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NEW BIOACTIVE COMPOUNDS OF PLANT ORIGIN

SHAHANA U. KAZMI[1], SAMINA NOORALI[1], RYED A JAMAL[2] and ATTAUR- RAHMAN[2]
[1]Department of Microbiology, University of Karachi, Karachi-75270, Pakistan.
[2]H.EJ. Research Institute of Chemistry, University of Karachi, Karachi-75270, Pakistan.

ABSTRACT

Crude extracts of *Withania somnifera* Ws and *Polygonum equisetiforme* (PE) as well as the pun compounds isolated therefrom were screened for antibacterial and antifungal activity against 20 bacterial and 17 fungal cultures. The crude extract of PE and WS inhibited the growth of *T. mentag*rophyte, *M. canis* and *A. boydii* at an MIC of 450.500 item) whereas the pure compounds inhibited the growth at MIC of 300-350 pg/ml. Species of Corynebacterium, Bacillus, Streptococcus and Streptococcus were found to be highly susceptible to both the crude and the pure compounds. MIC values of both crude extracts for different organisms tested were found to be higher (200-350 Mg/ml) than the pure compounds (150-170 Mg/ml). The crude extract of It did not inhibit the growth of *Ps. aeruginosa,* however, the pure compound was found to be bacteriostatic. Brine shrimp and BALE/c mice lethality test indicated that these extracts may be toxic at high concentration.

Introduction

Although Pakistan is endowed with a wide variety of medicinal plants, very little effort has been made so far towards a bioassay directed isolation of biologically active compounds from indigenous plants. It is desirable to exploit the therapeutic potential of higher plants to get new, less toxic, less expensive and more effective drugs, in view of the escalating cost of synthetic antibiotics and an emergence of antibiotic resistant strains. We report studies on the antibacterial and antifungal activity of the crude extracts of *Polygonum equisetiforme* and *Withania somnifera* as well as their pure compounds. These substances were also screened for animal toxicity and cytotoxicity. One of the plants used in this study was *Withania somnifera* (WS) which belongs to the family Solanaceae and is commonly found in India, Pakistan, Iraq, Syria, and Turkey (Yasin *et al,* 1985; Jafri, 1966). A number of reports describe the therapeutic use of the products derived from this plant such as the treatment of carbuncles, rheumatism, for loss of memory and lumber pains (Nadkarnis, 1976). The other plant extract which was screened for its antibacterial activity was *Polygonum equisetiforme* (PE) which belongs to the family Polygonaceae. It is mainly found in Philistean, Upper and Lower Jordan Valley and in the Dead Sea area (Zohary, 1966) but the therapeutic use and biological activity of

PE is not known. Other species of this genus have been used in the treatment of bronchitis, whooping cough, colic pains, snake bites, jaundice and penumonia (Naryan, 1986). The crude extract of *Withania somnifera* and *Polygonum equisetiforme* and the respective pure compounds, 2,3-dihydrowithanferin-A and a new diterpene, were tested for antibacterial and antifungal activity against local clinical isolates and standard ATCC bacterial and fungal cultures. Crude extracts of both the plants were also tested for their toxicity in BALB/c mice and Brine shrimp lethality assay.

Materials and Methods

Butanolic extract of *Polygonum equisetiforme* and chlorformic extract of *Withania* somnifera were prepared by the standard published procedure (Baba *et al*, 1989).

In vitro antibacterial activity

The antibacterial activity was determined by the Agar Well Diffusion Method proposed by Jaffer *et al.* (1988). Briefly, one loop full of a 24 hours old culture containing approximately $l0^4$ to 10^6 cells was spread on the surface of Mueller Hinton Agar plates. Wells were dug in the medium with the help of a sterile borer. Since the crude extract and pure compounds were soluble in dimethyl sulfoxide (DMSO) only, stock solution of crude extract of both the plants and their pure compounds in concentration of 1 mg/ml was prepared in DMSO. Dilutions of stock solution containing 50, 100 and 200 pg were prepared in DMSO. 100 pi of each dilution was poured in the respective wells. Control wells received only 100 pl of DMSO.

In vitro antifungal activity

Sabroud Dextrose Agar (SDA) slants supplemented with various concentrations of the test substance were prepared by the following procedure. First, melted agar was poured in culture tubes when the temperature of the medium was 45°C, 1 ml of the required concentration of the crude extract or pure compound (100-500 pg) was added to each tube. Control tube received 1 ml of DMSO instead of the test substance. After solidification of the medium, fungal cultures were inoculated with the help of a sterile fungal needle. The slants were incubated at 30°C for 7-10 days (Brass *et al*, 1979).

