of L-tryptophane and incubated at 25 ± 2 °C for 72 h. After completing the incubation period, the isolates were centrifuged at 3000 rpm for 30 minutes. Then, 2 ml of supernatant was mixed with 2 drops of orthophosphoric acid and 4 ml of the Salkowski reagent, which contains 50 ml, 35% of perchloric acid, and 1 ml of a 0.5 M FeCl3 solution. The appearance of a pink colour signifies the production of IAA (Singh et al., 2022). The optical density was measured at 530 nm using spectrophotometer. The amount of IAA produced was calculated using a standard curve for authentic IAA (Himedia) (Alkahtani et al., 2020).

3.4.4 Salt tolerance ability

Salt tolerance ability of isolated endophytic bacteria was performed by following the method of (Sharma & Mallubhotla, 2022). For the test, different concentrations (0-10%) of NaCl were added in LB agar medium, and then 24-hour-old endophytic bacterial

strains were inoculated in those plates and incubated at 30 °C for 5 to 7 days. After 24 hours, observations were taken as good growth, moderate growth, low growth, or no growth.

Objective 5: To screen the ability of isolated endophytic bacteria to produce extracellular/hydrolytic enzymes: Hydrolytic enzymes production ability of endophytic bacteria was performed by following the method of (Dogan & Taskin, 2021) with minor modifications.

3.**5.1 Amylase activity:**

The isolated endophytic bacteria were inoculated on LB agar supplemented with 1% (w/v) starch to evaluate their amylase activity. After inoculation, the plates were inoculated at 25℃ for 48 hours. To perform the test, the plates containing isolates were treated with iodine solution after 48 hours of incubation. The unstained zone around the colonies means positive for amylase activity.

3.5.2 Lipase activity:

To perform the lipase activity test of the isolated endophytic bacteria, isolates were inoculated in a culture medium containing: Luria Bertani broth (8 g), CaCl2 H2O (0.1 g), agar (15 g), pH 6.0, and 20 ml Tween 20. Total volume was 1L, and Tween 20 was mixed after sterilisation of the media. After inoculation, the culture plates were incubated at 25 °C for 72 hours, and on the final day, the plates were kept at 4 °C for 30 minutes. Lipase-positive isolates were determined by examining the opaque zone surrounding the colonies.

3.5.3 Cellulase activity:

For the cellulase activity test, isolates were inoculated in the culture medium containing NaNO3 (1 g), K2HPO4 (1 g), KCl (1 g), MgSO4 (0.5 g), yeast extract (0.5 g), glucose (1 g), carboxymethylcellulose (CMC) (5 g), and agar (15 g) in 1L distilled water. For six to eight days, the plates were incubated at 25 °C. Petri dishes containing isolates were treated with 0.2% (w/v) Congo Red solution at the end of the incubation period and were left at room temperature for 20 minutes. The plates were then rinsed Petri dishes with a 5 M NaCl solution and incubated at normal temperature for an additional half hour

to get rid of extra colour. Cellulase-positive colonies were identified by the presence of a light-yellow zone surrounding the colony on a red background.

3.5.4 Protease activity:

For the protease activity test, media preparation was done by mixing milk powder of 1g per 100ml of distilled water and sterilised at 110℃ for 5 minutes and cooled down to 45℃. And then mixed with already sterilised nutrient agar medium under sterilised laminar air flow. Protease activity of isolated endophytic bacteria was analysed by inoculating the isolates on LB Agar containing 1% (g/l) skimmed milk powder. Then incubated for 3 to 4 days at 25 ℃. Formation of clearing zone around the colonies means positive for protease activity test.

3.5.5 Pectinase activity:

For pectinase activity test, a culture medium was prepared which contains yeast extract (2 g), ammonium sulfate (2 g), Na2HPO4 (6 g), KH2PO4 (3 g), pectin (5 g), and agar (15 g) and distilled water 1L. The isolated endophytic bacteria were then added to the medium and incubated for three days at 25°C. Following incubation, the Petri dishes were allowed to stand at room temperature for ten minutes, following the addition of 1% (w/v) cetyltrimethylammonium bromide (CTAB) solution. An activity of pectinase was demonstrated by the production of clear area surrounding the colony.

3.5.6 Xylanase activity:

For xylanase activity test, isolates were inoculated on the medium containing NaNO3 (1 g), K2HPO4 (1 g), KCl (1 g), MgSO4 (0.5 g), yeast extract (0.5 g), glucose (1 g), agar (15 g), and xylan (5 g) and distilled water (1L). The plates were then incubated at 25°C for three to four days. Following the incubation period, the Petri dish was stained with 0.1% (w/v) Congo Red solution for 20 minutes. To remove the excess dye, a 5 M NaCl solution was added to the Petri dishes, which were then incubated at room temperature for half an hour. Xylanase activity was demonstrated by formation of a zone of light colour on a red background.

Objective 6: To evaluate anti-microbial activities of isolated endophytic bacteria

3.6.1 Endophytic bacterial extract preparation

The 16 isolated bacterial endophytes were inoculated in 100 mL of LB broth in 16 different conical flasks and incubated for 15 days at 30℃ at 120 rpm. After the incubation period the broth cultures were centrifuged at 10,000 rpm for 15 minutes. Then the supernatant was mixed with methanol (methanol: supernatant – 2:1) and incubate for 24 hours at normal temperature for extraction. Further, it was concentrated by freeze drying using lyophilizer. The concentrated extract was dissolved in sterilised distilled water in 400 mg/ml concentration. Afterwards, an antibacterial activity test was conducted using this dissolved extract (War Nongkhlaw & Joshi, 2017).

3.6.2 Anti-microbial activity test

Anti-microbial activity test was done against 6 pathogenic bacteria namely, *Staphylococcus aureus* (MTCC 737), *Escherichia coli* (MTCC 443), *Bacillus subtilis* (MTCC 441), *Enterobacter aerogenes* (MTCC 2822), *Pseudomonas aeruginosa* (MTCC 1688) and *Klebsiella pneumonia* (MTCC 109). The test was performed by disc diffusion method: 100 µl of the 24 hours old pathogenic bacteria were spread on the MHA media plate using sterilised glass spreader and 6mm sized sterilised filter paper disc were placed on it and endophytic bacterial extract of 50 µl of 400 mg/ml extract concentration pipette over it. After that the plates were incubated at 30℃ for 24 hours. The zone of inhibition was then measured for isolates that demonstrated efficacy against harmful microorganisms. Different standard antibiotics were used as a positive control, ampicillin (AMP 10) was used for *S. aureus*, *E. coli*, *B. subtilis*, and *P. aeruginosa*; gentamicin (GEN 10) was used for *K. pneumonia*; ciprofloxacin (CIP 5) was used for *E. aerogenes*.

3.6.3 Data analysis

Every experiment was conducted in triplicate. SPSS software was used for the statistical analysis. Additionally, MEGA 11, BLAST, BioEdit 7.2and Clustal W were used for sequence analysis.