Pakistan Journal of Pharmaceutical Sciences
6(1) 61-66 (1993)

AN ANTITUMOR CARDENOLIDE WITH INHIBITORY ACTIVITY AGAINST *PSEUDOMONAS PSEUDOMALLEI*

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ABSTRACT

The antibacterial activity of a new cardenolide, 7B, 14B - dihydroxy-5-card-20(22) enolide has been studied and it has been found to be active against Pseudomonas pseudomallei. The new cardenolide also exhibited antitumor activity in a potato disc assay

INTRODUCTION

Calotropis procera is a succulent, erect shrub occurring wild in Asia and Africa, which has medicinal use (Akhter *et al.*, 1992). A literature survey of Calotropis procera revealed that the plant contains many cardenolides (Allen and Sprunt, 1978), besides steroids and triterpenes (Dymock, 1883). From the hexane insoluble fraction of this plant a new free cardinolide named proceragenin has been isolated and its struture was elucidated through spectral studies (Akhter *et al.*, 1992). The medicinal importance of Calotropis procera prompted us to carry out antibacterial activity of proceragenin. It was found to be active against *Pseudomonas pseudomallei a* causative agent of melioidosis.

MATERIAL AND METHODS

Isolation Procedure

The freshly collected plant material (94 kg) was extracted with MeOH at room temperature.The methanolic extract was evaporated under reduced pressure and the gummy-residue thus obtained was partitioned between $CHC1_3$ and H_2O. The $CHC1_3$ soluble fraction was evaporated and divided into hexane soluble and insoluble portions. The hexane insoluble portion was chromatographed over silica gel and eluted with various solvent gradient of increasing polarity. The eluate from $CHCI_3$ - MeOH (95:5) was further subjected to preparative TLC on silica gel plates using solvent system hexane-chloroform (1:9). Proceragenin was crystallized from methanol (15 mg), m.p 254 - 255.

Pseudoniadei Antibacterial Activity

The antibacterial activity of proceragenin was determined by Agar well diffusion method (Jailer *et al*, 1988). One loop full of a 24 hour old culture containing $l0^4$ - 10^6 cells was spread on the surface of the Mueller-Hinton Agar plates. Welk were dug in the medium with the help of a sterile borer. Stock solution of the pun compound, 2 mg/ml was prepared in dimethylsulphoxide (DMSO). Dilutions of the stock solution containing 50, 100 and 200ug were prepared in DMSO. 100 ul of each dilution was added to the respective wells. Control well recieved only 100 ul of DMSO (Kazmi *et al*, 1991). The antibacterial activity of proceragenin was compared with different standard antibiotics (Table 1). A loop full of each group of organism was spread on seprate Mueller-Hinton Agar plates , discs of antibiotics were placed Plates were incubated at 37C for 18 - 24 hours and zone of inhibition was measured in mm.

Determination of MIC and MBC

Minimum inhibitory concentration (MIC), was determined by the tube dilution method using Mueller-Hinton broth (Allen and Sprunt, 1978). The tubes were inoculated with susceptible organisms which was found to be sensitive to proceragenin by the agar well diffusion method and incubated at 37C for 24 hours. After the incubation period, culture was diluted upto 10_{-8} CFU/ml, 0.1 ml of the diluted culture was added into the tubes having different concentrations of the compound and then 0.8 ml of Mueller-Hinton broth. Tubes *were* incubated at 37C for 18 - 24 hours. Results were recorded after the incubation period. Bactericidal and bacteriostatic concentration of the pure compound was determined by diluting 1 ml of culture from MIC tube as well as 1 ml from the tubes above and below the MIC level. The culture was washed three times with thioglycolate broth, 0.1 ml of a washed broth culture was transferred to a sterile petriplate mixed with 20 ml of Mueller-Hinton Agar and incubated at 37C for 18 - 24 hours (Akhter *et al.*, 1992). Appearance of growth was recorded after incubation.

Antitumor Activity

Growth medium *f*or *Agrobacterium tumefaciens* was prepared by adding 0.5 g sucrose (Merck), 0.8 g nutrient broth (Difco) and 0.1 g yeast extract (Difco) to 100 ml of distilled water in a 250 ml flask. This medium was autoclaved for 15 minutes at 121C . One loop full of A. mmefaciens was inoculated and incubated at 27C for 48 hours in a shaking water bath. Bacto agar (1.5 gm/100 ml) was prepared for each sample and after sterilization it was poured in sterile petriplates and allowed to solidify. Red skin potatoes were washed first with tap water and then soaked in bleach solution (chlorox) for 30 minutes. 4 mg of sample was dissolved in 1 ml of DMSO and lml of DMSO was taken as blank standard. Samples were prepared by adding 1.5 ml water, 2.0 ml bacteria and 0.5 ml sample to the *scr*e*w* capped tubes. The blank standard was prepared by replacing the sample with 0.5 ml of DMSO. Potatoes were taken out from the bleach and

Table 1
Antibacterial activity of proceragenin.

