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The Potential Activity of Hydroxychavicol against Pathogenic Bacteria

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Abstract

Hydroxychavicol isolated from Piper betle leaves, showed inhibitory activity against gastrointestinal pathogens. It exhibited inhibitory effect on the entire gastrointestinal pathogens tested (MICs of 200-400 µg/ml) with an MBC, which was twofold greater than the inhibitory concentration. Hydroxychavicol exhibited concentration dependent killing of *Staphylococcus aureus* and *Escherichia coli* to 4 x MIC, that the hydroxychavicol would be a useful compound for the development of antibacterial agents against gastro intestinal pathogens and has great potential for use in treatment of gastrointestinal infections.

Keywords: MIC; Hydoroxychavicol; Time killing curve

Introduction

Infectious diseases account for about half of the deaths globally prior to the discovery of antibiotics. Antibiotics play a major role in treating a number of life threatening diseases. But parallel to antibiotic usage, bacterial resistance to antimicrobial agents has been emerged [1]. Therefore due to alarming increase in the rate of infections with antibiotic resistant microorganism and due to side effects of some of synthetic antibiotics there is an increasing interest in medicinal plants as a natural alternate to synthetic drugs [2]. The gastrointestinal tract is a major ecological site for various bacteria that can reach neighbouring sterile sites and cause invasive diseases. Human gastrointestinal tract is an important reservoir of multiple bacteria and there is evidence that it also provides an important source for transmission and dissemination of these organisms [2].

Escherichia coli is found widely in nature, including the intestinal tracts of humans and warm-blooded animals. Disease-causing strains, however, are a frequent cause of both intestinal and urinary-genital tract infections. *E.coli* causes severe cramps and diarrhoea.

SalmoEscherichia coli hogenic bacteria predominately found in the intestinal lumen. Its toxicity is due to an outer membrane consisting largely of lipopolysaccharides (LPS) which protect the bacteria from the environment. In the last 30 years, several reports of outbreaks of *Salmonella* gastroenteritis in hospitalized patients have been published [3-10].

Streptococcus pyogenes is a frequent human pathogens capable of producing a wide variety of infections which range from suppurativesequelae like pharyngitis, impetigo, streptococcal toxic shock-like syndrome (STSS), necrotizing fasciitis to more severe and life-threatening post streptococcal nonsuppurativesequelae like acute rheumatic fever (ARF), acute glomerulonephritis (AGN). In past decade, there has been an increase in reports of serious streptococcal infections and the sequelae worldwide [11]. In India, prevalence of rheumatic heart disease and pharyngitis varies from 1 to 5.4/1,000 [12] and 4.2% to 13.7% [13,14] school-age children respectively, which is comparable to the rates reported from developed countries [15].

Staphylococcus aureus and the coagulase negative *Staphylococcus* species (CoNS) are among the most common causes of mortality and morbidity in the hospital setting worldwide. About nearly 20% of the human population are long-term carriers of Staphylococcus aureus causing a range of illnesses such as pimples, impetigo, boils cellulitis folliculitis, carbuncles, scalded skin syndrome, and abscesses to life threatening diseases like pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome (TSS), bacteremia, and sepsis. It is still one of the most common causes of nosocomial infections [16], often causing postsurgical wound infections.

Shigellosis still remains a public health problem in most developing countries because of the poverty, poor sanitation, personal hygiene and poor water supply [17]. Literature review shows that about 140 million people suffer from shigellosis with estimated 600,000 deaths per year world-wide [18-20]. It is a major cause of dysentery/diarrhea in children and others. Many of them are hospitalized immediately after the onset of the disease. Though, oral rehydration is the principal means of management, because of the enteroinvasiveness antibacterial treatment may be necessary.

Presently many plant components have been extracted, analysed and are being synthesized in large laboratories for use in pharmaceutical preparations. Piper betel plant which being used in the present investigation is one of the important medicinal plants.The Betel (Piper betel) belongs to the Piperaceae family the betel vine, dioecious creeper represent goodness of wealth in Hindu mythology.In India Piper betleleaves are used for chewing and to improve appetite [21,22]. Many scientific research have been carried out and various beneficial bioactivities were discovered in this plant including antimutagenic, anticarcinogenic, antioxidant, antidiabetic, and anti-inflammatory [23-27].

So far less data about the antibacterial activities of these plants

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were reported. In the present study antibacterial activities of the hydroxychavicol was investigated.

