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Research Article

Antibacterial and Cytotoxic Activities of *Acacia nilotica* Lam (Mimosaceae) Methanol Extracts Against Extended Spectrum Beta-Lactamase Producing *Escherichia coli* and *Klebsiella* Species

Saba Riaz[1,2]*, Muhammad Faisal[1], Shahida Hasnain[1] and Naveed Ahmed Khan[2]

[1]Department of Microbiology and Molecular Genetics, University of the Punjab, Lahore, Pakistan 54590, [2]School of Veterinary Medicine and Sciences, University of Nottingham, Sutton Bonington, England, LE12 5RD, UK.

Abstract

Purpose: *To investigate lysates from Acacia nilotica pods for their antimicrobial and cytotoxic activities against a variety of extended spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae as well as methicillin resistant Staphylococci aureus (MRSA).*
Methods: *ESBLs-producing E. coli and Klebsiella spp were isolated from clinical and environmental specimens and incubated with Acacia nilotica pod lysates. The bactericidal activity and cytotoxic effects of the lysates were evaluated while fast protein liquid chromatography (size exclusion FPLC) was used to identify the various compounds showing bactericidal activity.*
Results: *The lysates showed remarkable bactericidal properties, killing almost 100 % of the bacteria they were tested against, including neuropathogenic Escherichia coli, MRSA, and Klebsiella spp. The bactericidal activity was heat-resistant and showed minimal cytotoxic effects on human brain microvascular endothelial cells. FPLC revealed eight peaks, with three of them representing compounds that had maximum bactericidal activity against all the tested isolates, but showed < 30 % host cell cytotoxicity.*
Conclusion: *The lysate of Acacia nilotica pods is a potentially good candidate for the therapy of antibacterial-resistant bacteria, and would therefore require further studies.*

Keywords: *Acacia nilotica, ESBLs, MRSA, E. coli, Klebsiella, Antibacterial resistance, Cytotoxicity*

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Corresponding author:* ***Email:* *sabbb1502@yahoo.com, saba@mmg.pu.edu.pk;* ***Tel:*** *+92-42-99231248;* ***Fax:*** *+92-42-99231249*

INTRODUCTION

Extended spectrum beta-lactamase (ESBL) is an enzyme that hydrolyzes the beta-lactam ring in antibiotics and readily inactivates first-line drugs such as cephalosporins, penicillins and monobactams [1]. With the limited availability of alternative antibiotics and the increase in antibiotic resistance worldwide, ESBLs-producing *Enterobacteriaceae* is a serious threat to current beta-lactam therapy [2]. In the United States, for example, a survey of the intensive care units of 400 hospitals between 1990 and 1993 recorded an increase from 3.6 to 14.4 % in ESBL-producing strains of *Klebsiella* spp [3]. In Pakistan, the prevelance of ESBLs-producing bacteria among nosocomial isolates is as high as 48 % [4]. Moreover, before the 1990s, *K. pneumoniae* isolates typically produced a single beta-lactamase [5] but today, these bacteria produce three to six beta-lactamases [6].

The use of natural products, such as medicinal plants [7], as therapy against infectious diseases, is an age-long practice, especially in developing countries. *Acacia* is an important plant genera that is commonly used in a variety of infections. It is widely distributed in Asia, Australia and America and its efficacy has been demonstrated in the treatment of gonorrhoea, leucorrhoea, diarrhoea, dysentery and wounds [8]. The objective of the present study is to investigate the antibacterial properties of crude methanol lysates of *Acacia* pods against multidrug resistant ESBLs and MRSA *in vitro* and also to determine whether the lysates had any cytotoxic effects on human cells.

EXPERIMENTAL

Plant material

Acacia nilotica Lam (Mimosaceae) was obtained from Dr M Faisal, Botany Department, University. of Punjab, Pakistan. For future reference, a voucher specimen was deposited in the Herbarium of Punjab University with voucher specimen no. 69306.

