CHAPTER 4

MATERIALS & METHODS

4 Material and methods

4.1 Collection and identification of plant material

The plant material was collected from the Panbari area of district Kamrup (26°11′41″N; 91°59′05″E), Assam, India. The plant material was identified as *N. herpeticum* by Dr. Devanjal Bora, Scientist-2 and Research Officer (Botany), North-East Herbarium of Ayurveda Research (NEHAR), Central Ayurveda Research Institute (CARI), Guwahati, Assam, India (Accession no. DB-21-7612) and deposited in the herbarium of the institute for future reference. The plant name has been verified with http://www.mpns.kew.org/ and http://www.worldfloraonline.org/ as accessed on November 15, 2021. Further, the plant sample was subjected to DNA barcoding analysis.

4.2 DNA Barcoding

4.2.1 Extraction of DNA

A leaf sample of the medicinal plant was used for the extraction of DNA as per the manufacturer's instructions outlined in the NucleoSpin®Plant II Kit (Macherey-Nagel). In a microcentrifuge tube containing 100 mg of a fresh plant sample, 400 µl of buffer PL1 was added and vortexed for one minute. It was then inverted, and 10 µl of RNase A solution was added to it. After 10 minutes of incubation at 65°C, the homogenate was transferred to a Nucleospin filter and centrifuged at 11000xg for 2 minutes. 450 µl of PC buffer was added to the collected flow through liquid, which was then thoroughly mixed. The solution was transferred to a Nucleospin Plant II column and centrifuged for one minute. The flow-through liquid was then discarded. 400 µl of buffer PW1 was added to the column, which was then centrifuged at 11000xg for one minute, and flow-through liquid was discarded once more. An additional 700 µl of PW2 was added, the mixture was centrifuged at 11000xg, and the flow-through liquid was discarded. Finally, 200 µl of PW2 was added, and the silica membrane was

centrifuged at 11000xg for 2 minutes to dry it. The column was then transferred to a new 1.7 ml tube, 50 µl of buffer PE was added, and it was incubated for 5 minutes at 65°C. The DNA was then eluted by centrifuging the column at 11000xg for one minute. The extracted DNA was kept at 4°C.

Using agarose gel electrophoresis, the purity of the isolated DNA was examined. 5 µl of DNA was mixed with 1 µl of 6x gel loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH 8.0). The samples were deposited onto a 0.8% agarose gel prepared in 0.5x Tris-Borate-EDTA (TBE) buffer with 0.5 g/ml ethidium bromide. At 75V, electrophoresis was conducted with 0.5x TBE as the electrophoresis buffer until the front of the bromophenol dye migrated to the bottom of the gel. The gels were viewed in a UV transilluminator (GeNei) and the image was obtained using the Gel documentation system (Bio-Rad) under UV light.

4.2.2 PCR Amplification

Chloroplast gene rbcL was the marker of choice, as in 2009, the Consortium for the Barcode of Life (CBOL) Plant Working Group had proposed it as the core barcodes of plant species. Easy amplification, unanimity, and comparability are the characteristics supporting its proposal as a barcode fragment [52].

About 25 µl of reaction mixture included 12.5 µl of 2x Phire master mix, 0.4 µl of forward primer, 0.4 µl of reverse primer, 3.0 µl of DNA, and 8.7 µl of sterilised water. Thermocycling was carried out in PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) under the following conditions: (i) initialization: heating at 98°C for 30 seconds, (ii) denaturation and annealing: succeeded by 40 cycles of heating at 98°C for 5 seconds, 58°C for 10 seconds, and 72°C for 15 seconds, and (iii) extension: exposure to 72°C for 60 seconds. After PCR amplification, the samples were stored at 4°C for subsequent use.

On 1.2% agarose gels prepared in 0.5x TBE buffer containing 0.5 g/ml ethidium bromide, the PCR products were analysed. 1 µl of 6x loading dye was combined with 4 µl of PCR products, and electrophoresis was performed at 75V power supply with 0.5x TBE as the electrophoresis buffer for approximately 1-2 hours, until the bromophenol blue front had migrated almost to the bottom of the gel. 2-log DNA ladder (NEB) served as the molecular standard. The gels were viewed in a UV transilluminator (GeNei) and the image was obtained using Gel documentation system (Bio-Rad) under UV light.

The PCR product (5 µl) was combined with 0.5 µl of ExoSAP-IT (GE Healthcare) and incubated at 37°C for 15 minutes, followed by 5 minutes of enzyme inactivation at 85°C. ExoSAP-IT contains two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer for the removal of unneeded primers and dNTPs from a PCR product mixture without interfering with subsequent applications.

