Materials and Methods

3.0 MATERIALS AND METHODS

Plants have been an important source of medicine for thousands of years and an impressive number of modern drugs have been isolated from natural sources, many based on their use in traditional medicine (Preethi *et al.*, 2010). Plants are used in the treatment of bacterial and fungal infections for its wide range of bioactive molecule (Sajad *et al.*, 2011).

The present study was aimed to screen the effect of silver nanoparticles conjugated with *A.adenophora* leaf extract for their antimicrobial activity and its compound Eupalitin was subjected to *in silico* studies for its efficacy against the target protein dihydrofolate reductase of the most susceptible organisms namely *Escherichia coli, Staphylococcus aureus* and *Bacillus anthracis* and the fungal strain of *Candida albicans* and *Candida glabirata*. The experimental procedure employed in the present study is discussed below.

COLLECTION OF PLANT SAMPLE

The leaves of the plant sample were collected from in and around the Nilgiri district of Tamil Nadu. The plant sample is authenticated from Tamil Nadu Agricultural University (TNAU), Coimbatore.

PREPARATION OF METHANOLIC EXTRACT

Fresh leaves of *A. adenophora* were collected and cleaned to remove adhering dust particles, washed under running tap water, gently blotted dry between folds of tissue paper. 10g of leaf sample was weighed, cut into small pieces and added to 100ml of methanol. This was stored in dark with mild shaking for 24 hrs. The mixture was then filtered through Whatman No. 1 filter paper. The final extract was stored at 4°C for further experiments.

PREPARATION OF SILVER NANOPARTICLES

Silver nanoparticles were prepared from the methanolic extract of *A. adenophora* leaves. To 10 ml of the leaf extract 90 ml of 1mM silver nitrate solution was added (Donda *et al.*, 2013). The extent of nanoparticles synthesis was monitored by measuring the absorbance at 400-600nm.

ROOM TEMPERATURE

The samples and silver nitrate solution were incubated at room temperature for 72 hrs (Paulkumar *et al.*, 2014).

HEATING IN WATER BATH

The methanolic extract of *A. adenophora* leaves in the presence of silver nitrate was heated for various durations (5, 10, 15 and 20 min) in a water bath at a temperature of 60° C (Gulcin *et al.*, 2011; Mubarakali *et al.*, 2011).

HEATING BY MICROWAVE

The mixture of methanolic extract of leaves with silver nitrate solution was heated in microwave for various durations namely 10, 20, 30 and 40 seconds (Nooroozi *et al.*, 2012).

EXPOSURE TO SUNLIGHT

The methanolic extracts of *A. adenophora* with silver nitrate solution were exposed to sunlight for various durations (5, 10, 15 and 20 min) with silver nitrate solution (Sulaiman *et al.*, 2013).

SEPARATION OF SILVER NANOPARTICLES

To separate the synthesized silver nanoparticles, samples were centrifuged at 13,000 rpm for 20 min under refrigeration and washed 3 times with deionized water. A dried powder of the silver nanoparticles was obtained by freeze drying.

CHARACTERIZATION OF SILVER NANOPARTICLES

The synthesized silver nanoparticles were characterized as per the methods explained below.

UV- VISIBLE SPECTRA

The light nanoparticles like silver (Ag) exhibit unique and tunable optical properties on account of their Surface Plasmon Resonance (SPR). A volume of 100µl of synthesized nanoparticles sample was diluted with 900µl silver nitrate solution and subjected to spectral analysis using an UV-Visible nanophotometer (Optizen, Korea) in a scanning range of 400 nm to 600 nm.

SCANNING ELECTRON MICROSCOPY

Silver nanoparticles synthesized were characterized using high resolution scanning electron microscopy (SEM). The samples were prepared by simple drop coating of the suspension of silver nanoparticles separately on a carbon- coated copper grid, by simply dropping a very small amount of the sample on the grid and the excess solution was removed by blotting. The film on the scanning electron microscopy grid was then allowed to dry under a mercury lamp for 5 minutes. It was then subjected to SEM analysis.

EDAX SPECTRUM MEASUREMENTS

A.adenophora leaf extract– reduced silver solutions were dried, drop coated on to carbon film, and tested using EDAX analyses.