Preparation of *Acacia nilotica* lysate

Ten grams of shade-dried *Acacia nilotica* pods were ground and soaked in 100 mL of methanol (100 %). Extraction was performed in a water bath at 25 ℃ for one week with continuous shaking using Cole_Parmer Orbital shaker. The extract was filtered, concentrated at 40 ℃ under reduced pressure, dried in a freeze-dryer and stored at 4 ℃ pending further use. The extract was re-suspended in water at a concentration of 100 mg/ml prior to use [9].

Bacterial isolates

Clinical specimens (mostly urine, blood, sputum, foley tip and wound swabs) were obtained from Citilab and Research Center, Lahore. The specimens were processed according to standard operating procedures [10]. The clinical samples were plated on blood agar plates. Environmental samples (from sewage, soil and standing water) were collected from hospital surroundings. All the bacterial isolates were characterized by colony morphology and biochemical testing. *Escherichia coli* and *Klebsiella* spp were selected and their identity confirmed using 20 E API (BioMerieux). *Klebsiella pneumoniae* ATCC 700603 [11] and *E. coli* ATCC 25922 [12] were used as reference strains.

The bacterial isolates were tested for the presence of beta-lactamases using double-disk synergism (DDS) test method which entailed the application of cefpodoxime, ceftazidime, aztreonam, cefotaxime or ceftriaxone [1,2,13]. To further confirm beta-lactamase properties, Epsilometer test was carried [2]. Briefly, drug-impregnated AB Biodisk strips were used (Solna, Sweden), one end of which contains a gradient of ceftazidime (MIC test range, 0.5 to 32 µg/ml) and on the opposite end, a gradient of ceftazidime plus a constant concentration of clavulanate (4 µg /ml). The MICs of both ends

are interpreted as the point of intersection of the inhibition ellipse with the E-test strip edge [2]. *E. coli* K1 [14] and MRSA [15] were used in cytotoxicity assay. All the selected strains were preserved in glycerol stocks [16].

Antibiotic susceptibility testing

Antibiotic evaluation test was performed on Muller Hinton agar using Kirby bauer method as per Clinical Laboratory Standards Institute [10]. Briefly, antibiotic disks containing amikacin 30 µg, ampicillin 10 µg , amoxicillin-clavulanic acid (20 µg/10 µg) 30 µg, carbencilin 100 µg, cephredine 30 µg, cefaperazone 75 µg, ciprofloxacin 5 µg, gentamicin 10 µg, nitrofurontoin 300 µg, imepenum10 µg, meropenum10 µg, norfloxacin 10 µg, cotrimexazole 25 µg, ceftizoxime 30 µg, cefotaxime 30 µg, cefoxitin 30 µg, cetazidime 30 µg, ceftriaxone 30 µg, cefixime 5 µg, aztreonam 10 µg, and pipracilin-tazobactam 100/10 µg [17] were tested. The results are listed in Table 1.

Bactericidal assays

A 100 µl aliquot of the crude extract was incubated with approx. 10^6 bacterial cells (*E. coli, Klebsiella,* K1 or MRSA) suspended in 10 µl. Tubes containing lysate alone, or phosphate buffer saline (PBS) alone were used as controls. The tubes were incubated for 2 h at 37 °C, and bacterial counts determined by making serial dilution and plating on nutrient agar plates as previously described [18]. The number of colony-forming units (cfu was determined after an overnight incubation at 37 °C. Bactericidal effect (BE) was determined as in Eq 1.

BE (%) = (100 – cfu in lysate)/(total bacterial cfu) x 100 …………………………… (1)

Total bacterial cfu were calculated by incubating bacteria alone without the lysate. In some experiments, different concentration of lysate (0.01, 0.05, 0.1, 0.5, 1, 2.5, 5, and 10 mg/ml) were used for the bactericidal assay. In all assays, the total volume was adjusted to 100 µl with PBS and bactericidal effect determined as above. To determine the nature of antimicrobial compound(s), the lysate was heated at 100 ℃ for 10 min in the presence or absence of sodium dodecyl sulfate (SDS), followed by bactericidal assay.

