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Original Article

Activity of Some Plant Extracts Against Multi-Drug Resistant Human Pathogens

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Abstract

Plants used for traditional medicine contain a wide range of substances which can be used to treat various infectious diseases. Hence, antibacterial activities of ethanolic extracts of 19 plant species were studied against multi-drug resistant clinical isolates using agar well diffusion method. Extracts of *Liquidambar orientalis*, *Vitis vinifera*, *Rosmarinus officinalis*, *Punica granatum*, *Cornus sanguinea*, *Euphorbia peplus*, *Ecballium elaterium*, *Inula viscosa* and *Liquidambar orientalis* showed broad-spectrum antibacterial activity with inhibition zones ranging from 8 to 26 mm. The most resistant organisms were *Escherichia coli* (*E. coli*) (Ampicillin-, amoxycillin- and sulfamethoxazole-resistant), *Stenotrophomonas maltophilia* (*S. maltophilia*) (Amoxycillin- and nalidixic acid-resistant) and *Klebsiella pneumoniae* (*K. pneumoniae*) (Ampicillin-, amoxycillin- and aztreonam-resistant), and the most susceptible species were *Staphylococcus aureus* (*S. aureus*) (Penicillin G- and oxacillin-resistant), *Streptococcus pyogenes* (*S. pyogenes*) (Penicillin G-, erythromycin- and clindamycin-resistant) and *Pseudomonas aeruginosa* (*P. aeruginosa*) (Sulfamethoxazole- and novobiocin-resistant), respectively. Minimum Inhibitory Concentrations (MIC) of crude extracts were determined for the seven highly active plants showing activity against methicillin resistant *S. aureus* (MRSA), *E. coli*, *P. aeruginosa*, *S. pneumoniae* and the reference bacteria (*E. coli* ATCC 11229 and *Kocuria rhizophila* ATCC 9341 NA). MICs of active extracts ranged from 8 to 14.2 mg/mL against one or other test bacteria.

Keywords: Antibacterial activity; Clinical isolate; Drug-resistant; Medicinal plants; Turkish.

Introduction

One of the more alarming recent trends in infectious diseases has been the increasing frequency of antimicrobial resistance among microbial pathogens causing nosocomial and community-acquired infections. Numerous classes of antimicrobial agents have become less effective as a result of the emergence of antimicrobial resistance, often as a result of the selective pressure of antimicrobial usage. Among the more important emerging resistance problems are oxacillin resistance in staphylococci, penicillin resistance in streptococci, vancomycin resistance in enterococci (and eventually staphylococci), resistance to extended-spectrum cephalosporins and fluoroquinolones in Enterobacteriaceae, and carbapenem resistance in *P. aeruginosa* (1). For example, in clinical isolates of *S. pneumoniae* resistance to antibiotics routinely used to treat infections is now at 40%

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in some European countries. Similarly, a high level of ampicillin resistance is very significant in *E. coli*, while it would be natural in most other enterobacteria. *Escherichia coli* and *Klebsiella* spp. are the only ones generally susceptible to narrow-spectrum cephalosporins (2). Also, MRSA, gained much attention in the last decade, is a major cause of hospital-acquired infections (3). During the last two decades a renewed interest in *Corynebacterium* species and other non-spore-forming Gram-positive bacilli has emerged among clinicians and microbiologists alike. Infections caused by these organisms are emerging, new species are being recognized, and infections by toxigenic and nontoxigenic *Corynebacterium diphtheriae* strains are also being described with increasing frequency, indeed, in countries where diphtheria had been totally or almost eradicated (4).

Herbal medicines have been important sources of products for the developing countries in treating common infectious diseases and overcome the problems of resistance and side effects of the currently available antimicrobial agents (5). The World Health Organisation (WHO) estimates that 80% of the people living in developing countries almost exclusively use traditional medicines. This means approximately 3300 million people use medicinal plants on a regular basis. Medicinal plants used in traditional medicine should therefore be studied for safety and efficacy (6).