Determination of MIC, MBC and MFC

Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and Minimum Fungicidal concentration (MFC) of WS, PE and their respective pure compounds, 2,3-dihydrowithaferin- A and *a new* diterpene, (D-1) were determined

by the tube dilution method using Mueller Hinton Broth and Sabouraud Dextrose Broth (Oxoid,U.IC). In case of bacterial cultures the tubes were incubated at 37°C for 18 to 24 hours. Bactericidal / bacteriostatic concentration of each substance was determined by diluting 1 ml of culture from the MIC tube as well as 1 ml from the tubes above and below MIC level. Each of the culture with substance was washed 3 times in thioglycolate broth, 0.1 ml of the washed broth culture was transferred to a sterile petri plate, mixed with 20 ml of Mueller Hinton Agar, and the plates were incubated at 37°C for 18-24 hours. A similar method was followed for fungal cultures but fungal cultures were incubated at 30°C for 7-10 days, 1 ml of the culture broth with substance was diluted and washed in SD broth. 1 ml of washed broth culture was added to sterile plate, mixed with 20 ml of SDA medium and then incubated at 30°C for 7-10 days. After the incubation period the results were recorded (Allen and Sprunt, 1978).

Effect on bacterial and fungal growth

In order to observe the effect on the growth of *Streptococcus agalactiae, Streptococcus fecalis and Pseudomonas aeruginosa* cultures were grown with and without the plant extract and reduction in colony forming units per ml was determined by the agar plate method at an interval of 1 hour for 24 hours (Donachie *et al,* 1973). Effect of PE and WS on the growth of *Trichophyton* mentag*r*ophyte and *Microspore* cards was also monitored by generating growth curves with and without the plant extract by dry weight method (V^{i}ncent, 1958).

Toxicity tests

Each of the plant extract was tested for its toxicity by Brine shrimp and BALB/c mice lethality assay (Meyer *et al*., 1982). In BALB/c mice lethal dose 50 (LD5o) was calculated by the Reed and Muensh method (Reed and Muench, 1938). Six groups of mice were injected intraperitoneally with different concentrations of plant extract. One group of animals received only DMSO while the rest of the 5 groups received different concentrations (WS concentration 10, 50, 100, 150 and 200 mg/kg; PE concentration 2,4,6,8 and 10 mg/kg body weight) of plant extracts. The animals were kept in observation for one week and deaths of animals were recorded each day.

Results and Discussion

Butanolic extract of *Polygonum equisetiforme* (PE), chloroformic extract of *Withania somnife*ra and the pure compounds isolated from this designated as the new diterpene, (D-1), and 2,3- dihydrowithaferin-A respectively were screened for antibacterial and antifungal activity against 20 bacterial and 17 fungal cultures. The crude

extract of PE exhibited antibacterial activity only against gram positive bacteria whereas the pure compound, D- 1, was found to be active against both gram positive and gram negative bacteria including *Pseudomonas aeruginosa,* a very important human pathogen, which was otherwise resistant to as high as 300 pg/m1 of the crude extract. Strains of *Pseudomonas* aeruginosa were also resistant to most of the standard broad spectrum antibiotics like ampicillin (AMP), nalidixic acid (NA), cepthalothin (KF), cephaloridin (CR), erythrocin (ER), tetracycline (TE), velosef (V), kanamycin (K) and chloramphenicol (C). Most of the susceptible strains gave a zone of inhibition of 26-35mm with 150 pg/well of crude substance and produced a similar size of zone with only 100 pg/well of the pure compound (Table 1). The moderate increase in the spectrum of antibacterial activity of pure compound as compared to the crude extract of PE suggests that the active ingredient was some other yet unidentified compound, and that the pure compound isolated was only partly responsible for the activity observed in the extract.

Chloroformic extract of WS showed antibacterial activity against 6 gram positive and one gram negative human pathogen, Pseudomonas aeruginosa. As shown in Table 2, this extract inhibited the growth of species of Corynebacterium, Bacillus, Staphylococcus, Streptococcus and Pseudomonas. 2,3-Dihydrowithaferin-A obtained from WS was more inhibitory against the same bacteria in addition to another very important human pathogen – *strept. agalactiae* which was found to be resistant to high concentrations of the crude extract as well as standard antibiotics like azactam (AZT), nalidix is add (NA), tobramycin (NN), erythromycin (ERY), tetracycline (TE), cephaloridin (CR) and cephalothin (KF). Susceptible strain used exhibited a zone of inhibition of more than 20mm with 100 pg/well of crude substance. With the exception of *Ps. aeruginosa*, all other gram negative bacteria were resistant to more than 2110 pg/well of pure compound as well as crude extract (Table 2). The crude extract of WS was very effective in inhibiting the growth of *Swept fecalis* producing a zone of 36mm at 100pg/ well. In comparison to the crude extract, the pure compound of this plant gave bigger zones of inhibition with most of the susceptible strains tested (30mm zone) at a concentration of 100 pg/well except for *Strept. agalactiae* (18mm, Table 2).