4.2.3 DNA Sequencing and analysis

Following the manufacturer's instructions, the sequencing reaction was performed in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems, USA). The sequencing PCR mixture contained sequencing buffer (1.9 µl), forward primer (0.3 µl), reverse primer (0.3 µl), sequencing mixture (0.2 µl), ExoSAP-IT treated PCR product (1 µl), and sterile water (6.6 µl). The sequencing PCR amplification was performed under the following conditions: initial heating at 96°C for 2 minutes, followed by 30 cycles of heating at 96°C for 30 minutes.

The integrity of the sequence was evaluated using Sequence Scanner Software v1 (Applied Biosystems). Using Geneious Pro v5.1, sequence alignment and necessary modification of the obtained sequences were performed (**Fig. 4**) [53].

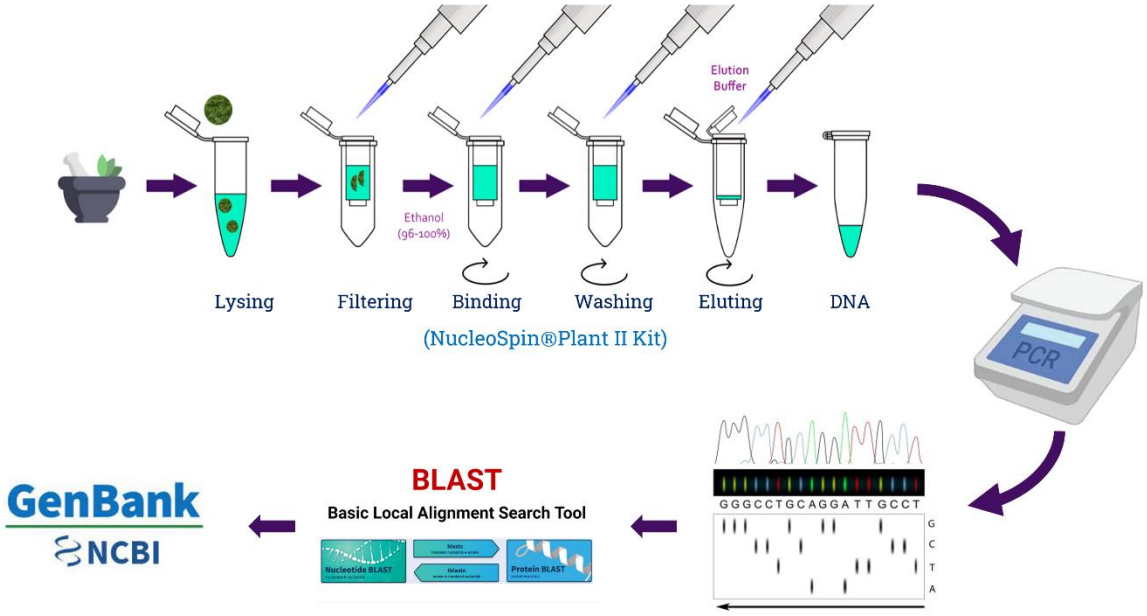

Fig. 4 A schematic representation of the DNA barcoding protocol

4.3 Quality control assessment

4.3.1 Morphological and microscopical study

The microscopic and macroscopic characteristics of the authenticated plant material and crude extract were examined, as mentioned in the literature. Sensory evaluation was done to study the morphological features like shape, size, colour, surface characteristics, texture, fracture characteristics, and appearance of the cut surface. Plant parts were sectioned and prepared for histological examination as per standard methods [54].

4.3.2 Physicochemical analyses

As per the WHO guidelines on quality control methods for medicinal plant materials, the various physicochemical constants like loss on drying, total ash, acid insoluble ash, water soluble ash, alcohol soluble extractive, water soluble extractive, foaming index, haemolytic index, and volatile oil content of the air-dried plant material were determined. The readings were taken in triplicate, and the results were expressed in terms of mean ± S.D. [54].

4.3.3 Loss on drying

The gravimetric determination for loss on drying determines both water and volatile matter. The air-dried material (5 g) was precisely weighed and put in a previously dried and tared flat weighing bottle. The sample was then dried in an oven at 100-105°C until the difference between two consecutive weights did not exceed 5 mg. The loss of weight in mg per g of air-dried material was calculated [54].

4.3.4 Ash value

The ground, air-dried material (4 gm) was placed in a previously ignited and weighed silica crucible. The material was spread in a uniform layer and ignited by progressively increasing the temperature to between 500-600°C until it turned white, indicating the absence of carbon. It was then cooled and weighed in a desiccator. The total ash content was calculated in mg per gram of air-dried material [54].