Using plants for medicinal purposes is an important part of the culture and the tradition in Turkey. Therefore, this in vitro study was aimed at screening selected plants for their antibacterial activity and evaluating their potential use in treating infections caused by multi-drug resistant clinical bacteria.

Experimental

Plant materials and preparation of the ethanolic extracts

Plants were collected in different sites of Manisa province and arounds of Turkey. Voucher specimens were deposited in the Herbarium of Botany, Department of Biology, Celal Bayar University. The used parts were leaves, stems, flowers, roots, young branches and, in some cases, fruits (Table 1).

The plant parts were separated, washed with distilled water, dried and then powdered finely using a blender. Thirty grams of ground air-dried plant material were shaken in 150 mL 96% weight/volume (w/v) ethanol (EtOH 96°) at room temperature for 60 h (180 cycles/min). The insoluble material was filtered by filter paper (Whatman No. 4) and evaporated to dryness in a water bath at 50°C. The extract was weighed and dissolved in EtOH 96° at a concentration of 200 mg/mL and stored at +4°C for further experiments.

Bacterial strains

Clinical isolates of the following: bacteria MRSA (Penicillin G- and oxacillin-resistant, and clindamycin-, vancomycin-, erythromycin-, sulfamethoxazole- and teicoplanin-sensitive), *E. coli* (Ampicillin-, amoxycillin- and sulfamethoxazole-resistant, and gentamicin-, cefuroxime-, levofloxacin-, imipenem-, aztreonam- and netilmycin-sensitive), *P. aeruginosa* (Sulfamethoxazole- and novobiocin-resistant, gentamicin- and netilmycin-intermediate, and piperacillin-, aztreonam-, imipenem- and tobramycin-sensitive), *S. maltophilia* (Amoxycillin- and nalidixic acid-resistant, and sulfamethoxazole- and levofloxacin-sensitive), *K. pneumoniae* (Ampicillin-, amoxycillin- and aztreonam-resistant, and imipenem-, netilmycin- and gentamicin-sensitive), *S. pyogenes* (Penicillin G-, erythromycin- and clindamycin-resistant, and oxacillin-sensitive), *S. pneumoniae* (Sulfamethoxazole- and penicillin G-resistant, and oxacillin- and lincosamine-sensitive) and *Corynebacterium* sp. (Erythromycin-, vancomycin- and nalidixic acid-resistant, and fusidic acid and clindamycin-sensitive) were kindly provided by the Department of Medical Microbiology, Faculty of Medicine, Osmangazi University (Eskisehir/Turkey). Also, Gram-negative *Escherichia coli* ATCC 11229 and Gram-positive *Kocuria rhizophila* ATCC 9341 were used as reference strains for comparison of MIC and inhibition zones.

Cultures of bacteria

All bacteria were cultured on Nutrient Agar plates, except for *S. pyogenes*, *K. pneumoniae*

Table 1. List of the studied plants.

Studied plants	Family	Plant part (s)	Rate	Collection time	Origin
Nerium oleander L.	Apocynaceae	LF[a], FL	2:1	24/06/2005	Campus
Pyracantha coccinea M. Roem.	Rosaceae	LF, RF	2:1	24/06/2005	Botanical Garden (BG)
Cornus sanguinea L.	Cornaceae	LF, FL, S	2:1:1	25/06/2005	Campus
Artemisia arborescens L.	Compositae	LF, FL, S	2:1:1	25/06/2005	BG
Thuja orientalis L.	Cupressaceae	LF, RF	2:1	24/06/2005	BG
Carpobrotus acinaciformis (L.) L. Bolus	Aizoaceae	LF, S	2:1	27/06/2005	BG
Punica granatum L.	Punicaceae	LF, FL	2:1	28/06/2005	BG
Conyza canadensis (L.) Cronquist.	Compositae	LF		07/07/2005	BG
Mirabilis jalapa L.	Nyctaginaceae	LF, FL, S	2:1:1	26/06/2005	BG
Euphorbia peplus L.	Euphorbiaceae	LF, FL, S, RT	2:1:1:1	08/07/2005	Campus, Yagcýlar
Citrus reticulata L.	Rutaceae	LF, RF	2:1	06/07/2005	BG
Vitis vinifera L.	Vitaceae	LF, RF, S, YB	2:1:1:1	24/06/2005	BG
Liquidambar orientalis Mill.	Hamamelidaceae	LF		24/06/2005	BG
Inula viscosa (L.) Aiton.	Compositae	LF		10/07/2005	Muradiye
Rosmarinus officinalis L.	Lamiaceae	LF		27/06/2005	Campus
Hypericum perforatum L.	Guttiferae	LF, FL, S	2:1:1	27/06/2005	Campus, Yagcýlar
Lonicera japonica Thunb.	Caprifoliaceae	LF		27/06/2005	BG
Ecballium elaterium A. Richard	Cucurbitaceae	LF, FR	1:1	30/06/2005	Yagcýlar
Eucalyptus camaldulensis Dehnh.	Myrtaceae	LF		05/07/2005	Campus