Cytotoxicity assay

Human brain microvascular endothelial cells (HBMEC) were routinely grown in RPMI-1640 containing 10 % foetal bovine serum, 10 % NuSerum, 2mM glutamine, 1 mM pyruvate, penicillin (100 U/ml), streptomycin (100 U/ml), non-essential amino acids and vitamins (Invitrogen, Paisley, UK), as previously described [14].

To determine whether the lysate exhibits toxic effect on HBMEC, cytotoxicity assay was performed, as previously described [19]. Briefly, HBMEC grown to confluency in 24-well plates were incubated with various volumes of lysates including 25, 50, 100, 200 µl and the final volumes adjusted to 500 µl with RPMI-1640. The plates were incubated for 24 h at 37 ℃ in a 5 % CO_2 incubator. Following incubation, the supernatant of each well was collected and cell death determined using a cytotoxicity detection kit (Roche Applied Science, Lewes, UK). This assay is based on measuring lactate dehydrogenase (LDH) release. Lactate dehydrogenase (LDH) is a soluble cytosolic enzyme, found in all cells and is released into the culture medium only by damaged cells. Thus, the release of LDH is considered as an estimate of cell death. There are two steps of reaction which determine LDH. In the first step, the reaction involves reduction of nicotinamide adenine dinucleotide $(NAD)^+$ to nicotinamide adenine dinucleotide (reduced form, NADH) by oxidation of lactate to pyruvate. In the second step, the catalysed diaphorase, transfers 2 hydrogen from $NADH^+ + H^+$ to the terazolium salt (INT), which is subsequently reduced to formazan. Formazan dyes are artificial chromogenic substrates for dehydrogenases and reductases; they are water soluble and show a broad absorption maximum at about

500 nm while the tetraxolium salt (INT) shows no significant absorption at this wavelength. Thus, an increase in the amount of plasma membrane-damaged cells results in an increase of LDH enzyme activity in the culture supernatant. Cytotoxicity (Ct) was determined as in Eq 2.

Ct (%) = (Test value – control value)/(total LDH release – control value) x 100 ….. (2)

Control values were obtained from HBMEC incubated alone and total LDH release was measured from HBMEC-treated with 5 % Triton X-100 for 1 h at 37 °C. Triton X-100 is a detergent that permeabilizes eukaryotic cells and results in HBMEC damage.

Fast Protein Liquid Chromatography (FPLC)

FPLC was performed on the lysate using a Bio-Rad Biologic Workstation system. Sterile water was used as the solvent at a flow rate of 0.5 ml/min with fractions collected into sterile microcentrifuge tubes. A size exclusion column (3 kDa – 75 kDa) packed with Sephadex and agarose beads (GE Healthcare) was used in order to fractionate the crude lysate on the basis of molecular weight. Thereafter, the different fractions were screened for antibacterial activity (see Fig 1)

Statistical analysis

Statistical analysis was carried out using SPSS 17 software. Standard error mean (SEM) was calculated based on three independent experiments. Chi square test was used to calculate statistical significance. Other data are presented as percentages.

RESULTS

Antibacterial activity of *Acacia nilotica* lysate

The results of antibacterial activity of the *A. nilotica* lysates are listed in Table 1. The findings revealed that aliquots of 100 μl lysate (equivalent to10 mg of *A. nilotica* lysates) exhibited > 99 % bactericidal activity against MRSA (see Table 1). At 10 mg/ml concentration, the extract exhibited > 80 % bactericidal activity against all the clinical isolates of *E. coli*, but only 52% against *E. coli* isolated from sewage (Table 1). *A. nilotica* lysates exhibited > 90 % bactericidal activity against all *K. pneumonia* isolates at a concentration of 10 mg/ml except *K. pneumonia* folly tip isolate against which it showed 42 % (Table 1). The solvent alone had no effect on the viability of any of the bacterial isolates (data not shown).

