INTRODUCTION

Infectious diseases are caused by pathogenic microorganisms, such as bacteria, viruses, parasites or fungi. Infectious diseases are the world’s leading cause of premature deaths, killing almost 50,000 people every day. Infections due to variety of bacterial etiologic agents such as pathogenic Escherichia coli, Salmonella sp., and Staphylococcus aureus are most common. Among the various drug resistant pathogens, MRSA (Methicillin Resistant Staphylococcus aureus), VRSA (Vancomycin resistant Staphylococcus aureus) and ESBL (Extended spectrum β-lactamase) strains are in major concern. Extended spectrum β-lactamase (ESBL) is enzymes that mediate resistance to extended spectrum cephalosporin, cefotaxime, ceftiriazone and ceftazidime and the monobactam aztreonam. Such enzymes are commonly found in Escherichia coli, Klebsiella sp., Pseudomonas aeruginosa and Acinetobacter baumannii.

To solve this problem all over the world scientists are searching various living resources for lead compounds for the development of novel drugs against multidrug resistant pathogens. A large portion of the world population depends mainly on traditional system of medicine for variety of diseases. Several hundreds of plants are used as of medicine and are a source of very potent and powerful drugs which is used for a long time and still being in use today.

Plants produce a diverse range of bioactive molecules, making them rich source of different types of medicines. Most of the drugs today are obtained from natural sources or semi synthetic derivatives of natural products and used in the traditional systems of medicine. Medicinal plants are finding their way into pharmaceuticals, cosmetics, and neutraceuticals. In pharmaceutical field medicinal plants are mostly used for the wide range of substances present in plants which have been used to treat chronic as well as infectious diseases.

A Siddha system of medicine is the oldest holistic management system with meticulously documented medicines and being practiced by a large population in south India. In Siddha system of medicine, herbs and herbal formulations are considered as an initial choice of drugs followed by higher order medicines of parpams and chendurams prepared from minerals and metals, if the patient is not responding well to herbal medicines. Choornam is defined as a fine powder of drug or drugs in Ayurvedic system of medicine. Drugs mentioned in patha, are cleaned properly, dried thoroughly, pulverised and then sieved.

The churna is free flowing and retains its potency for one year, if preserved in airtight containers. Churna formulation is similar to powder formulations in Allopathic system of medicine. In recent days churna is formulated into tablets in order to fix the dose easily. It is prescribed by the Ayurvedic physician for treating conditions such as diabetes, indigestion, constipation etc. Ayurvedic churnas combine all six of the ayurvedic tastes: sweet, sour, salty, pungent, bitter, and astringent. They are created through the combination of a number of different fresh herbs, and can be added to almost any food stuff. With this view the present study was evaluated the antimicrobial activity of selected choornams against drug resistant pathogens.
MATERIALS AND METHODS

Bacterial test pathogens

Bacterial test pathogens were obtained from Centre for Laboratory Animal Technology and Research, Sathyabama University. Antibiotic susceptibility of strains were done on Mueller Hinton Agar plates using Kirby-Bauer disc diffusion method according to CLSI guidelines (CLSI, 2012) using antibiotics Methicillin, Amikacin, Gentamicin, Cefotaxime, ceftazidime, imipenem, chloromphenicol Ceafepine and Pipercillin/Tazobacattam (Himedia).

Antifungal susceptibility testing was performed by NCCLS M44-A disc diffusion method (NCCLS). Briefly, antibiotic discs containing Itraconazole (10 mcg), Ketoconazole (10 mcg), Clotrimazole (10 mcg), Fluconazole (25 mcg), Amphotericin-B (20 mcg) and Nystatin (100 units) were tested. The zones measured only that is showing complete inhibition and the diameters of the zones recorded to the nearest millimeter.

Confirmation of ESBL pathogens

Extended spectrum beta lactamase production among gram negative bacteria was detected using combination disc method. In this test, 0.5 McFarland’s standard test bacterial culture was inoculated on the surface of Muller Hinto Agar (MDA) plate. The cefotaxime (30 μg) and cefotaxime-clavulanic acid (30 μg/10 μg) discs were placed 20 mm apart on the agar surface. Similarly, the ceftazidime (30 μg) and ceftazidime-clavulanic acid (30 μg/10μg) (Himedia Laboratories, Mumbai) discs were also placed for detection of esbl production. After incubating for overnight at 37°C, a ≥5 mm increase in the zone of inhibition diameter was measured and interpreted as positive for ESBL production. The Positive and negative control strain used for this study is K. pneumoniae ATCC 700603 and E. coli ATCC 25922 respectively.