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 a150ml of chloroform as a solvent for 2days.The plant extracts were filtered through what man No.1 filter paper into vials and stored at 4°C for further use.

Bacterial strains and culture condition

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

Escherichia coli, *Salmonellatyphi*, *Shigella dysentrie*, *S. aureus*, *S.pyogenes*, *Pseudomonas aeruginosa*. All the gram positive bacteria were maintained on Blood agar before processing and gram negative bacteria maintained on Nutrient agar before to process.

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 crystalized from benzene petroleum ether. And the purity of the hydroxychavicol is estimated by the HPLC and found 98% pure.

Screening for the antibacterial activity of hydroxychavicol

The bacterial cultures were grown in peptone water medium and incubated at 37°C after 6hrs of growth, bacteria were at a concentration of 106 cells/ml were inoculated on the surface of Mueller-Hinton agar plates subsequently, filter paper disc(6mm in diameter) saturated with extract (50µg/ml) was placed on surface of each inoculated plate. To evaluate the efficiency of the methodology, 50µl of the extract was inserted simultaneously in a hole made in newplates. The plates were incubated at 37°C for 24hrs and inhibition zone was observed. Cultured bacteria with halos equal to or greater than 7mm were considered susceptible to the hydroxychavicol. The hydorxychavicol dissolved in 2% DMSO. 2% DMSO served as a negative control. The hydroxychavicol was later tested to determine the Minimal Inhibitory Concentration (MIC) for each bacterial sample.

Determination of Minimum Inhibitory Concentration

The antibacterial studies of hydroxychavicol was analysed 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 $1.5x10^8$ CFU/ml. The twofold serial dilutions of hydroxychavicol was prepared in Muller Hinton broth, 100µl of the bacterial inoculum was added to each well of the plate, resulting in a final inoculum of $5x10^5$ 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 inoculum 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 24h. The minimum concentration of compound that showed ≥99.9% reduction of the original inoculum was recorded as the MBC.

Time kill studies against *E.coli* and *S.aureus*

E.coli and *S.aureus* were grown in nutrient broth at 37°C for 24hrs. The turbidity of the suspension was adjusted to 0.5McFarland standard in sterile normal saline, 20µl of this suspension was inoculate 2ml of Nutrient Broth containing increasing concentration of hydroxychavicol ranging from 100 to 1000µg/ml. Dimethysulfoxide controls were also included in the study. Suspension were incubated at 37°C, and the number of colony forming units (CFU) was determined on Luria Bertani Agar plates using a serial dilution method at various time point [29].

Results

The antibacterial activity of chloroform extract was examined byAgar cup plate method. The chloroform extract of the piper betle exhibited a potent antibacterial activity towards all the bacteria.

To evaluate the antimicrobial activityof hydroxychavicol, it was examined by the micro broth dilution assay against microorganisms the MIC of the hydroxychavicol range from 800-200µg/ml against tested pathogenic bacteria. *S.aureus* and *S. pyognes* showed potent and broad-spectrum antibacterial activity (i.e 200µg/ml) against hydroxychavicol. *E.coli*, *Salmonellatyphi* and *Shigelldysentriae* showed activity (i.e 400µg/ml) towards a higher concentration of hydroxychavicol. MIC was determined and the results were showed in (Table 1). Hydroxychavicol are effective on gram positive bacteria compare to the gram negative bacteria and activity of hydroxychavicol was not observed in *pseudomonas aeruginosa*.

Minimum Bactericidal Concentration was determined and the concentration for the *S.aureus* and *S.pyogenes* ranges from 400µg/ml and MBC for *E.coli*, *Shigelladysentria*, and *Salmonella typhi* ranges from 800µg/ml.

Time-kill studies

The time-kill kinetics studies were specifically performed against *S. Aureus* and *E.coli* owing to its importance in the initiation of infections. The results of the time-kill studies are shown in (Figure 1). The MIC of hydroxychavicol (200µg/ml) showed a 3-log reduction in growth in10hrs, compared to the untreated control, while 400µg/ml and 800µg/ml reduced the CFU count of *S.aureus* with in short period of time. The results of the time-kill studies are shown in (Figure 2). The MIC of hydroxychavicol (400µg/ml) showed a 3-log reduction in growth in 10hrs, compared to the untreated control, while 400µg/ml and 800µg/ml could reduce the CFU count of *S.aureus*ATCC. The kill kinetics study showed that hydroxychavicol exhibited a time- and concentration-dependent killing effect against *S. aureus* and *E.coli*.

