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Research Article

IN VITRO ANTIMICROBIAL ACTIVITY OF THIOPHENE DERIVATIVE PITC-2 OF PLUCHEA INDICA AND ITS MECHANISM OF ACTION

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ABSTRACT

Extracts of various parts of plants have been used traditionally as component medicines for many years by several communities throughout India. With this background the plant *Pluchea indica* was selected and a pure compound was extracted from the roots of the plant with the help of methanol following an elaborate procedure. Purity of the compound was confirmed by NMR and was labeled as PITC-2. Antimicrobial action of this specific agent was determined by agar dilution technique with the help of standard international methods; the action was compared with the penicillin and streptomycin. PITC-2 was found to inhibit the action of DNA polymerase which was confirmed by agarose gel electrophoresis.

Keywords: Antimicrobial activity, *Pluchea indica,* DNA synthesis inhibition, PITC-2

INTRODUCTION

Antibiotics and different types of antibacterial chemotherapeutics have definite therapies for the treatment of many diseases caused by several species of microorganisms during the last seven decades. Such achievements have simultaneously presented the inevitable threat of drug resistances among the microbial pathogens. It may be pointed out that the frequency and spectrum of multidrug resistant pathogenic bacteria have increased immensely during the past few decades (Cohen,1992). The species of *Enterococcus,Psedomonas, Acinatobacter, Spreptococcus,Staphylococcus* and *Mycobacterium* are now resistant to almost all the existing therapeutic agents including antibiotics (Spratt,1994). Under such circumstances there is an urgent need to meet the challenges that are being caused by the immergence of multidrug resistant infectious agents. From time immemorial human beings have practiced plant based therapy for different types of ailments. It may be mentioned here that traditional medical systems based on plant products are still continued as folk medicine among different tribes in India and various other countries of Asia,Africa and South America. Ayurveda is the science of life and is believed to be the oldest healing science in existence among Asians. In ayurveda about two thousand plant species have been considered as potential source of medicines. According to World Health Organization (WHO), medicinal plants are the best sources for evaluating variations in formulation of drugs. Hence investigations may be carried out with the help of plants for evaluating their properties,safety and efficacy[3]. However provision of scientific background in support of folk medicines and identification of a rather cheap therapeutic substance from the plants with least side effects have become essential now. Thus efforts are required to determine the antimicrobial property of a defined substance obtained from a plant in-vitro and then establish the exact mechanism of action at molecular level. The present study describes the antibacterial action of the thiophene derivative termed as PITC-2 obtained from the roots of the plant *Pluchea indica.* This plant was selected on the basis of earlier studies by Biswas et al (2005)[4].

MATERIALS AND METHODS

Bacteria

A total of 22 bacterial isolates belonging to gram positive and gram negative bacterias were tested. The strains were obtained from the Department of microbiology, Burdwan, West-Bengal.

Collection of plant and preparation

Pluchea indica (L.) Less.of the family (Asteraceae) is a large, evergreen shrub found abundantly in salt marshes and mangrove swamps in Sunderbans (India), Bangladesh, Myanmar, China, Philippines, Malaysia, other Tropical countries of Asia and Australia.

Chemicals

Chemicals and reagents used for the study of sub-acute toxicity study were purchased from Sigma-Aldrich (USA) while other chemicals, solvents and reagents used in chromatography were purchased from Merck, India.

Isolation of PITC-2 (a thiophene derivative) from *Pluchea indica*

Plant material- The roots of tissue cultured *P.indica*, (8 months matured) based on the protocol (Pramanik et al.,2007)[5],were separated, washed, oven-dried at 60ºC, powdered by micro pulverizer and sieved through 100 meshes (0.0254 cm diameter). Fibres and unwanted debris were discarded after sieving. The powder was preserved in an airtight container for further use. The plant was taxonomically identified and authenticated by Botanical Survey of India, Shibpur, West-Bengal, India.