Collection and description of chooranam

Chooranams were procured from SKM Siddha and ayurvedha company (INDIA) limited, Erode. The ingredients and organoleptic characters of the chooranams were noted.

Confirmation of drug resistant pathogens

The test bacterial pathogens were collected from Centre for Laboratory Animal Technology and Research, Sathyabama University. The test pathogens such as Staphylococcus aureus, Bacillus cereus, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella typhi and Proteus vulgaris were confirmed by standard procedures.

Isolation and characterization of Candida albicans

The Candida albicans samples were inoculated on the Sabouraud Dextrose Agar (SDA) medium and the plates were incubated at room temperature for 24-48 hours. After incubation, the culture plates were examined for the appearance, size, color and morphology of the colonies. Wet mount preparation, Germ tube formation and growth on corn meal agar were carried out according to standard techniques.

Preparation of chooranam extract

Extraction methods involve separation of medicinal active functions of chooranams from inactive/inert components, by using selective solvents and extraction technology. Solvents diffuse into chooranams and solubilize compounds of similar polarity. Quality of chooranam extract depends on choice of solvents and the extraction.

Preparation of aqueous and solvent extracts

5 grams of fresh chooranam powder were taken dissolved in 25ml of sterile distilled water in 50ml beaker. The aqueous chooranam mixture was covered with aluminium foil and kept at room temperature for 24 hours. This procedure was adopted for the preparation of aqueous extracts for all the chooranam powder as well as for solvents. This content was mixed well and the beaker was covered with aluminium foil and kept for extraction at room temperature for 24 hours.

Separation of crude extracts

After extraction, the aqueous and solvent mixture of chooranam powder was filtered by passing through muslin cloth to remove debris. Further the filtered liquid portion is centrifuged at 5000 rpm for 15 minutes to remove fine debris which are not removed by filtration. The aqueous and solvent extracts were transferred to clean 25ml beaker. Crude compounds were concentrated by evaporation at room temperature and stored in small vials at 4°C until further use.

Antimicrobial activity of aqueous extracts

The antimicrobial activity of chooranam aqueous extracts was studied by well diffusion method using Muller Hinton agar (MHA) plates. About 18 hours old bacterial culture was prepared and inoculated into MHA plates. 5 mm diameter well was cut on plates. Each 10μl of aqueous plant extracts were added in wells using micropipette. 10 μl sterile distilled water was used as a control well. All the plates were incubated at 37°C for 24 hours and plates were observed for zone of inhibition.

Antimicrobial activity of solvent extracts

The antimicrobial activity of solvent extracts was studied by disc diffusion method using MHA plates. About 18 hours old bacterial cultures were inoculated into MHA plates. 0.25mg of crude extracts were added into sterile filter paper disc (5 mm diameter) and allowed to dry at room temperature for few minutes. Crude chooranam extract impregnated discs were placed on MHA plates inoculated with test bacterial strains. Sterile empty disc was used as a control. All the plates were incubated at 37°C for 24 hours. After incubation the plates were observed for zone of inhibition.
Partial purification of active compound by thin layer chromatography

The crude compound was purified by using silica gel thin layer chromatography using method described by Saravanan et al.\textsuperscript{12}. To find out the best solvent system to separate the crude compound, solvents such as methanol, chloroform, acetic acid, n-butanol, n-hexane and water were used in different proportions. After running, the sheet was kept at room temperature for the complete drying of the plate and the separated spots were visualized in iodine chamber. Rf value of the spots on the TLC plate was determined by;

\[
\text{Rf value} = \frac{\text{Movement of the solute from the origin}}{\text{Movement of solvent from the origin}}
\]

Bioautography

The bioautography method was for the detection of active compound separated in TLC. Chromatogram developed was placed in a sterile bioassay petri dish containing nutrient agar medium inoculated with \textit{Staphylococcus aureus} and \textit{E. coli}.\textsuperscript{2} Active compound from methanol extract of parangipattai chooranam was further purified from preparative TLC and tested for antimicrobial activity by disc diffusion method as described earlier.

Spectral analysis of purified compound

Spectral analyses of purified compound were carried out at Centre for Ocean Research, Sathyabama University, Chennai, Tamil Nadu.

UV spectral analysis

Ultraviolet (UV) spectrum of the purified compound was determined using Shimadzu UV-1800 series. One milligram of sample was dissolved in 10mL of methanol and the spectra were recorded at a wavelength of 200 – 800nm.