4.3.4.1 Acid-insoluble ash

To the crucible containing the total ash, 25 ml of hydrochloric acid (70 g/l) was added and covered with a watch-glass. It was then boiled cautiously for 5 minutes. The watch-glass was rinsed with hot water (5 ml) and the same water was added to the crucible. The insoluble matter was collected on an ashless filter-paper and cleansed thoroughly with hot water until the filtrate was neutral. The filter paper containing the insoluble substance was transferred to the crucible, allowed to dry on a hotplate, and ignited to a constant weight. The residue was allowed to cool in a suitable desiccator for 30 minutes, then weighed without delay. The content of acid-insoluble ash was calculated in mg per g of air-dried material [54].

4.3.4.2 Water-soluble ash

To the crucible containing the total ash, water (25 ml) was added and boiled for 5 minutes. The insoluble matter was collected in a sintered-glass crucible. After washing with hot water, it was

ignited in a crucible at a temperature not exceeding 450°C for 15 minutes. The weight of the residue (mg) was subtracted from the total ash weight. The amount of water-soluble ash per gram of air-dried material was determined [54].

4.3.5 Extractive value

The extractable matter was determined using the cold maceration method. In a conical flask with a glass stopper, 4 gm of air-dried, coarsely pulverised material was placed. The plant material was macerated in 100 ml of the specified solvent (alcohol/water) for 6 hours with vigorous stirring, then allowed to stand for 18 hours. 25 ml of the filtrate was transferred to a tared, flat-bottomed dish and evaporated in a water-bath until dry. It was dried at 105°C for 6 hours, cooled in a desiccator for 30 minutes, and immediately weighed. The amount of extractable matter per gram of air-dried material was calculated in milligrams [54].

4.3.6 Foaming index

Plant material (1 gm) was reduced to a coarse powder (sieve size no. 1250), accurately weighed, and transferred to a 500 ml conical flask containing boiling water (100 ml). Boiling was carried out at a moderate rate for 30 minutes, cooled and filtered into a 100 ml volumetric flask, and then diluted to volume with water through the filter. The decoction was poured into 10 stoppered test tubes of height 16 cm and diameter 16 mm in increments of 1-10 ml, and the volume in each tube was made to 10 ml using water. The tubes were stoppered and shaken lengthwise for 15 seconds, at a rate of two shaking per second. The foam was allowed to stand for 15 minutes before measuring its height.

The results were assessed as follows-

- If the froth height in each tube was less than 1 cm, the foaming index was less than 100.

- If 1 cm of foam was measured in any tube, the volume of the decoction of plant material in this tube (a) was used to determine the index. If this was the first or second tube in a series, an intermediate dilution was prepared to acquire a more accurate result.
- If the height of the froth in each tube exceeded 1 cm, the foaming index was greater than 1000. In order to acquire a result, repeat the determination using a new series of dilutions of the decoction.

The foaming index was calculated using the following formula:

$$\frac{1000}{a}$$

where a = the volume in ml of the decoction used for preparing the dilution in the tube where foaming to a height of 1 cm is observed [54].

4.3.7 Heavy metals and aflatoxins analyses

Heavy metals like copper (Cu), argon (Ar), zinc (Zn), cadmium (Cd), mercury (Hg), and lead (Pb) were determined by atomic absorption spectroscopy (AAS). Mycotoxins like aflatoxin B1, B2, G1, and G2, and total aflatoxin content were determined by using the LC-MS-MS method [54].

4.4 Preparation of plant extract

The freshly collected aerial parts of *N. herpeticum* were shade dried under room conditions for a few weeks. It was then ground to coarse powder (2000/355) by mechanical means. The powdered plant sample (1000 gm) was then extracted using distilled water (5000 ml) as the solvent by cold maceration process for 72 hours. The extracts obtained were concentrated by removing the solvent using rotatory vacuum evaporator (42 mbar vacuum at 30º C) and subsequent lyophilization. The dried extracts (89.91 gm) were stored in desiccators until further use.

4.5 Animal selection and care

The animal studies were conducted in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and were approved vide Dean/2019/IAEC/1644; dated 17/11/2019, by the Institutional Animal Ethics Committee. Female albino Wistar rats (150±20 g) were obtained from the Central Animal House, Banaras Hindu University (BHU), Varanasi, India. The animals were housed in well-ventilated animal house under controlled environmental conditions with a 12-hour light/dark cycle in polypropylene cages, along with free access to food (standard food pellets) and water *ad libitum*. Prior to the experiments, the animals were acclimated to laboratory conditions for five days.