[a] LF: leaves, FL: flowers, FR: fruits, RF: raw fruits, RT: roots, S: stems, YB: young branches, NI: not informed

and *S. pneumoniae* which were cultured on Blood Agar plates, and were incubated for 24 h at 37°C. Few colonies from these cultures were inoculated into Mueller-Hinton Broth and incubated at 37°C for 24 h before use. Nutrient Agar (Merck) and Blood Agar were used to maintain the clinical isolates of the bacteria.

Agar well diffusion assay

The assay was conducted as described by Perez et al. (7) with slight modification according to the present experimental conditions. Bacterial strains grown on nutrient agar at 37°C for 18 h were suspended in a saline solution (0.85% NaCl) and adjusted to a turbidity of 0.5 MacFarland standards [10^6 Colony Forming Units (CFU)/mL]. Briefly, 50 µl inoculum was used to inoculate 90-mm diameter petri plates containing 25 mL Mueller-Hinton Agar (MHA), with a sterile non-toxic cotton swab on a wooden applicator. Wells with 6-mm diameter were punched in the agar and filled with 100 µl extract solution (4 mg/mL). The dissolution of the organic extracts (ethanolic) was facilitated with the addition of 5% (v/v) dimethyl sulfoxide (DMSO) which not affected the growth of microorganisms (as shown by our control experiments). The dishes were preincubated at 4°C for 2 h to allow uniform diffusion into the agar. After preincubation, the plates were incubated at 37°C for 24 h. The antibacterial activity was evaluated by measuring the inhibition zone diameter observed. In addition, ampicillin (10 µg) and gentamicin (10 µg) were used as positive control to determine the sensitivity of the strains by the disc diffusion method (8). The experiments were performed in triplicate.

Determination of minimal inhibitory concentration

The Minimum Inhibitory Concentration (MIC) was determined for the seven highly active plants which showed antibacterial activity against MRSA, *E. coli*, *P. aeruginosa*, *S. pneumoniae* and the reference bacteria. Broth technique with slight modification was used to determine MIC of extracts against selected test bacteria as described by the Clinical and

Laboratory Standards Institute (CLSI) (9). In brief, the cultures were diluted in Mueller-Hinton broth at a density adjusted to 0.5 McFarland turbidity and 0.5 mL of a bacterial suspension containing 1.5×10^6 CFU/mL was added to 4.5 mL of susceptibility test broth containing diluted extract solution which was already prepared by serial two-fold dilution from the extract stock solution starting from 30 to 0.8 mg/mL, in glass test tubes. Positive controls were made of broth and innoculum only. The first row of tube served as the negative control (broth plus innoculum plus solvent used to dilute the extracts). The contents of each tube were mixed on a shaker at 250 rpm for 1 min and then incubated at 37°C for 24 h before being read. MICs of ampicillin and gentamicin were used as standards determined in parallel experiments in order to comparison. The MIC was considered the lowest concentration of the sample that prevented visible growth. All samples were examined in two separate experiments.