Effect of denaturing conditions on the bactericidal activity of *A. nilotica* lysates

The findings revealed that heating the *A. nilotica* lysates at 100 ℃ for 10 min had no effect on its bactericidal properties (Table 2). A concentration of 1 mg/ml exhibited > 90 % bactericidal activity; boiling exhibited a similar bactericidal activity (Table 2). Similarly, lysates incubated with 1 % sodium dodecyl sulphate (SDS) and treated at 100 ℃ for 10 min exhibited > 85 % bactericidal activity, indicating that SDS had no effect lysate activity. The solvent alone had no effect on the viability of any of the bacterial isolates (Table 2).

Antibacterial activity of FPLC-derived fractions of *A. nilotica* lysate

The results of the use of Fast protein liquid chromatography (FPLC) to characterize and separate fractions of *A. nilotica* lysate and their antibacterial activities are shown in Fig 1. FPLC fractionation revealed the presence of 8 distinct peaks which showed bacteriocidal activity ranging from 50 to 93 %. This suggests the presence of multiple antibacterial substances molecular weight < 10 kDa. When the FPLC fractions were tested on HBMEC in order to assess their cytotoxicity (LDH release), cytotoxicity ranged

Table 1: Antibacterial activity of *Acacia nilotica* lysates (mean ± SEM, n= 3)

Bacteria	Specimen type	% Bactericidal activity of lysate	Antibiotics to which there was resistance*
E. coli K1	CSF	89±0.57	AMC,AMP,CAR,CE,CIP,CN,FN,NOR
MRSA	Blood	99±0.8	AMC,AMP,CAR,CAZ,CE,CIP,CN,CRO,CXM,FN,NOR
K. pneumonia	Pus	93±0.3	AMC,AMP,CAR,CAZ,CE,CIP,CN,CRO,FN,NOR,SXT
K. pneumonia	Standing water	95±0.91	AMC,CAR,CAZ,CFP,CE,CIP,CN,CRO,CXM,CFP,NOR,SXT,ZOX
E. coli	Pus	82±0.67	AMC,CAR,CAZ,CFP,CE,CIP,CN,CRO,CXM,CFP,NOR,SXT,ZOX
E. coli	Urine	96±0.53	AMP,CAR,CAZ,CE,CFP,CN,CRO,CXM,FN,NOR,ZOX
E. coli	Sewage	82±0.66	AMC,AMP,CAR,CAZ,CE,CIP,CN,CRO,FN,NOR,SXT
E. coli	Sewage	52±1.2	AMC,CAR,CAZ,CFP,CE,CIP,CN,CRO,CXM,CFP,NOR,SXT,ZOX
E. coli	Urine	92±0.33	AMP,CAR,CAZ,CE,CFP,CIP,CRO,CXM,FN,NOR,SXT,ZOX
K. pneumonia	Urine	94±0.57	AMP,CE,CFP,CAR,CAZ,CIP,CN,CXM,CRO,FN,NOR,SXT,ZOX
E. coli	Urine	86±0.89	AMP,CE,CFP,CAR,CAZ,CXM,CRO,FN,NOR,SXT,ZOX
E. coli	Blood	88±1.6	AMP,CE,CFP,CAR,CAZ,CIP,CXM,CRO,FN,NOR,SXT,ZOX
K. pneumonia	Folly tip	42±1.45	AMP,CE,CFP,CAR,CAZ,CIP,CN,CXM,CRO,FN,NOR,SXT,ZOX

AMP = Ampicillin, AMC = Amoxycillin/Clavulanic acid, CAR = Carbencilin, CAZ = Ceftazidime, CFP = Cefoperazone, CE = Cephredine, CIP = Ciprofloxacin, CN = Gentamicin, CRO = Ceftriaxone, CXM = Cefuroxime, CFP = Cefoperazone, NOR = Norfloxacin, SXT = Trimethoprim/sulfamethoxazole, ZOX = Ceftizoxime. FN = Nitrofurontoin.