4.6 Acute toxicity study

Acute toxicity study was conducted as per the limit test of the Organization for Economic Cooperation and Development 420 (OECD-420) guideline. Non-pregnant and nulliparous female rats (n=10) were used for the experiment. Using computer-generated randomization tables, rats were randomly assigned to groups (i.e., test and control) in order to minimise weight differences between groups. The test group (n = 5) orally received a testing dose of 5000 mg/kg body weight, while the other group served as the control and received saline orally. Following the treatment, animals were closely monitored individually once during the initial 30 minutes, and special attention was given during the first four hours. Further, the animals were observed every 24 hours for the next 14 days. The pathological evidences were examined and LD_{50} was determined as per the OECD guideline [55].

4.7 Repeated dose 28-day oral toxicity study

The repeated dose oral toxicity study was completed in 28 days as per the OECD Test Guideline 407 [56]. Animal group allocation was done randomly using computer generated randomization tables. Each group contains five male and five female animals, which were kept separately to prevent mating. The groups were as follows: Group 1 served as control (received saline), while Groups 2-4 received oral administration of different extract doses (500, 1000, and 2000 mg/kg) for 28 days, respectively. A satellite group was kept for an additional 14 days for follow-up observation, as recommended. On the last day of the experiment, the blood from the animals was collected for biochemical analyses, while the major organs such as the lungs, liver, heart, and kidney were harvested using standard protocol for further histological examination.

4.8 Qualitative analysis using LC-MS

The crude aqueous extract of *N. herpeticum* was analysed for the identification of compounds by Quadrupole Time-of-Flight (QToF) Mass Spectrometer (Agilent Technologies, USA). Dual Agilent Jet Stream Electron Spray Ionization (Dual AJS ESI) (Model: G6550A; Agilent Technologies) probe, at both positive and negative modes, was opted for detection through injection with needle wash (injection volume: 5.0 µl). The medium isolation width of ~ 4 a.m.u. and mass range of 120-2000 m/z were selected with a sample flow rate of 100 µl/min. 100 µl/min was the sample flow rate. The flow rate and pressure limit of the binary pump (Model: G4220B; Agilent Technologies) were 0.3 ml/min and 0-1200 bar, respectively. Water and acetonitrile (95:5) (few drops of formic acid) was used as the mobile phase [57].

4.9 Network Pharmacology-based prediction

4.9.1 Compound target prediction and in-silico drug-likeness screening

QToF-MS identified compounds (21 compounds) were assessed for drug-likeness property using the free web tool SwissADME (http://www.swissadme.ch/; accessed on March 21, 2022). Compounds obeying Lipinski's rule of five and with an oral bioavailability score > 0.5 were considered for further investigation (**Fig. 5**) [58]. Restricting the search to *Homo sapiens*, we predicted the targets of bioactive compounds using SwissTargetPrediction (http://www.swisstargetprediction.ch/; accessed on March 30, 2022) and STRING (https://string-db.org/; accessed on April 15, 2022 [59, 60]. The UniProtKB database (https://www.uniprot.gov/, accessed on April 20, 2022) was referred to validate the target names [61].

4.9.2 Antibacterial potential prediction

4.9.2.1 Mining of bacterial infection associated targets

The targets relevant to bacterial infection were collated from the GeneCards (https://www.genecards.org/; accessed on April 25, 2022) and DisGeNET (https://www.disgenet.org/search; accessed on April 27, 2022) databases [62, 63]. UniProt database (https://www.uniprot.org/; accessed on May 10, 2022) was consulted for identifying the UniProt ID of disease targets and standard gene names [61].

4.9.2.2 Establishment of protein-protein interaction complex and its analysis

Acquisition of data pertaining to protein-protein interaction of all the targets was reaped from the STRING database (https://string-db.org/, accessed on May 15, 2022) [60]. The compound-target network, bacterial infection associated-target network, and compound target-infection target network were constructed based on the interaction data. Cytoscape v3.9.1 software was used to construct, analyse, and visualize the networks [64]. Evaluation of the central attributes

of the nodes in the network was based on the calculation of topological parameters like density of maximum neighbourhood component (DMNC), degree, edge percolated component (EPC), radiality, maximal clique centrality (MCC), multinetwork clustering (MNC), eccentricity, stress, bottleneck, betweenness centrality, clustering coefficient, and closeness centrality [65]. Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Enrichment analysis was done using the STRING database within the genome limiting to *H. sapiens* [66]. The gene function annotation and its role in the metabolic pathway of target-associated disease can be known through the KEGG pathway [67].