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Bacterial strains	MIC µg/ml range	MBC µg/ml range
Staphylococcus aureus	200	400
Streptococcus pyogenes	200	400
Escherichia coli	400	800
Salmonella typhi	400	800
Shigelladysentriae	400	800
Pseudomonas aeruginosa	-	-

Table 1: Antimicrobial activity of hydroxychavicol against different pathogens.

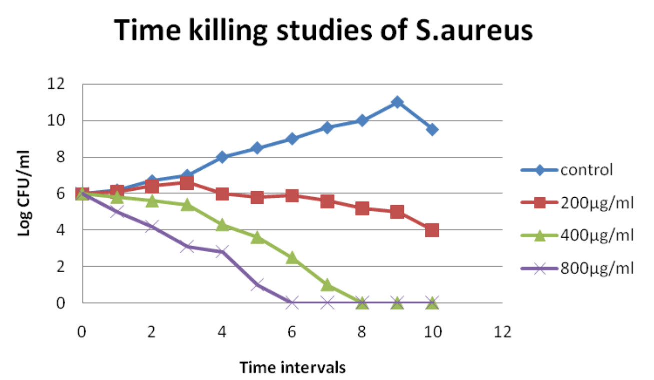

Figure 1:

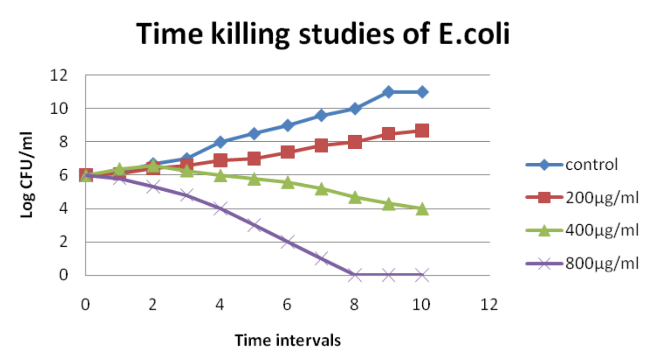

Figure 2:

Discussion

Hydroxychavicol isolated from the piper betel was tested against the pathogenic microbes for its inhibitory effect. Hydroxychavicol demonstrated the bacteriostatic activity and bacteriocidal activity towards five bacterial species except the *pseudomonas aeruginosa*.

In India betle leaves were extensively consumed and hydroxychavicol is one of the major constituents of *P.betle*. The first preliminary report of antimicrobial activity of Piper betle extract was demonstrated against bacteria30. The antibacterial properties of Piperbetle L. against all the pathogens, *Ralstonia, Xanthomonas*, and *Erwinia* and found that the hydroxychavicol present in the plant play a major role for antimicrobial activity. The activity of piperbetle extract on bacteria by using the crude aqueous extract of Piper betle on *streptococcus* mutans and found that theextract causes plasma cell membrane damage and coagulation of the nucleoid31. The bacteriostatic effect of Piper betle and Psidiumguajava extracts were detected on Dental plaque bacteria and found the activity at 4mg/ml against the *S.mitis*, *S.sanguinis* and less acivity against actinomyces spp [32]. The hydroxychavicol from chloroform extract of piper betle leaves and showed inhibitory activity against oral cavity pathogens [33].

In our study we demonstrated that hydroxychavicol present in the betle leaves has MICs of 200µg/ml for *S.aureus* and *S.pyogenes* for *E.coli*, Salmonelltyphi and Shigelladysentriea MICs is of 400µg/ml.

Piper betle showed a remarkable antibacterial activity on almost all test organisms except Pseudomonas aeruginosa, in accord with Lirio and Chanda and later in our study on the endophytes of the piper betle we identified that the pseudomonas aerugniosa is one of the endopyte present in the plant.

Conclusion

Piper betle leaf has a significant antimicrobial activity against broad spectrum of micro organisms (gram postivie bacteria dn gram negative bacteria). Locally available and easily cultivated. The antibacterial activity of hydroxychavicol against *E.coli*, *Shigella dysentrie*, *Salmonella typhi, S.aureus* and *S.pyogenes* 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 of hydroxychavicol showed the most promising antimicrobial properties indicating the potential for the discovery of new novel drugs from plants. Further phytochemical studies are required to determine the types 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.

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