Statistical treatment of the results

The mean values were analysed with the MINITAB Release 13.20 program statistically by the general one-way (unstacked) analysis of variance (ANOVA) to find out the most effective plants and the most sensitive test organisms.

Results and Discussion

Antibacterial activity of nineteen plants belonging to seventeen botanical families was evaluated in vitro against eight drug-resistant clinical isolates and against two reference bacteria which are known to cause pneumonia, mucosal, respiratory, skin, soft tissue and urinary tract infections in humans.

The antibacterial activity of the extracts and their potency was assessed by the presence or absence of inhibition zone as given in Table 2. Results showed that the most susceptible organisms were MRSA (clinical isolate) which was sensitive to 17 extracts, *P. aeruginosa* and *Corynebacterium* sp. being sensitive to 15 plant extracts, *S. pneumoniae* being sensitive to 14 plant extracts, and *S. pyogenes* being sensitive to 13 plant extracts. The most resistant species were *E. coli* being resistant to 11 plants, *S. maltophilia* being resistant to 9 plants, and *E. coli* ATCC 11229 which was resistant to 7 plants. Maximum inhibitions were observed with the extract of *Cornus sanguinea* against *S. aureus* (26 mm) and that of *R. officinalis* against *S. pyogenes* (26 mm). The inhibition zone against *E. coli* were produced by the extract of 8 plants, i.e. *L. orientalis*, *R. officinalis P. granatum*, *Conyza canadensis*, *E. peplus*, *Citrus reticulata*, *V. vinifera* and *E. elaterium*, in which the first and second ones with a inhibition zone of 16 mm apperead to be highly active. However, negative control (DMSO, 100 µl) could not inhibit test bacteria (Table 2).

Similar report by Erdogrul on antibacterial activities of *R. officinalis* leaves showed various inhibitory effects against Gram-positive and Gram-negative bacteria (7–16 mm inhibition zone), except the acetone extract against *Yersinia enterocolitica* (10). In another study, ethanol extract of *P. granatum* against *P. aeruginosa*, *Bacillus cereus* and *S. pyogenes* developed imhibition zones of 12, 24 and 26 mm, respectively, while *Nerium oleander* was found to be less active against 14 pathogenic bacterial species (11). Our results confirm these studies.

Sensitivity of test strains, in decreasing order, was as follows: *S. aureus* > *P. aeruginosa* > *S. pyogenes* > *S. pneumoniae* > *Corynebacterium* sp.> *K. pneumoniae* > *K. rhizophila* ATCC 9341> *E. coli* ATCC 11229 > *S. maltophilia* > *E. coli* (Figure 1). Gram-negative bacteria were less sensitive than Gram-positive bacteria, which may be due to their differences in the cell wall composition (3). It was interesting to note that antibiotic-resistant bacteria showed more sensitivity to the investigated plant extracts. This has clearly indicated that antibiotic resistance does not interfere with the antibacterial action of plant extracts and these extracts might have different modes of action on test organisms.

Most of the studied plants are potentially rich sources of antimicrobial agents. However, the plants differ significantly in their activity against test bacteria. The most active plants were *V. vinifera*, *L. orientalis*, *R. officinalis*, *E. elaterium*, *P. granatum, C. sanguinea*, *I. viscosa, E. peplus* and *Eucalyptus camaldulensis* showed broad-spectrum antibacterial activity against resistant bacteria. On the other hand, the least

Table 2. Antibacterial activity of the ethanolic extracts of plants against multi-drug resistant bacteria and the reference bacteria.