4.9.3 Anti-inflammatory potential prediction

4.9.3.1 Extraction of inflammation-associated targets

The databases GeneCards (https://www.genecards.org/) and DisGeNET (https://www.disgenet.org/search) served to compile the targets pertaining to inflammation. From the UniProt database (https://www.uniprot.org), disease targets' UniProt IDs and common gene names were taken.

4.9.3.2 Network construction and analysis

As described previously, all of the targets' protein-protein interactions were established in the STRING database (https://string-db.org/). Based on the interaction data, the compound-target network, inflammation-associated-target network, and prospective compound target-inflammation target network were built. The networks were generated, examined, and visualised using Cytoscape v3.9.1. The topological approach was used to analyse the central network. Based on the calculation of topological parameters, the central characteristics of the nodes in the network were evaluated [65]. Using ShinyGO 0.77, the KEGG Pathway Enrichment analysis was carried out.

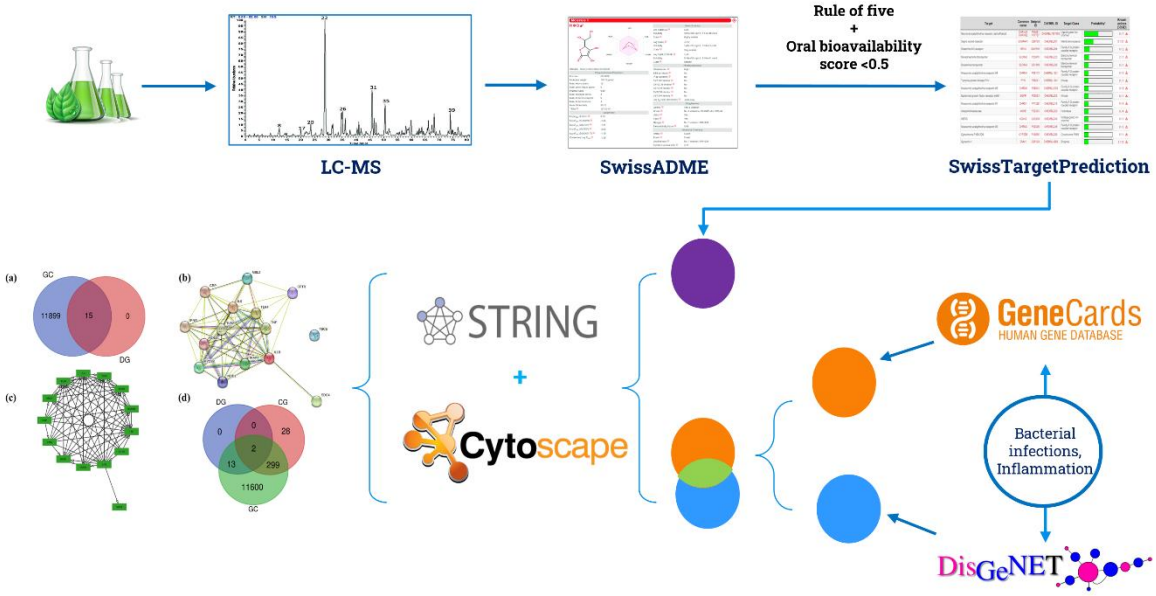

Fig. 5 A diagrammatic representation of the Network-pharmacology protocol employed in this study

4.10 In vitro antibacterial study

4.10.1 Microbial strains and culture media

Representative Gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacteria were used to evaluate the antimicrobial activity of the plant. Mueller-Hinton agar medium was used for the microbial cultures of *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 33677, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853. The standard microbial strains were obtained from HiMedia Laboratories Pvt. Ltd.

To make pure bacterial colonies, an aliquot from the standard cultures was streaked onto petriplates with selective culture medium using a sterile inoculating loop. At 37°C for 24 hours, the streaked agar plates were incubated. After transferring a pure colony from the earlier incubated plates into Mueller-Hinton broth (MHB) containing tubes, it was then incubated at 37°C for 24 hours to allow microbial growth. McFarland standards were used for comparison

of the concentration of viable broth cultures and to obtain a working culture of 10^6 CFU/ml. Repeated culturing was performed weekly throughout the period of study to obtain viable working cultures [68].

4.10.2 Sample preparation

A stock solution (100 µg/ml) of the extract was prepared in MHB and filtered using a 0.22 µm syringe-filter. A series of dilutions (100, 50, 25, 12.5, and 6.25 µg/ml) in MHB were made under sterile conditions. The antibacterial activity of these concentrations of the extract was evaluated using well diffusion and microtiter plate dilution methods.

