

IN VITRO ANTIMICROBIAL POTENTIALITIES OF CHLOROFORM EXTRACTS OF ETHANOMEDICINAL PLANT AGAINST CLINICALLY ISOLATED HUMAN PATHOGENS

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ABSTRACT

Resistant bacterial infections are a threat to the world due to the favorable outcome of the common infections in hospitals and community. Betalactamase producing strains in gram negative bacteria show resistant to penicillins and cephalosporins, which leads to the production of the Extended Spectrum Betalactamases (ESBLs). These enzymes confer resistance towards the cephalosporins. Though a number of new antibiotics are being developed bacteria are developing resistance to these drugs. During the last decades resistance to these drugs by the bacteria has increased which has the ability to transmit and acquire resistance to drugs. During the last decade lot of work is carried on the plants to study the action towards the microorganisms. In India most of the people from ancient times use the traditional medicines which are derived from medicinal plants. Most of the plants were studied to detect for their antimicrobial compounds. But less work is done on the piper betle. Our main aim of this study is to investigate the antimicrobial activity of the piper betle leaves extracts against the clinically isolated ESBL producing bacteria. Chloroform extract of piper betle leaves was examined against 6 human pathogens like *Klebsiella*, *Citrobacter* and *Proteus* spp. We found profound antimicrobial activity (>11mm inhibition zone). MIC (400 -1200µg/ml). The organic extracts of these plants could be a possible source to obtain new and effective herbal medicines to treat infections which may cause by multi-drug resistant (MDR) strains of micro organisms from community as well as hospital settings. The study for the first time justified the ethnic uses of plant parts against infectious diseases.

Keywords: Piper betle, ESBL, MIC

INTRODUCTION

The resistant bacterial infections are becoming threat to the present world due to the favorable outcome of the common infections in hospitals and community. β -lactamase producing strains in the gram negative and gram positive bacteria shows resistance to the penicillins and cephalosporins. There is a belief that cephalosporins can acts towards the β -lactamases producing organisms. It is wiped off by isolation of the organisms which shows resistance towards the cephalosporins. This leads to the production of the Extended Spectrum Betalactamases (ESBLs). Due to the wide spread usage of the third generation cephalosporins and aztreonam is believed to be the major cause of the mutations in these enzymes that leads to the emergence of the ESBLs¹. These enzymes mediate resistance towards the cefotaxime, ceftazidime and other broad spectrum cephalosporins and to monobactams such as aztreonam, but have no detectable activity against cephamycins and imipenem. Due to the greatly extended substrate range these enzyme are called Extended Spectrum β -Lactamases. Though throughout the world so many pharmacological industries were producing a number of new antibiotics in the last decades and the resistance to these drugs by the bacteria has increased which has the ability to transmit and acquire resistance to drugs⁸. These cases have been mainly increased due to suppressed immunity in the patients and developing of the new bacterial strains which are multi-resistant that results in high mortality in the hospitals. The problem of the resistance to different drugs is increasing day by day and the outlook for the use of drugs in future aspect is uncertain. Actions must be taken to reduce this problem and the ultimate goal is to provide efficient drugs to the patient.

From the ancient period of time plants have been a good source of natural products for human health. During the last decade lot of work is carried on the plants to study the action towards the microorganisms. According to studies done on the plants it is proved that the plants would be the best source of variety of drugs⁶.

In India most of the people from ancient times use the traditional medicines which are derived from medicinal plants. Therefore it is very important to us to better understand their properties of plant by which we can develop different variety of antibiotics. Plant extracts and phytochemical, both are known to act on the microorganisms, which can be having a great therapeutic application. Many plants have been used due to their antimicrobial traits, which are secondary metabolites of its own. Most of the plants

were studied to detect for their antimicrobial compounds. But less work is done on the piper betle. Our main aim of this study is to investigate the antimicrobial activity of the Piper betle (P.betle) leaves extracts which are used in the folk medicine our present work is focused on the action of the piper betle extract on the urinary tract pathogens, ESBL producing bacteria.

MATERIALS AND METHODS

Preparation of Chloroform extract

Fresh leaves of P.betle (1kg) were washed under running tap water and shade dried for 2days and the leaves were powdered. Ten grams of the powder was subjected to soxhlet apparatus by using a 150ml of chloroform as a solvent for 2days. The plant extracts were placed into vials and stored at 4°C for further use.