Extraction and isolation

The pulverized powder (500gm) was soaked overnight with petroleum ether (Merck) at 60ºC-80ºC for synthesis, dried at room temperature and was extracted with methanol solvent using a soxhlet extractor to obtain the methanolic root extract of tissue cultured *P.indica*. The solvent was then evaporated under reduced pressure using a rotary evaporator (Model no. HS-2005V) to obtain a semisolid residue. The yield of the extract was 8.7% (w/w).A portion (43.5gm) of the crude methanolic extract was partitioned between n-butanol and water. The butanol and water fractions were separately evaporated.The butanol fraction was then shaken with ethyl acetate to obtain an ethyl acetate soluble part and ethyl acetate insoluble part. The ethyl acetate soluble part was concentrated in a rotary vacuum evaporator and then dried to obtain a crude residue, 10 gms of which was subjected to column chromatography using silica gel (60-20 mesh,300 gms) as an adsorbent using petroleum ether-ethyl acetate mixtures of increasing polarity as eluants. About 10 ml of fraction were collected and mixed on the basis of their TLC behaviour. Elution with petroleum ether, ethyl acetate mixture (6:4,4:6) afforded the fraction A (1.5 grams) with trace materials in the TLC chamber 1(benzene:chloroform :ethyl-acetate=6:3:1).Fraction-A was further chromatographed over silica gel (45 grams) yielded a yellowish solid named B(0.8 grams) in the chamber1 while eluting with petroleum ether:ethyl acetate (8:2,7:3). This B fraction was further chromatographed over silica gel (24grams) by elution with petroleum ether:ethyl acetate (8.5:1.5, 8:2) to yield a single prominent spot which was further characterized by NMR, IR and MASS spectra studies that finally yielded PITC-2.

Antibiotics

The antibiotics benzyl penicillin and streptomycin were obtained as pure, dry powder from their manufacturers in INDIA.

Media

The liquid medium used for this study was Mueller Hinton broth (MHB,Himedia). Solid medium was Mueller Hinton agar (MHA) obtained by solidifying the liquid medium with 1.2% (w/v) agar (Himedia). The pH was maintained at 7.2/7.4 for both the media.

Determination of Minimum Inhibitory Concentration(MIC) of PITC-2 and antibiotics

PITC-2 was added at concentration of zero(control),10,25,50,100 and 200 µg/ml in the molten MHA and poured into petridishes according to CLSI guidelines (2009)[6]. The antibiotics penicillin and streptomycin were added at concentration of zero (control),2,5,10,15 and 20 µg/ml in the molten MHA as described above for PITC-2. The organisms were grown in MHB for 18 hours and harvested during the stationary growth phase. A direct suspension of the organisms were prepared in 5ml sterile distilled water. The turbidity of the suspension was adjusted to match a 0.5Mc Farland's standard (20) with a spectrophotometer (Chemito UV2600 double beam UV-VIS spectrophotometer,Mumbai,India) at 625nm which corresponded to $2.4x10^8$ colony forming units (cfu)/ml. These were spot inoculated on to the MHB plates containing increasing amounts of the drug, including a control. The plates were incubated at 37°C, examined after 24 hours and incubated further for 72 hours, where necessary. The lowest concentration of the drug in a plate that failed to show any visible microscopic growth was considered as its MIC.

Mechanism of antibacterial action of PITC-2

The MIC of PITC-2 against *S.aureus* 342 was found to be 25 µg/ml. At the logarithmic growth phase of the cultures, the cfu counts of the strains were taken and twice the MIC of PITC-2 (50µg/ml) was added to each culture. Subsequently these cfu counts of the cultures were obtained after 2,4,6 and 18 hours after adding ther drug.

Agarose gel electrophoresis

Plasmid DNA was isolated from *E.coli* 22 following standard procedure (Birnboim,1983)[7]. The extracted DNA was mixed with 2 different concentrations of PITC-2,5 µg/ml and 15 µg/ml in two eppendrof tubes. A part of the DNA was heated and to the heated single stranded DNA were added DNA primer, template DNA, DNA polymerase and dNTPs in a separate eppendrof tube. In the same way a part of the plasmid DNA was treated with all the above mentioned four elements plus PITC-2. The treated DNA samples along with the original plasmid DNA were mixed with the loading dye and 20 µl of each sample with the dye was loaded in to agarose gel trough containing 0.7% agarose (Sigma). A gel was run for 45-60 minutes and the gel was stained with ethidium bromide solution, destained with distilled water and placed under Bio-Rad gel docking system for detecting presence or absense of plasmids (Tambekar et al,2007;Baserisalehi and Bahador,2008[8].

RESULTS

Antimicrobial activity of PITC-2 and antibiotics

Among the six strains of *S.aureus* the MIC varied between 2 µg/ml and 10 µg/ml with respect to penicillin, while the MIC of streptomycin in the bacteria was between 5-10 µg/ml level. The same organisms could be inhibited by PITC-2 at 25 µg/ml to 100µg/ml level. In this way it was found that most of the test organisms had MIC values with respect to penicillin and streptomycin were distinctly low. However strains of *bacillus subtilis*, *Shigella* and *vibrios* were sensitive to PITC-2. It may be noted that strains of *S.aureus* and *E.coli* were much less sensitive to PITC-2. Bacteriostatic action of PITC-2 at the logarithmic growth phase of the culture of *S.aureus* 342, the number of variable cells were $2x10^8$. Subsequently the number of variable cells were $6.0x10^5$ after 2 hours,$5.2x\ 10^4$ after 4 hours,$9.6x10^2$ after 6 hours and $4x10^2$ at the end of 18 hours.

