Research Article

Antimicrobial Activity of Selected Chooranams (Poonaikali, Ponnavarai, Gunma Uppu, Parangipattai) against Drug Resistant Pathogens

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ABSTRACT

Antimicrobial activity of Poonaikali, Ponnavarai, Gunma Uppu, Parangipattai extracts was assessed on seven bacterial and one fungal drug resistant pathogens. The methanol extracts from paringipattai chooranam showed maximum zone of inhibition 10-12mm against bacteria and fungi pathogens. Thin layer chromatography and bioautography assay in paringipattai choornam methanol extracts demonstrated well defined inhibition zone against *S. aureus* at Rf value ranging from 0.58 and 0.75. The minimum inhibitory concentration of active compound was 6.25µg/ml against *Staphylococcus aureus.* The results of FTIR analysis confirmed the presence of phenol, alkanes, aldehyde, secondary alcohol, amino acid, aromatic amines compounds which showed major. The result of UV and FTIR analysis revealed the existence of few compounds with potent biological activity, but tended to be present in the formulation even at low concentration levels.

Keywords: Paringipattai, Poonaikali, Gunma uppu, Ponnavarai, drug resistant, Methanol.

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, ceftriazone and ceftazidime and the monobactum aztreonam. Such enzymes are commonly found in *Escherichia coli, Klebsiella sp., Pseudomonas aeruginosa and Acinetobacter baumanii*[1].

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[2].

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[3]. 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 [4].

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[5]. 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[6]. 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[7]. 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 [8]. 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 Cefepime and Piperacillin/Tazobacctam (Himedia).

Antifungal susceptibility testing was performed by NCCLS M44-A disc diffusion method[9] (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 [10]. 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[11].

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 choornam 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 [2].

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 [2].

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 [2].

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 [2].

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* [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 *Staphylococcus aureus* and *E.coli* [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^{-1} at a resolution of 1.0cm^{-1}. Potassium bromide (AR grade) was dried under vaccum at 100^{o}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 [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 8th tube.

Inoculum preparation

Freshly grown colonies of bacterial cultures such as *Staphylococcus aureus, 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 9th and 10th tubes and marked as C1 and C2 respectively. 500µl of working stock solution was added to the 1st 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) [14].

RESULTS AND DISCUSSION

Infections due to a variety of bacterial agents such as pathogenic *E. coli, Vibrio cholera, Shigella spp., Salmonella spp., Pseudomonas spp., Klebsiella spp.* and *Staphylococcus aureus* are the most common diseases causing microorganisms [14].

Traditional medicine has been improved in developing countries as an alternative solution to health problems and costs of pharmaceutical products [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 [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 [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 paringipattai choornam though simple and cost effective has diverse medicinal properties and used in the treatment of various diseases like Granthi, soolai, Megam, Vettai, Vandukadi, Padaigal, Viranangal kandamalai. Sivaranjini *et al*[18] studied organoliptic character and assess the quality of drug Paringipattai chooranam by conducting physicochemical analysis, preliminary phyto chemical 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 chooranms were observed (Table 1 & 2). The test pathogens used in this study were identified and confirmed by standard procedures.

Table 1: Ingredients of Chooranams

Chooranams	Ingredients	Botanical Name	Parts Used
Parangipattai	Purified parangipattai Sugar	*Smilax china* *Saccharum officinasum*	Roots
Ponnavarai	Ponnavarai Leaves	*Cassia senna*	Leaves
Poonaikali	Velvet bean	*Mucuna prurita*	Seeds
Gunma uppu	Sottru uppu Savattu uppu Valaiyal uppu Kallu uppu Vedi uppu Indhu uppu Butter milk Varikumatti pazha saaru	*Sodium chloride* *Alkaline earth salt* *Sandevere glass salt* *Artifical salt* *Potassium nitrate* *Rock salt* *Bos indicus* *Citrullus colocynthus*	-