FT-IR analysis

The Infrared (IR) spectrum of the purified compound was determined using Shimadzu. The spectrum was obtained using potassium bromide (KBr) pellet technique in the range of 450 to 4000cm\textsuperscript{-1} at a resolution of 1.0cm\textsuperscript{-1}. Potassium bromide (AR grade) was dried under vaccum at 100°C and 100mg of KBr with 1mg of purified sample was used to prepare KBr pellet. The spectrum was plotted as intensity versus wave number.\textsuperscript{13}

Minimum inhibitory concentration (MIC) of the purified compound

Working stock preparation

Eight microfuge tubes of 2 ml capacity were taken and marked as 1-8. In the first tube, 900µl of distilled water was added and 500µl of distilled water was added to each of the remaining tubes. Hundred microlitre of crude compound from the stock solution was transferred to the first tube and mixed well. Five hundred microlitre of the diluted antibiotic was transferred from the first tube to the second tube and thus the dilution was continued up to the 8\textsuperscript{th} tube.

Inoculum preparation

Freshly grown colonies of bacterial cultures such as \textit{Staphylococcus aureus}, \textit{E.coli} grown on nutrient agar plates were suspended in 2ml of sterile Mueller Hinton Broth. Turbidly of the inoculum was adjusted to 0.5 McFarland Standard.

Broth dilution

About 10 sterile glass tubes were taken and marked as 1-10. Each tube 500µl of Mueller Hinton Broth was added in the first eight tubes. Each 900µl of Mueller Hinton broth was added to the 9\textsuperscript{th} and 10\textsuperscript{th} tubes and marked as C1 and C2 respectively. 500µl of working stock solution was added to the 1\textsuperscript{st} tube. From the first tube 500µl was transferred to second tube and then serially transferred up to eight tubes. The one tube marked as C1 was the culture control and other tube marked as C2 was the broth control. 500µl of bacterial inoculums was transferred to all the tubes except C2. All the tubes were incubated at 37°C for 18-24 hours. After incubation all the tubes were observed for growth inhibition. The lowest antibiotic concentration which showed no turbidity was taken as minimal Inhibitory concentration (MIC).\textsuperscript{14}

RESULTS AND DISCUSSION

Infections due to a variety of bacterial agents such as pathogenic \textit{E. coli}, \textit{Vibrio cholera}, \textit{Shigella spp.}, \textit{Salmonella spp.}, \textit{Pseudomonas spp.}, \textit{Klebsiella spp.} and \textit{Staphylococcus aureus} are the most common diseases causing microorganisms.\textsuperscript{14} Traditional medicine has been improved in developing countries as an alternative solution to health problems and costs of pharmaceutical products.\textsuperscript{15} Plant based antimicrobials represent a vast untapped source for medicines and further exploration of plant microbial need to occur. Contrary to the synthetic antibiotic, antimicrobials of plant origin are not associated with many side effects and have an enormous therapeutic potential to heal many infectious diseases.\textsuperscript{16} The use of medicinal plants as a basis for relief from sickness can be traced back over five millennia to written documents of the early civilization in India, China and the Near east, but it is doubtless an art as old as mankind.\textsuperscript{17} The people of India have a very long-standing tradition in the use of natural medicines and the local practices are still quite common in the treatment of diseases.

The parangipattai choornam though simple and cost effective has diverse medicinal properties and used in the treatment of various diseases like Granthi, soolai, Megam, Vettaii, Vondukadi, Padaigal, Viranangal kandamalai. Sivaranjini et al.\textsuperscript{18} studied organoliptic character and assess the quality of drug Parangipattai chooranam by conducting physicochemical analysis, preliminary phytochemical analysis and other analytical techniques. In this present study also four different Choornams were studied...
for antimicrobial evaluation, and its characterization. The ingredients and organoleptic characters of all the chooranams were observed (Table 1 & 2). The test pathogens used in this study were identified and confirmed by standard procedures.