Plants	Test bacteria[a]									
	Ec	*Kp*	*Sm*	*Pa*	*Sy*	*Sp*	*Cy*	*Sa*	RB	
									EsC	*SARL*
Nerium oleander L.	6[b]	8	6	10	6	6	14	18	6	12
Pyracantha coccinea M. Roem.	6	6	6	6	6	8	6	6	8	6
Cornus sanguinea L.	6	12	14	16	18	14	12	26	6	20
Artemisia arborescens L.	6	12	6	6	18	20	12	15	6	14
Thuja orientalis L.	6	8	20	10	20	10	8	22	6	12
Carpobrotus acinaciformis (L.) L. Bolus	6	8	6	6	6	12	6	8	8	6
Punica granatum L.	10	15	12	16	20	12	16	20	6	14
Conyza canadensis (L.) Cronquist.	8	8	6	12	10	6	6	10	16	6
Mirabilis jalapa L.	6	10	6	8	6	6	14	18	6	6
Euphorbia peplus L.	14	14	16	18	8	16	14	14	6	10
Citrus reticulata L.	10	10	6	8	6	6	8	10	10	6
Vitis vinifera L.	10	20	18	24	18	18	24	20	16	20
Liquidambar orientalis Mill.	16	20	22	20	20	24	16	16	14	18
Inula viscosa (L.) Aiton.	6	8	8	22	22	16	10	14	10	16
Rosmarinus officinalis L.	16	16	10	18	26	18	16	24	20	18
Hypericum perforatum L.	6	10	6	6	10	6	16	20	10	16
Lonicera japonica Thunb.	6	6	6	8	6	10	6	6	10	6
Ecballium elaterium A. Richard	14	8	10	20	16	12	14	14	24	18
Eucalyptus camaldulensis Dehnh.	6	14	8	18	16	14	8	18	16	12
Ampicillin (10 µg/disk)	8	6	10	12	12	6	14	14	10	22
Gentamicin (10 µg/disk)	26	30	24	20	24	26	32	28	28	32
Negative control (DMSO, 100 µl)	6	6	6	6	6	6	6	6	6	6

[a] Bacterial strains; *Ec*: *Escherichia coli*, *Kp*: *Klebsiella pneumoniae*, *Sm*: *Stenotrophomonas maltophilia*, *Pa*: *Pseudomonas aeruginosa*, *Sy*: *Streptococcus pyogenes*, *Sp*: *Streptococcus pneumoniae*, *Cy*: *Corynebacterium* sp., *Sa*: methicillin resistant *Staphylococcus aureus,* RB: Reference Bacteria, *EsC*: *Escherichia coli* ATCC 11229, *SARL*: *Sarcina lutea* ATCC 9341NA.
[b] Inhibition zone diameter in mm, including well diameter (6 mm), test medium: MHA, dose: 4 mg/ml, mean values n=3, DMSO: Dimethyl sulfoxide.

active plants were *Pyracantha coccinea*, *Lonicera japonica*, *Carpobrotus acinaciformis*, *Mirabilis jalapa*, *Citrus reticulate*, *N. oleander* and *C. canadensis*. However, *Hypericum perforatum*, *Thuja orientalis* and *Artemisia arborescens* were moderately active plants (Figure 2).

The antibiotic susceptibility pattern of the clinical bacterial strains was provided by Faculty of Medicine, Osmangazi University (Eskisehir/ Turkey), and only ampicillin and gentamicin were tested against test bacteria in our laboratory. With the exception of *K. rhizophila* which had an inhibition zone of 22 mm, other bacteria were resistant to ampicillin (10 µg/disk), indicating their multi-drug resistance phenotype. We could not use these antibiotics as therapeutic agents to treat diseases caused by the reference bacteria. A comparision on the inhibition zones of the pathogenic bacteria showed that gentamicin was effective against all ten bacterial species tested.

Significant antibacterial effects, expressed as MIC of crude extracts, were observed against MRSA, *E. coli*, *P. aeruginosa* and *S. pneumoniae* (Table 3). The maximal inhibition zones and MIC values for bacterial strains, which were sensitive to the plant extracts were in the range of 14-26 mm and 13.4–10.2 mg/mL, respectively. Extracts of selected plants were among the most active with the MIC values ranging from 8.0-14.2 mg/ mL. Among the plants tested, ethanolic extract of *R. officinalis* and *V. vinifera* showed very strong activity against MRSA with the best MIC (8.6