Organism	Concentration (μg/100 ul) of proceragenin used with the zone of inhibition (mm)			Control (DMSO) (ul)	Response
	50	100	200	100	
Micrococcus luteus	+	19	21	+	Bacteriostatic
Aeromonas sobriae (Arancase No. 15)	+	+	35	+	Bacteriostatic
Aeromonas caviae (Arancase No. 10)	+	20	22	+	Bacteriostatic
Pseudomonas pseudomalliae	+	21	25	+	Bacteriostatic
Escherichia coli (N-97-4)	+	+	29	+	Bacteriostatic
Streptococcus fecalis	+	+	30	+	Pacteriostatic
Vibrio cholarae (N.C-58	+	+	22	+	Bacteriostetic
Klebsiella peumoniae (D-671)	+	+	32	+	Bacteriostetic
Streptococcus agalactiae	+	20	30	+	Bacteriostetic
Corynebacterium pseudo-diphthericum	+	+	30	+	Bacteriostetic
Corynebacterium diphtheriae	+	+	28	+	Bacteriostetic
Bacillus subtilis	+	+	18	+	Bacteriostetic

cylindrical pieces of potatoes were bored out with the help of a sterile cork borer. Discs of potatoes were made by cutting cylindrical pieces of potatoes with the help of a sterile knife. In each solidified bacto agar plate five discs of potatoes were placed with the help of sterile a forcep. One drop (0.05 ml) of inoculum per disc was added and the plates were sealed with the parafilm strips and finally wrapped in tin foil. All the plates were incubated in dark at 27^0C for 12 - 21 days (Ferringi *et al*, 1982) After the incubation period percent inhibition of crown gall tumors was calculated using the following formula :-

$$\% \text{ Inhibition} = 100 - \frac{\text{Average no. of tumors of sample}}{\text{Average no. of tumors of control}} \times 100$$

RESULTS AND DISCUSSION

Cardenolide is a modified steroid with a β-unsaturated ketone system instead of a side chain (betenolide ring). Pseudomonal infections occur in debilitated, burned or inununosuppressed individuats. Proceragenin was found to be active against *Pseudomonas* pseudomallei and other gram negative organisms (Table 1) and showed no activity against strains of *Pseudomonas aeniginosa.* Hence proceragenin can be developed into a diagnostic disc to differentiate Pseudomonas pseudomallei from *Pseudomonas aeruginosa.* Proceragenin was found to be active against *Pseudomonas pseudomallei* showing a zone of inhibition of 21 mm and 25 mm with 100 µ*g* and 200 µg per well respectively (Table 1). The zone of inhibition of the standard antibiotics (30 Ug) for this organism was smaller as compared to proceragenin. However, no inhibition could be observed below 50 µg for proceragenin. The MIC of this susceptible organism was 90 µg and it had a bacteriostatic effect whereas the MIC of other organism ranged between 100 and 150 µg (Table 1). *Pseudomonas pseudomallei* is a gram negetive, lactose fermentig, motile rod which causes mehoidosis or pseudoglanders, an infectious disease of rodents in India and South East Asia, which can be transmitted to man. The charecteristic lesion is a small caseous nodule, found generally throughout the body, involving skin, heart, lungs, liver, bone, joints, lymph nodes and eyes. Latent infections, laboratory acqu[i]red infections and nosocomial infections are also found. The nodules then break down into an abcess. The symptoms vary accord[i]ng to the body area or organs involved. Proceragenin can be used to treat infections due to this highly infectious zoonotic agent of human disease if found non-toxic in human clinical trials.

A. turnefaciens causes tumor in plants, it is a gram negative bacterium containing Ti (Tumor inducing) plasmids which carry genetic information (T - DNA) that transforms normal plant cells into autonomous tumor cells. Plates were observed after 21 days for tumor formation. Since the tumors formed were not too prominent, the plates *were* reincubated for another week and were observed after one month. After an incubation of one month 5 tumors developed in the proceragenin supplemented agar plates whereas the number of tumors observed in the control plates with no proceragenin *were* 20 (Figure 1). The percentage of tumor inhibition was calculated by the formula mentioned in the Materials and Methods.

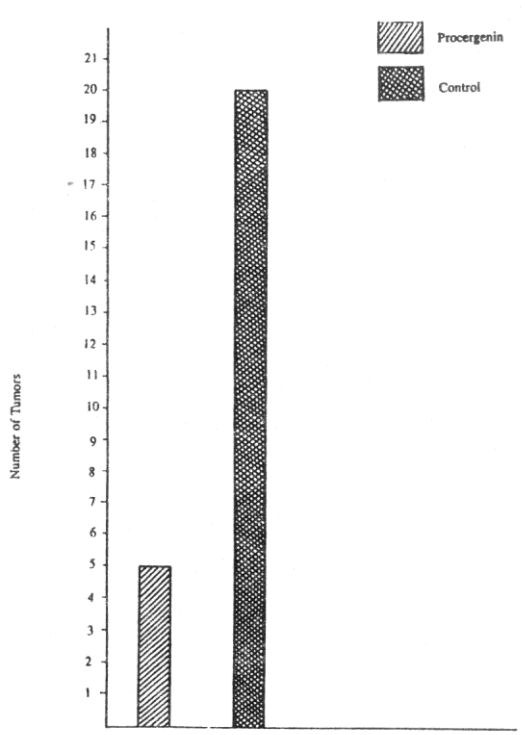

Figure 1. Antitumor activity of proceragenin from *Calotropis procera*

It has been reported that Quassia amara showed 45% inhibition (Pierre *et al,* 1980) and Tabernaemontana arborea showed 36% inhibition in tumor formation (Kingston, 1978). Whereas the percentage of tumor inhibition on potatoes in presence of proceragenin was found to be 75%, indicating that proceraginin can be used as a potential antitumor compound It is yet to be established whether procerageoin is active in the 3PS cell line (in viva, mouse, leukemia) antitumor assay.

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