**Table 1: Ingredients of Chooranams**

<table>
<thead>
<tr>
<th>Chooranams</th>
<th>Ingredients</th>
<th>Botanical Name</th>
<th>Parts Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parangipattai</td>
<td>Purified parangipattai Sugar</td>
<td>Smilax china Saccharum officinum</td>
<td>Roots</td>
</tr>
<tr>
<td>Ponnavarai</td>
<td>Ponnavarai Leaves</td>
<td>Cassia senna</td>
<td>Leaves</td>
</tr>
<tr>
<td>PoonaiKali</td>
<td>Velvet bean</td>
<td>Mucuna</td>
<td>Seeds</td>
</tr>
</tbody>
</table>

**Table 2: Organoleptic characters of chooranams**

<table>
<thead>
<tr>
<th>Chooranams</th>
<th>Colour</th>
<th>Consistency</th>
<th>Mixed or Pure</th>
<th>Odour</th>
<th>Taste</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parangipattai</td>
<td>Muddish Brown</td>
<td>Granulated</td>
<td>Mixed</td>
<td>Pungent</td>
<td>Slightly sweet</td>
</tr>
<tr>
<td>Ponnavarai</td>
<td>Olive green</td>
<td>Fine powder</td>
<td>Pure</td>
<td>Pungent</td>
<td>Bitter/Slightly sweet</td>
</tr>
<tr>
<td>PoonaiKali</td>
<td>Creamy white</td>
<td>Fine powder with black granules</td>
<td>Pure</td>
<td>Musky</td>
<td>Bitter</td>
</tr>
<tr>
<td>Gunma uppu</td>
<td>Light sandal</td>
<td>Granulated</td>
<td>Mixed</td>
<td>Pungent</td>
<td>Salty</td>
</tr>
</tbody>
</table>

All the chooranams used in this study were extracted for crude compounds using various solvents such as methanol, acetone, chloroform and aqueous. Saravanan et al.² extracted crude compounds from leaf powder using methanol, n-hexane, ethylacetate, dichloromethane and aqueous.

Antibiotic susceptibility of seven test bacterial pathogens against standard antibiotics is given in table 3. From the observation S. aureus showed resistance against more than four antibiotics including m ethicillin. K. pneumoniae and P. aeruginosa showed resistance to more than three antibiotics. E. coli, S. typhi showed resistance against four antibiotics tested. From the observation the S. aureus strains was identified as Methicillin resistant Staphylococcus aureus (MRSA) and E. coli, K. pneumonia, P. aeruginosa, S. typhi was identified as drug resistant pathogens.

After incubation in SDA medium, the yeast shows Gram positive ovoid cells, Chlamydospores production on Corn Meal agar, the germ tube production at 45°C were considered as Candida albicans. Antifungal test results indicate that the Candida albicans isolate was resistant to Nystatin Clotrimazole, Itraconazole Fluconazole and susceptible to Amphotericin-B, Ketoconazole.

Antimicrobial activity of aqueous extract was studied by well diffusion method. In this study aqueous extract of Poonakali, Parangippattai and Gunma uppu showed activity against Candida albicans and Pseudomonas aeruginosa. Among the aqueous extract prepared from four chooranams, extracts from Parangippattai, Gunma uppu, Poonakali showed activity against only Candida albicans and Pseudomonas aeruginosa. Aqueous extracts of Ponnavarai doesn’t showed activity against any of test pathogens (Table 4). Saravanan et al.² also studied antimicrobial activity of aqueous extract by well diffusion method.

In this present study, solvents such as ethyl acetate, methanol, n-hexane, acetone and dichloromethane were tested for extraction of crude compound. Among the various solvents tested, the crude compounds were extracted only in methanol and acetone but not in other solvents. Methanol extract of Parangippattai showed 14 mm zone of inhibition against S. aureus, and Candida albicans, 15 mm zone of inhibition against Proteus vulgaris and Bacillus cereus, 13 mm zone of inhibition against E. coli, 11mm zone of inhibition against Pseudomonas aeruginosa, 12 mm and 10mm against Salmonella typhi and Klebsiella pneumoniae respectively. Acetone extract of Parangippattai showed 10mm zone of inhibition against Candida albicans, 11mm against S. aureus, 12mm against S. typhi and P. aeruginosa, 13mm against P. vulgaris. Solvent extracts of remaining chooranams doesn’t showed activity against any of the bacterial test pathogens tested and results are reported in table 5. Selvaraj et al.¹⁵ studied antimicrobial activity of solvent extracts for seed powder by disc diffusion method. In his study, the methanol and acetone extracts showed activity against Salmonella sp., Shigella sp., and E. coli. In this present also the methanol and acetone extracts showed activity against Staphylococcus aureus, Salmonella typhi, Pseudomonas aeruginosa, Candida albicans and Proteus vulgaris.
Table 3: Antibiotic susceptibility of bacterial and fungal pathogen