4.10.3 Agar well diffusion method

The antibacterial activity of the crude aqueous extract of *N. herpeticum* was determined using the agar well diffusion method (**Fig. 6**). Sterile petridishes with Muller Hinton agar (MHA, 20 ml) media poured into them were let to solidify, and upon solidification, uniform spreading of microbial inoculum (100 µl) on the agar plate surface was done. Six wells were punched aseptically on the inoculated media containing agar plates, and molten agar was used to seal the bottom of the well. The serial dilutions (50 µl) of the extract and the positive control were prepared using two-fold dilutions and were added to the designated wells. The plates were further incubated for 24 hours at 37°C. Gentamicin (5 µg/ml) was used as a positive control. The experiment was conducted in duplicate [69].

4.10.4 Microtiter plate dilution method

This method was used to determine the bacteriostatic and bactericidal activity of the extract [69]. In a 96-well microplate, two-fold dilutions of the extract were added serially, followed by the addition of adjusted 100 µl bacterial cultures (1.5×10^8 CFU/ml) to each well. The plant extract was prepared with an initial stock concentration (100 µg/ml) using MHB. Except for the first, all wells were filled with MHB (50 µl). In the first set of wells, 100 µl of plant extract

was added using a multi-channelled micropipette), and serial two-fold dilutions were performed until the desired minimum concentration was achieved (**Fig. 6**). The medium (MHB) was used as a negative control, while gentamicin (0.625-10 µg/ml) was used as a positive control. The microplate was covered and incubated at 37°C for 24 hours. Turbidity was measured at 600 nm to assess the MIC. The experiment was performed in triplicate. Further, 10 µl aliquots from non-turbid wells were transferred into Eppendorf microtubes containing 2 ml of MHB. These tubes were incubated at 37°C overnight and observed for visual turbidity. The lowest concentration exhibiting no visible growth was considered the minimum bactericidal concentration (MBC).

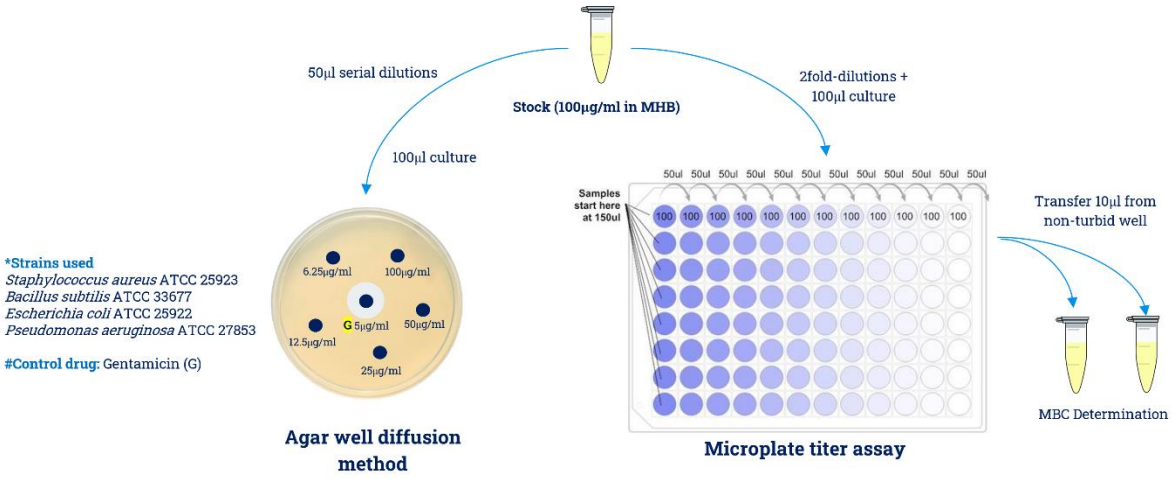

Fig. 6 A diagrammatic representation of antibacterial assays

4.11 Molecular Docking studies and MM-GBSA studies

4.11.1 Ligand selection and preparation

For molecular docking, 14 compounds with drug-likeness properties were selected. All the compounds were sketched in 2D using the ChemBio draw (Ver. 2016) software and converted into .mol format before initiating the molecular docking process. The structures were refined and converted to 3D using the LigPrep module in Schrodinger Maestro 13.3. Additionally, possible conformation and tautomeric states could be generated if any of the ligands were

generated using the Epik tool of LigPrep at the target pH (7.4). The ligands were further stabilised to their minimum energy conformation using the OPLS_2005 force field. The ligands were thus utilised for docking with selected proteins.