FTIR (FOURIER – TRANSFORM IR) ANALYSIS

FTIR analysis is done to obtain the infra red spectra of absorption, emission and to ensure the formation of silver nanoparticles. FTIR measurements help to identify the possible interactions between silver and bioactive molecules, which may be responsible for formation and stabilization (capping material) of silver nanoparticles. The advantage of using an FTIR is that it simultaneously collects spectral data in a wide spectral range.

TEST MICROORGANISM

The bacterial and fungal strains used in the present study were the clinical isolates maintained in our laboratory. The bacterial strains used were *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Shigella flexneri*, *Proteus vulgaris* and *Klebsiella pneumoniae*.

The fungal strains used were *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Candida albicans*, *Mucor oryzae* and *Rhizopus indicus*.

ANTIBACTERIAL ASSAY

Preparation of medium

Mueller Hinton Agar medium

The medium was prepared by dissolving 33.9g of the commercially available Mueller Hinton Agar Medium (HiMedia) in 1000ml of distilled water. The effects of silver nanoparticles conjugated with *A.adenophora* leaf extract on the several bacterial strains were assayed by agar well diffusion method. The medium was autoclaved at 15 lbs pressure at 121°C for 15 minutes. The autoclaved medium was mixed well and poured onto petriplates (25-30ml/plate) while still molten.

Preparation of the test culture

Inoculums of the microorganism were prepared from overnight culture grown in nutrient broth and the suspension was adjusted with a turbidity equivalent to that of 0.5 MacFarland standards.

Agar well diffusion method

Principle

The antimicrobials are allowed to diffuse out into the medium and interact in a plate freshly seeded with test microorganisms. The resulting zone of inhibition will be uniformly circular as there will be a confluent lawn of growth. The diameter of zone of inhibition can be measured in millimeters.

Procedure

Petriplates containing 20ml of Mueller Hinton Agar medium were seeded with the inoculums prepared from a broth that has been incubated for 6 hours, when the growth is in logarithmic phase, 100µl were spread in plates. Wells were cut in the agar and 10µl of the silver nanoparticles conjugated with *A.adenophora* leaf extract was added. The plates were incubated at 37°C for 24 hours. The antibacterial activity was assessed by the diameter of zone of inhibition formed around the wells (NCCLS, 1993). Chloramphenicol was used as standard antibacterial agent.

Antifungal assays

The effects of silver nanoparticles conjugated with *A. adenophora* leaf extract on the several fungal strains were assayed by Agar plug method.

Agar plug method

Principle

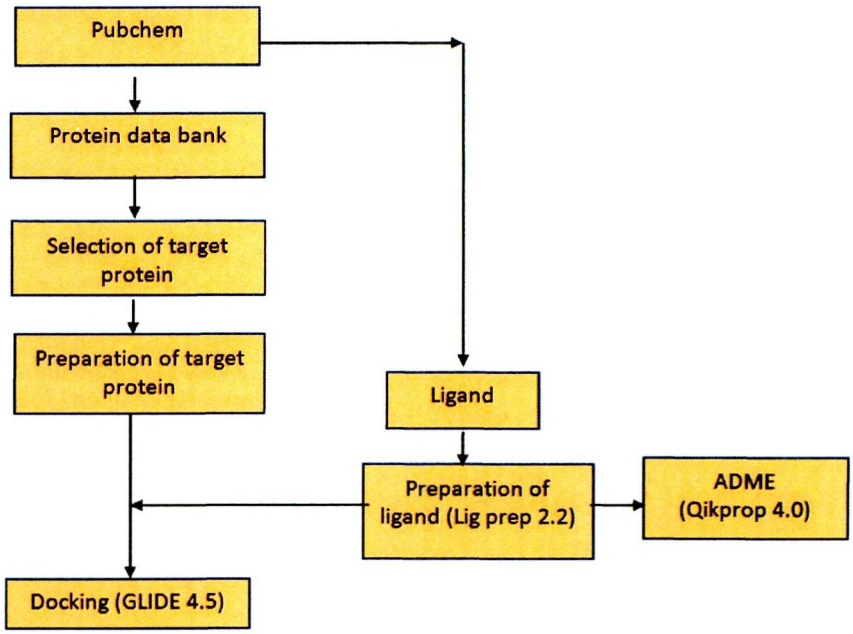
The fungicidal effect of the silver nanoparticles conjugated with *A. adenophora* leaf extract can be assayed by the inhibition of mycelial growth of the fungus and is observed as a zone of inhibition near the disc or the wells.