Table 2: Effect of heat and SDS on the bactericidal activity of *A. nilotica* lysate (mean ± SEM, n= 3)

Treatment applied	Bactericidal activity (%)
K1 + solvent	0
K1 + lysates	91.90±0.50
K1 + lysates (heated)	98.20±0.40
K1 + lysates (heated + SDS)	85.89±8.20
MRSA + solvent	0
MRSA + lysates	96.80±1.70
MRSA + lysates (heated)	99.85±0.07
MRSA + lysates (heated + SDS)	88.90±1.50

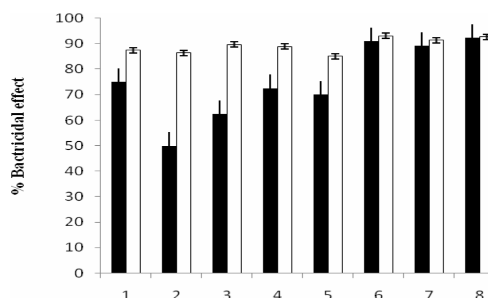

Figure 1: Antibacterial activity of the eight *A. nilotica* lysate fractions obtained by FPLC size exclusion chromatography. ***Note:*** Dark bars represent neuropathogenic *E. coli* K1 while transparent bars represent methicillin-resistant *Staphylococcus aureus* (MRSA).

from 8.1 – 29.0 %, depending on the fraction.

DISCUSSION

The presence of antimicrobial compounds in plants is a well known fact and they are ancient weapons in the defence against infection. Antimicrobials destroy or inhibit the growth of microbes such as bacteria, viruses, fungi or protozoa and more than 50% of the reported antimicrobial substances are derived from plants [20]. A previous study has shown that the stem, leaves and roots of *Acacia* are commonly used in the traditional therapy of various diseases [8]. At 10 mg/ml concentration, *A. nilotica* lysates exhibited > 95 % antimicrobial activity against all the bacteria tested including neuropathogenic *E. coli* K1, MRSA, and *K. pneumoniae*. This is remarkable as lysates demonstrated such potent bactericidal activity against bacterial isolates where several well-known antibiotics failed. It is also interesting that heat treatment of the lysates at 100 °C did not reduce potency of *A. nilotica* lysates.

FPLC is popular for separation of different compounds in plant extracts [21]. FPLC data suggest that there is more than one compound present in the lysates. The fact that the lysates exhibited potent bactericidal activity against both Gram-negative (neuropathogenic *E. coli* and *K. pneumonia*) as well as Gram-positive bacteria (MRSA) is an indication that the putative target(s) was conserved in the bacteria tested. Such broad-spectrum antibacterial activity will have tremendous potential in the treatment of bacterial infections. Furthermore, the lysates had limited cytotoxic effect on HBMEC, suggesting that putative target(s) for the active component(s) are absent in eukaryotes.

The present work, which is preliminary in this regard, suggests that the lysates interferred with important structural component of the bacterial membranes, thus leading to defective membranes, and hence increased permeability and leakage of ions from the cell. However, the precise identification of the compounds, their identity and mode of action are yet to be elucidated and are expected to be the subject of future studies. Further studies will need to be performed using individual FPLC fractions against a range of bacteria, protists and fungi in order to obtain a more detailed profile of this promising antibacterial material.

CONCLUSION

The results of this study indicate that the methanol lysate of *Acacia nilotica* pods contains active compounds capable of killing a range of bacteria types, including MRSA, neuropathogenic *E. coli* K1, as well as beta-lactamase positive *E. coli* and *Klebsiella* spp. Future work on this plant material should include identification of the nature of its constituent compounds, such as the chemical structure and properties, as well as its mode of action.

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