4.11.2 Protein selection and preparation

From the RCSB protein data bank, four bacterial targets were chosen: DNA gyrase subunit b (PDB: 1KZN), glucosamine-6-phosphate synthase (PDB: 2VF5), dihydrofolate reductase (DHFR, PDB: 2W9S), and penicillin-binding protein (PDB: 4CJN). Further, the protein preparation and refinement are done via the protein preparation workflow module of the Schrodinger software. Protein preparations include removing water molecules, adding missing amino acids and loops, adding hydrogen bonds, etc. Finally, the protein was re-optimized for stable energy conformation using force field OPLS_2005.

4.11.3 Grid generation

The grid includes a specified area where an effective ligand-protein interaction will occur. To understand the interaction pattern of selected ligands with the active domain of the protein(s), the grid generation tool of Schrodinger was employed. We utilised a co-crystallized ligand to generate the desired grid dimensions at the protein active site. All atoms possessing a distance of 5 Å from the co-crystallized ligand in the protein binding site were utilised for molecular docking.

4.11.4 Molecular docking

The prepared ligands and proteins were further subjected to molecular docking studies by using the Schrodinger glide module. To perform docking studies, we employed the Glide module in Extra Precession (XP) mode, a standard, precise mode of docking for a few compounds. The XP allows for identifying the putative leads by considering the glide or docking scores. The top-scoring ligands in each category were selected, and the final ligand-protein interaction

analysis was done. For validation of the docking protocol, the co-crystalized ligands were re-docked into the active site of the protein. In order to determine the free binding energy, the protein-ligand complex was further subjected to Molecular Mechanics Generalized Born Model and Solvent Accessibility (MM-GBSA) analyses. For calculating MM-GBSA, the prime module of the Schrodinger suite was used.

4.12 Molecular Dynamic simulation study

The flexibility of receptors is restricted in molecular docking, which hinders the effective mimicking of the actual biological system. To address this, molecular dynamics (MD) simulation, an alternative that employs proteins and drugs in solvated conditions was carried out. The compound with the topmost dock score was further subjected to MD simulation studies for a time period of 50 ns using the Desmond OPLS3 force field. The water model SPC (single point charge) was used to solvate the protein-ligand complex in the orthorhombic boundary box. The counterions (Na^+ and Cl^-) were introduced to neutralise the system. Energy minimization and NPT equilibration were done sequentially to minimise the system. After minimization of the system, the MD simulation was run for 100 ns with atmospheric pressure and temperature set at 1.013 bar and 310 K, respectively.

4.13 In vitro anti-inflammatory assays

4.13.1 Protein denaturation method

To obtain a reaction mixture, 0.2 ml of 1% bovine albumin was mixed with 4.78 ml of phosphate buffered saline (pH 6.4) and 0.02 ml of extract, which was incubated in a water-bath at 37°C for 15 minutes. Following the heating of the reaction mixture at 70°C for 5 minutes, it was allowed to cool, and then turbidity was measured at 660 nm using UV-Visible spectrophotometer using aspirin as a control (**Fig. 7**) [70]. The percentage inhibition (using the given formula) and IC_{50} were calculated.

$$\% \text{ inhibition of denaturation} = 100 \times (1-A_2/A_1)$$

where A_1 = absorption of the control sample, A_2 = absorption of the test sample

4.13.2 Proteinase inhibition

The reaction mixture (2 ml) was obtained by mixing 0.06 mg trypsin, 1 ml of 20 mM tris-HCl buffer (pH 7.4), and 1 ml of the test sample (0.02 ml extract + 0.980 ml ethanol). The mixed solution was incubated at 37°C for 5 minutes. 1 ml of 0.8% w/v casein was added. The reaction mixture was incubated again for 20 minutes. After the addition of 2 ml of 70% perchloric acid, it was centrifuged. The absorbance of the supernatant was recorded at 210 nm against buffer solution as the blank [70, 71]. Aspirin was used as a control. The percentage inhibition of proteinase (formula provided below) and IC_{50} were determined.

$$\% \text{ inhibition of denaturation} = 100 \times (1-A_2/A_1)$$

where A_1 = absorption of the control sample, A_2 = absorption of the test sample

4.13.3 Membrane stability

4.13.3.1 Preparation of erythrocyte suspension

The whole blood collected from a healthy subject was centrifuged at 3000 rpm for 5 minutes in a heparinized centrifuge tube, and triple washing was done with an equal volume of normal saline (0.9% NaCl) followed by centrifugation. The blood volume was measured and reconstituted as a 10% v/v suspension with an isotonic buffer solution (10 mM sodium phosphate buffer, pH 7.4) [71].