Extraction of Hydroxychavicol by Column chromatography and Thin layer Chromatography

The chloroform extract sample was passed through the column chromatography to separate the compound present in it by using the 1% of methanol in chloroform as eluting solvent and the samples collected at different time intervals were subjected to the thin layer chromatography. The thin layer chromatography showed the detection of hydroxychavicol from the chloroform extract of piper betle leaves by using methanol and chloroform 1:19 ratio mobile phase and spraying FolinCiocalteu (Phenol) reagent over the silica gel plate for the detection of hydroxychavicol. The fractions containing the pure hydroxychavicol were pooled and the desire compound was crystallized from benzene petroleum ether. And the purity of the hydroxychavicol is estimated by the HPLC and found 99.9% pure.

Bacterial strains and culture condition

The pathogenic bacterial strains were obtained from Sneha Diagnostics Clinical Laboratory, Ongole.

Escherichia coli, *Klebsiella pneumonia*, *Klebsiella oxytoca*, *Proteus vulgaris*, *Proteus mirabilis*, *Citrobacter koseri*, *Citrobacter freundii*, *ESBLproducingE.coli*, *K.oxytoca*, *K.pneumonia*. All gram negative bacteria maintained on Nutrient agar before to process.

Preliminary Phytochemical Analysis

Phytochemical screening was carried out on the powdered plant material based on standard protocol^{3,4}

Phenotypic Disc Confirmatory Test

The test was performed as recommended by CLSI. Disks of ceftazidime (CA) 30µg and ceftazidime-clavulanic acid (CAC) 20+10µg placed on MHA at a distance of 30mm between each other. Increase in zone diameter (=5mm) for CAC versus CA is confirmed as ESBL producing organisms²

Screening for the antimicrobial potential of the plant extracts

The bacteria cultures were grown in basal medium peptone water at 37°C. After 6hrs of growth, each microorganism, at a concentration of 10⁶ cells/ml, was inoculated on the surface of Mueller-Hinton agar plates. Subsequently, filter paper discs (6 mm in diameter) saturated with extract 50 µl was placed on surface of each inoculated plate. To evaluate the efficiency of the methodology, each extract was inserted simultaneously in a hole made 50 µl in new plates. The plates were incubated at 37°C for 24 h. After this period, it was possible to observe inhibition zone. Overall, cultured bacteria with halos equal to or greater than 7 mm were considered susceptible to the tested extract. DMSO 2% was used to dissolve the extracts. The controls were the solvents used for the extract and they showed no inhibitions in preliminary studies. The extract that showed antimicrobial activity was later tested to determine the Minimal Inhibitory Concentration (MIC) for each bacterial sample.

Determination of Minimum Inhibitory Concentration

The antibacterial studies of hydroxychavicol were analyzed by Micro broth dilution assay. The assays were repeated at least three times

Microbroth-dilution assay

The MIC was determined as per the guidelines of Clinical and Laboratory Standards Institute²⁸. All the bacteria used in this study were incubated for 24hrs at 37°C. Bacterial suspensions were prepared by suspending 24hrs grown culture in sterile normal

saline. The turbidity of the bacterial suspensions was adjusted to a McFarland standard of 0.5, which is equivalent to 1.5x10⁸ CFU/ml. The twofold serial dilutions of hydroxychavicol was prepared in Muller Hinton broth, 100µl of the bacterial inoculums was added to each well of the plate, resulting in a final inoculums of 5x10⁵ CFU/ml in the well; final concentration of the compound hydroxychavicol ranged from 1000µg/ml to 100µg/ml. The plates were incubated at 37°C for 24hrs. The minimum concentration of the compound that showed 100% reduction of the original inoculums was recorded as the MIC.

The **Minimum Bactericidal Concentration (MBC)** was determined by spreading a 100-µl volume on a LB agar plate from the wells showing no visible growth. The plates were incubated at 37°C for 24 h. The minimum concentration of compound that showed ≥99.9% reduction of the original inoculums was recorded as the MBC.