Table 2: Organoleptic characters of chooranams

Chooranams	Colour	Consistency	Mixed or Pure	Odour	Taste
Parangipattai	Muddish Brown	Granulated	Mixed	Pungent	Slightly sweet
Ponnavarai	Olive green	Fine powder	Pure	Pungent	Bitter/Slightly sweet
Poonaikali	Creamy white	Fine powder with black granules	Pure	Musky	Bitter
Gunma uppu	Light sandal	Granulated	Mixed	Pungent	Salty

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 methicillin. *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, Paringipattai and Gunma uppu showed activity against *Candida albicans* and *Pseudomonas aeruginosa*. Among the aqueous extract prepared from four chooranams, extracts from Parangipattai, Gunma uppu, Poonaikali 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 Parangipattai 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 Parangipattai 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* [19] 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

Antibiotics	Zone of inhibition in mm in diameter								
	Bacterial Pathogens							Fungal pathogen	
	S. aureus	*B.cereus*	*E. coli*	*S. typhi*	*P. aeruginosa*	*K. pnemoniae*	*P.vulgaris*	Antibiotics	*C. albicans*
Gentamycin	R	S	S	S	R	S	S	Itraconazole	R
Amikacin	S	R	S	S	S	R	R	Ketoconazole	S
Cefepime	S	R	R	R	R	R	S	Clotrimazole	R
Chlromphenicaol	R	R	R	R	I	S	R	Fluconazole	R
Imipenem	R	S	R	S	S	R	S	Amphotericin B	S
Ceftazidime	I	S	R	R	R	R	S	Nystatin	R
Cefotaxime	S	S	R	R	R	R	S		
Piperacillin/ Tazobacctam	R	S	S	S	S	S	S		
Methicillin	R	S	S	S	S	S	S		

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

Chooranams	Test Pathogens (Zone of inhibition in mm in diameter)							
	S. aureus	*B.cereus*	*E. coli*	*S. typhi*	*P. aeruginosa*	*K. pnemoniae*	*P.vulgaris*	*C. albicans*
Parangipattai	-	-	-	-	-	-	-	14
Ponnavarai	-	-	-	-	-	-	-	-
Poonaikali	-	-	-	-	8	-	-	18
Gunma uppu	-	--	-	-	-	-	-	12

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

Chooranams	Test Pathogens (Zone of inhibition in mm in diameter)															
	Methanol Extract								Acetone extract							
	S. aureus	*B. cereus*	*E. coli*	*S. typhi*	*P. aeruginosa*	*K. pnemoniae*	*P.vulgaris*	*C. albicans*	*S. aureus*	*B. cereus*	*E. coli*	*S. typhi*	*P. aeruginosa*	*K. pnemoniae*	*P.vulgaris*	*C. albicans*
Parangi pattai	14	15	13	12	11	10	15	14	11	-	-	12	-	12	13	10
Ponnavarai	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Poonaikali	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gunma uppu	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

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 [20]. 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

guided isolation. This method avoids the need of previous purification of the substance, reducing the cost of initial screening [11]. Among the different solvent systems tested in various proportions, chloroform: methanol in 60:40 showed good separation and two spots were observed when exposed into iodine vapor for Parangipattai choornam. The Rf value of the separated spots were calculated as 0.58 and 0.75, respectively for Parangipattai

Since the compound PPT-A and PPT-B showed close Rf values in TLC, it is very difficult to separate both of them by adopting column chromatography. Hence further purification of active pigment was carried out using preparative TLC. The major compound PPT-A was identified as active compound by bioautography. Based on the bioautography the present study showed PPT-A was active compound against *Proteus vulgaris* (20mm zone of inhibition) and *Staphylococcus aureus* (18mm zone of inhibition). Spot B did not show any activity against test organisms.

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 choornams in FTIR and reported that the Chundaivattral chooranam contains amide, alkynes, 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 paringipattai chooranam which shows activity against all the test pathogens. Structure elucidation and characterization of PPT A compound of Parangipattai choornam need further studies to confirm its potential. The parameters obtained from this study are sufficient to authenticate for standardization of Parangipattai chooranam.

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