The crude extract of PE was bactericidal for *Strept. agalactiae* at 250 pg/ml (Table 4). A new diterpene, designated as D-1, isolated from the same plant showed a very strong inhibitory activity against *Strept. agalactiae* at a much lower concentration (120 pg/ml). The bactericidal concentration of D-1 for other susceptible bacteria was between 120-170 pg/ml (Table 4).

Table 1

Comparison of antibacterial activity of crude extract of *Polygonum equisetiforme*, new diterpene (pure compound) and different antibiotics

Name	Concentration of crude extract of *Polygonum equisetiforme* (µg/100ml) with zones of inhibition (mm)		Concentration of Diterpene (D-1) (µg/100ml) with zones of inhibition (mm)		Antibiotic Resistance
	100	150	100	150	
C. diphtheriae	24	29	30	40	Resistant to ATM and NA 30
C. pseudodiphthericum	29	34	35	40	Resistant to ATM and NA 30
B. anthracis	25	31	30	37	Resistant to ATM
S. agalactiae	+	26	25	30	Resistant to ATM, NN30, NA30, Erythromycin, CR30, TE50, K30
S. fecalis	+	35	36	41	Resistant to ATM
S.epidermidis	21	27	25	30	Resistant to ATM and Erythromycin
Ps. aeruginosa	+	+	20	25	Resistant to AMPID, NA30, KF30, CR30, ER15, TE5, AMP10, V, K30, C30
B. subtilis	+	+	20	26	Sensitive to all

KEY: + = Growth.

ATM = Azactam
NA30 = Nalidixic Acid
KF30 = Cephalothin
CR30 = Cephaloridin
ER15 = Erythromycin
AMP10 = Ampicillin
K = Kanamycin
C30 = Chloramphenicol
TE5 = Tetracycline

Table 2

Comparison of antibacterial activity of crude extract of *Withania somnifera*, 2,3-dihydrowthaferin-A (pure compound) and different antibiotics

Name of Bacteria	Concentration of *Withania somnifera* extract (μg/100ml) with zones of inhibition (mm)		Concentration of 2,3-dihydro-withferin-A (μg/100ml) with zones of inhibition (mm)		Antibiotic Resistance
	100	150	100	150	
C. diphteriae	26	32	30	35	Resistant to ATM & NA 30
C. pseudodiphthericum	25	30	30	36	Resistant to ATM & NA30
B. anthracis	25	33	30	37	Resistant to ATM
B. subtilis	23	36	35	40	Sensitive to all
S. fecalis	36	42	40	44	Resistant to ATM
S. aureus	15	20	30	35	Resistant to ATM, NA30
Ps. aeruginosa	18	23	30	40	Resistant to NA30, KF30, CR30, ER15, TE5, AMP10, V,K30,C30
S. agalactiae	+	+	15	18	Resistant to ATM, NA30, NN30, Erythromycin, CR30, TE5, K30

KEY: + = Growth

ATM = Azactam
NA30 = Nalidixic Acid
KF30 = Cephalothin
TE5 = Tetracycline
CR30 = Cephaloridin
ER15 = Erythromycin
AMP10 = Ampicillin
K30 = Kanamycin
C30 = Chloramphenicol
V30 = Velosef

The antifungal activity of crude extracts of PE and WS and the pure compounds was determined by growing fungal cultures in Sabouraud Dextrose Agar supplemented with the test substances as well as without the substance. The crude extract of PE exhibited antifungal activity against pathogenic fungi *Trichophyton mentagrophyte* at MIC of 500 pg/ml and *Micros*porum cants at 450 pg/ml (Table 3) whereas the pure compound isolated from the same plant was found to have a strong fungicidal activity at a much lower concentration (300 pg/ml) against the same strains. The crude extract of Withania *somnife*ra exhibited antifungal activity at MIC of 500 pg/ml against 2 pathogenic fungal strains, *Trichophyton mentag*r*ophyte* and Anesthetic *boydii* but 2,3-dihydroxywithaferin-A obtained from it was more potentin killing susceptible fungi at a MFC of 350 pg/ml. The moderate enhancement of antibacterial and antifungal activity observed with the pure compound as compared to the crude extract indicated that other more active substances may be presented in the cured extract.