Characterization of PITC-2 by NMR and IR

PITC-2 was crystallized from C_6H_6 as light yellow needles (80 mg) and it showed green colour with Liebermann-Burchard reagent. The melting point of the compound was recorded to be 106 – 108°C. The sample PITC-2 in IR showed ν_{max}^{KBr} cm^{-1}: 3328 (br), 3104, 2956, 2923, 2872, 2150, 1778, 1451, 1322, 1186, 1080 (s), 1022, 946, 864, 805 (m), 688. The compound showed IR absorptions for alcoholic groups (strong bonds at 3328 and 1080 cm^{-1}). Small but significant peaks at 3104 and 2150 cm^{-1} were indicative of an unsaturated system with a triple bond. EI-MS showed signals at *m/z* (rel. abundance %): 230 (M^+, 90), 199 (100), 171 (33), 170 (32), 169 (33), 145 (22), 139 (20), 127 (50). The mass spectrum showed a strong molecular ion peak at *m/z* 230, the base peak at *m/z* 199 and another strong peak at *m/z* 169 which agreed with the presence of a CHOH-CH_2OH moiety. The In ^{1}H NMR δ^{TMS} ($CDCl_3$, 300 MHz): 1.64 (D_2O exchangeable, OH merged with solvent H_2O), 2.04 (3H, s), 3.78 (1H, dd, J=11.4, 6.3 Hz, H_A of CH-C<u>H</u>$_2$OH), 3.82 (IH, dd, J=11.4, 3.9 Hz, H_B of CH-C<u>H</u>$_2$OH), 4.69 (1H, dd, J=6.3, 3.9 Hz, C<u>H</u>OH-CH_2OH), 7.04, 7.18 (2H, m, thiophene-H). The ^{1}H NMR spectrum showed peaks for a methyl attached to unsaturation (δ 2.04, s), a CHX-CH_2Y unit and an aromatic system. Finally the compound was identified as 2-(prop-1-ynyl)-5-(5,6-dihydroxyhexa-1,3-diynyl)-thiophene **(Fig. 1)**.

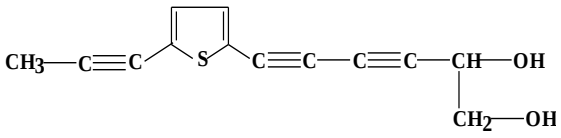

Figure 1: PITC-2, 2-(Prop-1-ynyl)-5(5,6-dihydroxyhexa-1, 3-diynyl)-thiophene

DISCUSSION

A pure compound was obtained through the elaborate extraction procedure of *Pluchea indica* root, that was grown in a tissue culture system. The compound was studied extensively for its antimicrobial potentiality with the help of 22 bacteria belonging to 2 gram-positive and 4 gram-negative types. Further studies revealed that PITC-2 is a bacteriostatic agent. The elaborate studies on its exact chemical nature showed that it is a thiophene derivative. In the data presented in Fig2 it was noted that the extracted plasmid DNA produced a distinct band in the electrophoresis system Fig2. The DNA was totally damaged by heating at 80°C and there was a definite action of 15µg/ml of PITC-2 on this DNA since there was no visible band at the same position(Fig 2). However addition of dNTPs, DNA polymerase, DNA primer, template DNA to the heated DNA helped to produce a distinct band at the same place. The same band was again inhibited by addition of 15µg/ml of PITC-2 (Fig2). A very faint band was observed in lane 3 was after addition of 15µg/ml of PITC-2 in the reconstructed DNA. In this way it may be concluded that PITC-2 was not only an active antibacterial agent but also is able to interfere and restrict the action of polymerase chain reaction for rebuilding of new DNA strands (Fig 2).

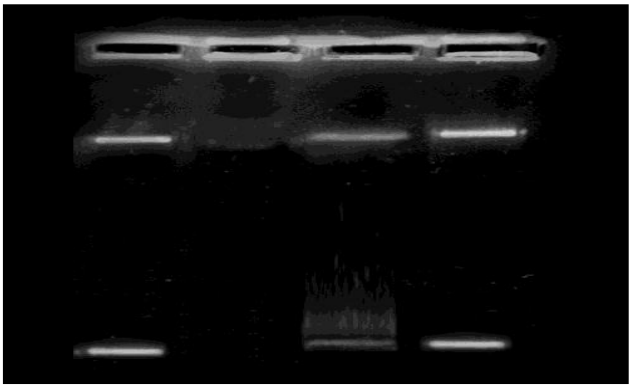

Figure 2:Gel picture showing the absence of DNA bands in presence of the drug PITC-2

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