RESULTS

In phytochemical analysis proved that the phenol compounds present in the piper betle plays a vital role in the bactericidal activity. The piper betle extract exhibited a broad spectrum activity towards the urinary tract pathogens. Microbroth dilution assay against the pathogenic bacteria were tested towards the hydroxychavicol, MIC of the hydroxychavicol ranged from 1200µg/ml to 200µg/ml. The bacteria belong to enterobacteriaceae family which causes the urinary tract infections exhibited 400-1000µg/ml MIC and the MBC varied from 800-1200µg/ml concentrations. MIC and MBC was determined in the table 1. It has broad spectrum activity towards the *citrobacter*, *klebsiella* and *proteus* species.

ESBL producing bacteria were also exhibited a wide range of activity towards the hydroxychavicol. MIC 400-1000µg/ml and MBC 800-1200µg/ml concentrations. The MIC and MBC of the ESBL producing bacteria were given in table 2.

Table 1: (MICs) Minimum Inhibitory concentrations and Minimum bactericidal concentrations (MBCs) on urinary tract pathogenic bacteria

	Bacterial isolates	MICs µ/ml	MBCs µg/ml
1	<i>C.fruendi</i>	400 – 1000	800 – 1200
2	<i>C.koseri</i>	400 – 1000	800 – 1400
3	<i>K.pneumoniae</i>	400 – 1200	800 – 1400
4	<i>K.oxytoca</i>	400 – 1200	800 – 1400
5	<i>Enterobacteraerogenes</i>	400 – 1200	800 – 1400
6	<i>P.vulgaris</i>	400 – 1200	800 – 1400
7	<i>P.mirabilis</i>	400 – 1200	800 – 1400
8	<i>Enterococcus faecalis</i>	200 – 400	800 – 1000

Table 2: (MICs) Minimum Inhibitory concentrations and Minimum bactericidal concentrations (MBCs) on ESBL isolated bacteria

	Bacterial isolates	MICs µ/ml	MBCs µg/ml
1	<i>E.coli</i> (26)	400 – 1000	800 - 1200
2	<i>K.oxytoca</i> (31)	400 – 1200	800 - 1400
3	<i>K.pneumoniae</i> (09)	400 – 1200	800 - 1400
4	<i>C.koseri</i> (13)	400 – 1000	800 - 1400

Phytochemical analysis

1	Alkaloids	--
2	Tannins	--
3	Saponins	--
4	Terpenes	--
5	Steroids	--
6	Triterpenoids	--
7	Cardiac glycosides	--
8	Phenol	+
9	Anthraquinones	--

DISCUSSION

The high level of resistance to the third generation cephalosporins and monobactams leads to the development of new drugs by using plant source. The yield of phenol compound extracts in the plant is due to the strong extraction capacity of chloroform. Some of these

metabolites may be responsible for antimicrobial activity associated with ethanomedicinal plants. It is well documented that the phenols will play a major role as antimicrobial agents.⁵ in addition to alkaloids and tannins⁶. In the present study the Piper betle plant tested for antimicrobial activity against ESBL producing *E.coli* and *K.pneumoniae*. The variations in the sensitivity of the bacterial species tested may be due to the difference in the type of ESBLs harboured by organisms since there is existing of more than 200 phenotypes.^{7,8}

CONCLUSION

Piper betle leaf has a significant antimicrobial activity against broad spectrum of micro organisms. The antibacterial activity of piper betle extract against ESBL producing strains of *E.coli*, *Klebsiella* spp. are reported for the first time. No previous report on the antibacterial activity of these species could be found in the literature. These microbial studies showed the most promising antimicrobial properties indicating the potential for the discovery of

new novel drugs from plants. Further NMR studies are required to determine the structure of active compounds responsible for the antibacterial activity of the piper betle and to development of new formulations are required. This plant could serve as useful sources for new antimicrobial agents. This plant provided as source of inspiration for the novel drug development. Phytomedicine can be used in treating of diseases as done in Unani and Ayurveda systems of medicines⁹. These findings on antibacterial activity support the claim of traditional healers that P.betle would be used against uropathogenic *E.coli*, *K.pneumoniae* and *K.oxytoca*. Hence in search of novel antimicrobial agent, the formulation of this extract may be used.

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