Table 3

Minimal inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of WS and PE extracts and pure compounds

Extract / Compound	Fungus	MIC μg/ml	MFC μg/ml
Withania somnifera extract	A. boydii	500	500
	T. mentegrophyte	500	500
2,3-dihydro withaferin-A	A. boydii	350	350
	T. mentrophyte	350	350
Polygonum equisetiforme extract	T. mentegrophyte	500	500
	M. canis 450 450		
New diterpene (D-1)	T. mentegrophyte	350	300
	M. canis	350	300

Table 4

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *Polygonum equisetiforme* extract and pure compound, new diterpene (D-1), for different bacterial isolates

Name of the Bacteria	MIC of *Polygonum equisetiforme* (μg/ml) extract	MBC of *Polygonum equisetiforme* μg/ml) extract	MIC of D-1 (μg/ml)	MBC of D-1 (μg/ml)
C. diphtheriae	200	static	170	cidal (150)
C. pseudodiphtericum	200	static	170	cidal (150)
B. anthracic	250	cidal (200)	170	cidal (150)
S. agalactiae	250	cidal (200)	150	cidal (120)
S. fecalis	200	static	170	cidal (150)
S. epidermidis	250	cidal (200)	170	cidal (150)
Ps. aeruginosa	+	+	150	static (150)
B. subtilis	+	+	170	static (170)

Key + = Growth observed.

Table 5

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *Withania somnifera* extrat and pure compound, 2, 3-dihydrowithaferin a, for different bacterial isolates

Name of the Bacteria	MIC of Withania somnifera (*µg*/ml)	MBC of Withania somnifera (*µg*/ml)	MIC of 2,3-Dihydro withaferin-A (*µg*/ml)	MBC of 2,3-Dihydrowithaferin-A (*µg*/ml)
C. diphteriae	250	static	170	cidal (150)
C. pseudodiphthericum	250	static	170	cidal (150)
B. anthracis	350	static	150	static (170)
S. agalactiae	+	+	170	static (170)
S. fecalis	250	static	170	cidal (150)
S. aureus	250	static	150	static (150)
Ps. aeruginosa	250	static	150	static (150)
B. subtilis	250	static	170	static (170)

Key + = Growth observed.

The antifungal and antibacterial activity, of natural plant products derived from Withania *somnifera* and *Polygonum equisetiforme* which are commonly used in traditional medicine, especially against pathogenic organisms lends scientific basis for their use in different ailments. Pure compounds were generally more inhibitory for susceptible organisms than the crude extracts. The zones of inhibition remained clear for more than a week of incubation, suggesting the bioactivity to be a stable characteristic of these products.

The inhibitory activity of WS and PE derived substances against human pathogens like *Staph. aureus, Ps. aeruginosa, strept agalactiae, C diphtheria, B. anthracis, T. mentegrophyte, M.canis* and *A. bodydii* provides sufficient justification for a possible use of these extracts or their active principles for the development of drugs to treat infections and reduce human suffering due to toxicity of synthetic drugs. Alternative sources of antimicrobial substances have to be explored due to the emergence of pathogenic strains of bacteria which have developed resistance to most of the antibiotics through plasmids and R-factors and due to the indiscriminate use of antibiotics in the hospitals. The use of antibiotics like chloramphenicol, tetracycline, erythromycin and others is probably limited because of the risk of serious side effects and their toxicity. We would like to suggest that the crude extracts or other natural products derived from *Withania somnifera* and *Polygon equisetiforrne* orate may be used to develop therapeutic drugs for the treatment and control of infectious diseases especially for Pseudomonad infections because the causative organisms, *Ps. aeruginosa,* has become resistant to most of the available antibiotics. However, these new natural plant products as well as others need to undergo carefully controlled clinical trials in order to evaluate their usefulness in daily medical practice. Animal toxicity studies of these products indicated that they may be toxic to the animal if administered in very high concentrations but for treatment purposes much lower doses may be required In Brine shrimp lethality test LD50 dose of WS and PE which killed 50% of the newly hatched Artemia Larvae was found to be 1000 pg/ml and 600 µg/ml respectively. LD dose of WS and PE which was lethal for half of the BALB/c mice treated with WS and PE was 100 mg/kg and 6 mg/kg of the body weight respectively. Toxicity at high concentration may be due to the presence of some toxic components in addition to the bioactive compound.

Susceptibility of pathogenic microorganisms to these natural products may prove to be important for both diagnostic and therapeutic purposes. Bioactivity directed isolation of other active components from the crude extracts of *Withania somnifera* and *Polygonum equisetforme* is in progress.

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