4.13.3.2 Heat-induced haemolysis

A mixture of 0.05 ml of blood cell suspension, 0.05 ml of plant extract, and 2.95 ml of phosphate buffer (pH 7.4) were incubated at 54°C for 20 minutes in a shaking water-bath. Upon incubation, the mixture was centrifuged at 2500 rpm for 3 minutes. Taking phosphate buffer

solution as a blank, the absorbance of the supernatant was recorded at 540 nm using UV-Visible spectrometer. Aspirin was used as a control in the experiment. Subsequently, the percentage inhibition of proteinase (formula provided below) and IC_{50} were determined.

$$\% \text{ inhibition of denaturation} = 100 \times (1-A_2/A_1)$$

where A_1 = absorption of the control sample, A_2 = absorption of the test sample

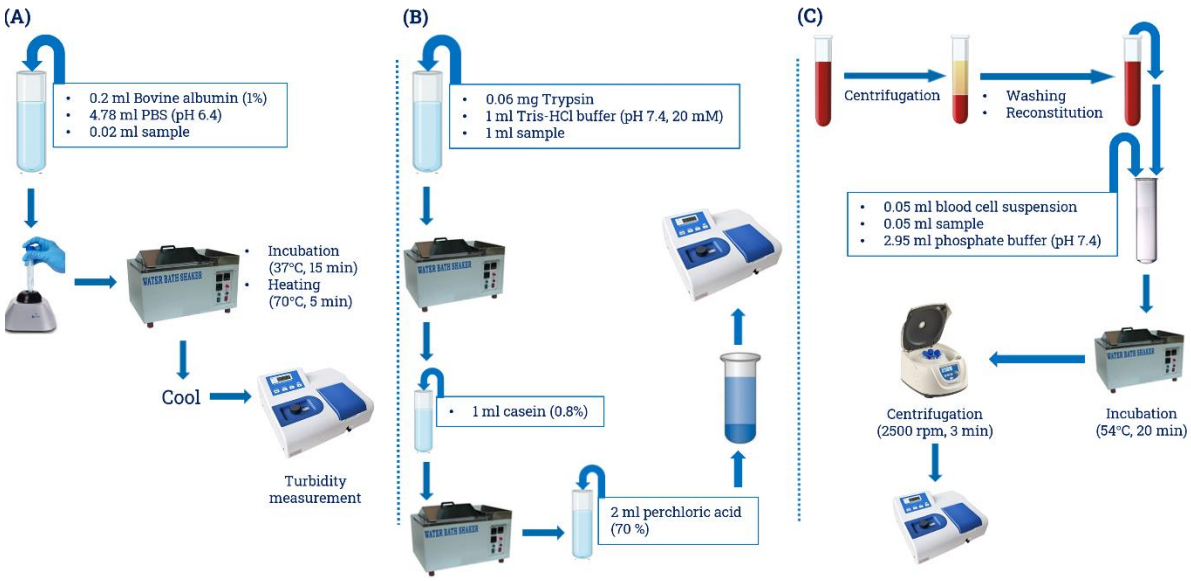

Fig. 7 A diagrammatic representation of in vitro anti-inflammatory assays

4.14 Carrageenan-induced paw oedema method

Rats were randomly allocated to five different groups using computer generated randomization tables. The groups (n = 6) are as follows: the control group received saline (2 ml/kg p.o.), the standard group received aspirin (100 mg/kg p.o.), and the other three groups received 125, 250, and 500 mg/kg p.o. doses of aqueous extract of *N. herpeticum*, respectively. All the groups were administered a single dose of their respective treatments 1 hour prior to the induction of inflammation. Inflammation was produced by injecting 1 ml of carrageenan (1% suspension in saline) into the sub-plantar region of the right hind paw. The paw volumes were measured using a digital plethysmograph (Orchid Scientific, India) by immersing up to a marked region of the lateral malleolus at different time points (0 hour, 1 hour, 3 hour, and 5 hour) following

carrageenan injection [72]. The percentage inhibition of paw oedema was calculated using the following formula:

$$\%\ \text{Inhibition of Paw oedema} = \left[1 - \frac{(V_t - V_o)_{treatment}}{(V_t - V_o)_{control}}\right] \times 100$$

where V_t = Paw volume of any group (control or treatment) at that particular time (t hour) and V_o = Paw volume of any group (control or treatment) at 0 hr

4.15 Statistical analysis

All the data from the conducted tests were recorded as the mean ± standard deviation (SD), where P <0.05 was considered statistically significant. GraphPad Prism, version 5.0 (GraphPad Software, USA), was used for statistical analyses. Percentage body weight gain and consumption of water and food were analysed using a two-way ANOVA followed by the Bonferroni test, whereas relative organ weight, biochemical parameters, and percentage paw oedema inhibition were assessed using a one-way ANOVA (Dunnet's multiple comparison test).