Procedure

Petriplates with 20ml of Potato Dextrose Agar were prepared. A fungal plug was placed in the center of the plate. Sterile discs impregnated with the silver nanoparticles conjugated with *A. adenophora* leaf extract were placed in the plates. Nystatin was kept as a positive control.

In silico Study

The methodology on molecular docking studies of eupalitin is shown in the following representation



Pubchem

The PubChem Compound Database contains validated chemical depiction information provided to describe substances in PubChem substance. Structures stored within PubChem Compounds are pre-clustered and cross-referenced by identity and similarity groups. Eupalitin structure was retrieved from the PubChem Compound Database.

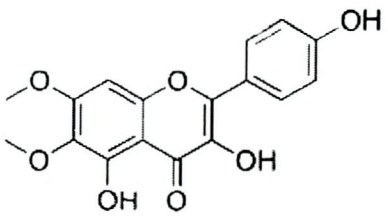
Protein data bank

The protein data bank (PDB) archive is the single worldwide repository of information about the 3D structure of large biological molecules, including proteins and nucleic acids. The structure in the archive ranges from tiny proteins and bits of DNA to complex molecular machines like the ribosome. The target proteins for the present study were obtained from the protein data bank.

Selection of target proteins

The component eupalitin was subjected to *in silico* studies for their efficacy against the target protein dihydrofolate reductase of the most different susceptible organisms namely the bacterial strains *Escherichia coli, Staphylococcus aureus* and *Bacillus anthracis* and the fungal strain of *Candida albicans* and *Candida glabirata*. The target proteins selected were 4PSS for *Escherichia coli*, 3F0Q for *Staphylococcus aureus*, 3S9U for *Bacillus anthracis*, 4HOG for *Candida glabirata* and 4HOE for *Candida albicans*.

Structure of Eupalitin



Molecular formula - $C_{17}H_{14}O_7$

Molar mass - 330.29 g mol^{-1}

Ligand

From the earlier studies on *Ageratina adenophora*, it has been reported by the spectral studies, that there are many phytochemical compounds, but the antimicrobial compound was found to be eupalitin which is a flavanoid. Hence eupalitin was chosen as the ligand for further studies.

Preparation of target protein structure

The protein preparation wizard panel allows you to take a protein from its raw state to a state in which it is properly prepared for use by Schrodinger.

Docking analysis using Maestro 9.3

Maestro

Maestro is the graphical user interface for all of Schrodinger products as Combi Glide, Epik,Glide, Impact, Liasion, Ligprep, Phase, Maestro model, Prime, Qikprop, Qsite. Maestro is a powerful and versatile molecular modeling environment and the portal to the most advanced science in computational chemistry.

Qikprop 4.0

Qikprop 4.0 module of Schrodinger is a quick, accurate, easy to use absorption, distribution, metabolism and excretion (ADME) prediction program. It predicts physically significant descriptor and pharmaceutically relevant properties of organic molecules. The ADME properties of eupalitin were detected by Qikprop.

Ligprep 2.2

The preparation of the ligand was done using LigPrep 2.2, a module on the Maestro window of Schrodinger is a robust quality, all atom 3D structures for large number of drug like molecules, starting with 2D or 3D structure in Maestro format. The target proteins namely 4PSS for *Escherichia coli*, 3F0Q for *Staphylococcus aureus*, 3S9U for *Bacillus anthracis*, 4HOG for *Candida glabirata* and 4HOE for *Candida albicans* were subjected to Ligprep. The output file was produced with extension "-out.mae".

GLIDE (Grid based ligand docking with energetic)

Glide searches for favorable interaction between one or more ligand molecules and a receptor molecule usually a protein. The ligand poses that are generated by Glide pass through a series of hierarchial filters which evaluate the ligand interaction with the receptor.

Docking

The ligands were then docked to the target proteins using Glide 4.5 module of Schrodinger. The docking was done in Standard Precision Mode (SP). The docked protein and the ligand were viewed with Glide Pose Viewer. The images of the best docked poses of the ligand and the protein were saved as .jpg files.

The above methodology was carried out and the results obtained are presented in the following chapter.