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Zone of inhibition in mm in diameter</th>
<th>Bacterial Pathogens</th>
<th>Fungal Pathogen</th>
<th>Antibiotics</th>
<th>C. albicans</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>S. aureus</td>
<td>B. cereus</td>
<td>E. coli</td>
<td>S. typhi</td>
</tr>
<tr>
<td>Gentamycin</td>
<td></td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Amikacin</td>
<td></td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Cefepime</td>
<td></td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Chloromphenicol</td>
<td></td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Imipenem</td>
<td></td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>Cefazidime</td>
<td></td>
<td>I</td>
<td>S</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td></td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Piperacillin/Tazobacctam</td>
<td></td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Methicillin</td>
<td></td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
</tbody>
</table>

Table 4: Antimicrobial activity of aqueous extracts of chooranams by well diffusion method

<table>
<thead>
<tr>
<th>Chooranams</th>
<th>Test Pathogens (Zone of inhibition in mm in diameter)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S. aureus</td>
</tr>
<tr>
<td>Parangipattai</td>
<td>-</td>
</tr>
<tr>
<td>Ponnavarai</td>
<td>-</td>
</tr>
<tr>
<td>Poonaikali</td>
<td>-</td>
</tr>
<tr>
<td>Gunma uppu</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 5: Antimicrobial activity of solvent extracts of chooranams by well diffusion method

<table>
<thead>
<tr>
<th>Chooranams</th>
<th>Test Pathogens (Zone of inhibition in mm in diameter)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Methanol Extract</td>
</tr>
<tr>
<td></td>
<td>S. aureus</td>
</tr>
<tr>
<td>Parangi pattai</td>
<td>14</td>
</tr>
<tr>
<td>Ponnavarai</td>
<td>-</td>
</tr>
<tr>
<td>Poonaikali</td>
<td>-</td>
</tr>
<tr>
<td>Gunma uppu</td>
<td>-</td>
</tr>
</tbody>
</table>

Thin layer chromatography reveals the partial purification of crude extracts for the detection of active compounds. The bioassay guided fractionation procedure used to identify bioactive natural products is often perceived as rate limiting and resource intensive. The early detection of active compound in the stage of initial separation is a time and resource saving process.

Bioautography allows localizing antimicrobial activity of an extract on the chromatogram; it supports a quick search for new antimicrobial agents through bioassay.
Physicochemical characterization of active compound is the prerequisite for its structural identification. The general property features of a molecule that are useful in the early stage might include: colour, solubility, acid/base properties, stability and size. If the target is an unknown molecule, it is probable that little is known about the nature of the compound. Compound PPTA is brown in colour, powdery in consistency. It is well soluble in methanol, poorly soluble in other solvents. Phenolic compounds constitute one of the main classes of secondary metabolites. They display a large range of structures and they are responsible for the major organoleptic characteristics of plant-derived foods and beverages, particularly color and taste properties and they also contribute to the nutritional qualities of fruits and vegetables. In this present study, in preparative TLC, 1 gm of methanol extract yielded 70 mg of active compound. The active compound was powdery in consistency and brown in colour.

The active compound present in PPT-A was spectrally analyzed by UV and FTIR which shows two peaks in UV analysis at 342 and 647nm wavelength. The FTIR spectrum frequencies reveal various functional groups present in the compound PPT A. The FTIR spectrum was used to identify the functional groups of the active components present in Parangipattai chooranam.

Shreedevi et al 21 reported that Chundaivattral chooranam having bioactive compound against Salmonella typhi and Shigella flexneri bacteria. She also analysed the choorams in FTIR and reported that the Chundaivattral chooranam contains amide, alkyne, alkanes, carboxylic acids, alkenes, aromatics, aliphatic amines and alkyl halides compounds. In this study also the parangipattai chooranam shows antimicrobial activity against drug resistant pathogens and the results of FTIR analysis confirmed the presence of phenol, alkanes, aldehyde, secondary alcohol, amino acid, aromatic amines. The MIC of compound Spot-A against Staphylococcus aureus and Proteus vulgaris were determined as 6.25μg/ml, 12.5 μg/ml respectively.

CONCLUSION
Finding of the present work evidenced that the Chooranams investigated in this study deserves the potential for drug resistant pathogens especially the parangipattai chooranam which shows activity against all the test pathogens. Structure elucidation and characterization of PPT A compound of Parangipattai chooranam need further studies to confirm its potential. The parameters obtained from this study are sufficient to authenticate for standardization of Parangipattai chooranam.

REFERENCES


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