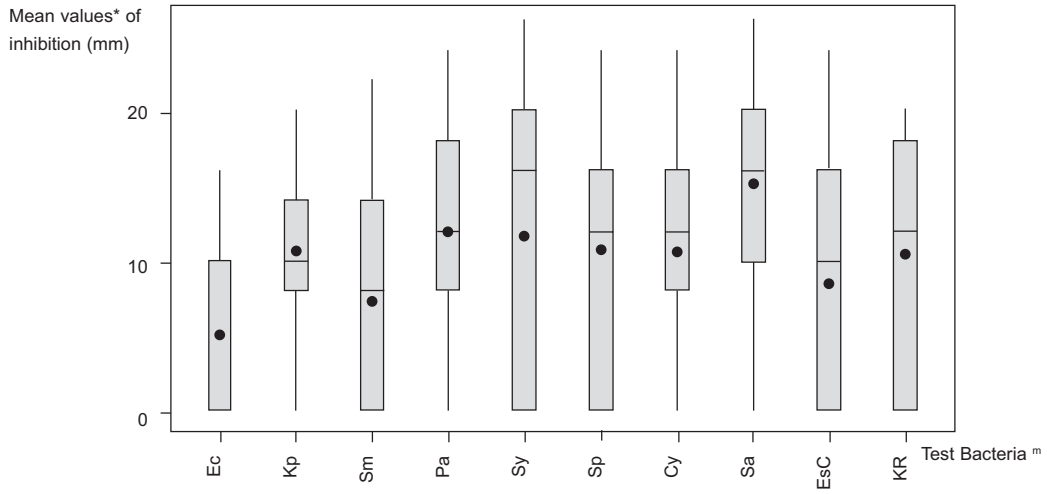

Figure 1. Mean values of inhibition (mm) of bacteria in relation to their susceptibility to the plant extracts.
* means are indicated by solid circles.
[m] See Table 2 for abbreviations of test bacteria.

and 9.4 mg/mL, respectively). The lowest MIC obtained with *V. vinifera* and *I. viscosa* extract, was 8.0 mg/mL for *P. aeruginosa*, whereas the highest MIC was 14.2 mg/mL for *V. vinifera* and *L. orientalis* extracts against *E. coli* ATCC 11229. MIC values of *R. officinalis*, *L. orientalis* and *P. granatum* extracts for *E. coli* were 8.6, 10.2 and 11.8 mg/mL, respectively.

Plant extracts have been studied against bacteria for years in the last three decades. During this period, a lot of antimicrobial screening evaluations have been published based on the traditional use of Turkish plant (15, 16, 19). Yet, a comparative study of the MIC of plant extracts against drug-resistant bacterial isolates have not been previously reported. Also, little information is available about the activity of plants against drug-resistant hospital isolates. Many previous researchers (13, 14, 17) reported the antibacterial activity of medicinal plants but their findings were different from those of present study. This discrepancy could be due to differences in the plants physiological state, seasonal variation, environmental condition, studied part of the plants, extraction procedure, concentration of crude extracts and strains of test bacteria.

Several studies have shown that the occurrence of resistance is closely related to the medical use of a drug, even though the association may be variable. This association has also been demonstrated for antimicrobial agents used for growth promotion. Also, in the hospital environment, antimicrobial use plays an essential role in the emergence of resistant bacteria causing the spread of resistant clones (2). Staphylococci are an important cause of both nosocomial and community-acquired infections. In the last decade, staphylococcal infection has reemerged as a cause for concern because of its numerical increase, the spread of MRSA isolates in the community, and the emergence of isolates not susceptible to vancomycin (18).

With the increase in resistance of microorganisms to the currently used antibiotics and the high cost of production of synthetic compounds, pharmaceutical companies are now looking for alternatives. Medicinal plants could be those alternatives because most of them are safe with little side effects if any, cost less, and affect a wide range of antibiotic resistant microorganisms (11, 19).

The demand for new effective antimicrobials is urgent and of great importance in the clinical health. Allied with this demand is the need for assays to detect new and previously undiscovered antimicrobials from plant sources. From this study, the plant extracts were found to have antibacterial activity against drug-resistant clinical bacteria. However, to explain the mode of action, the active phytocompounds of these

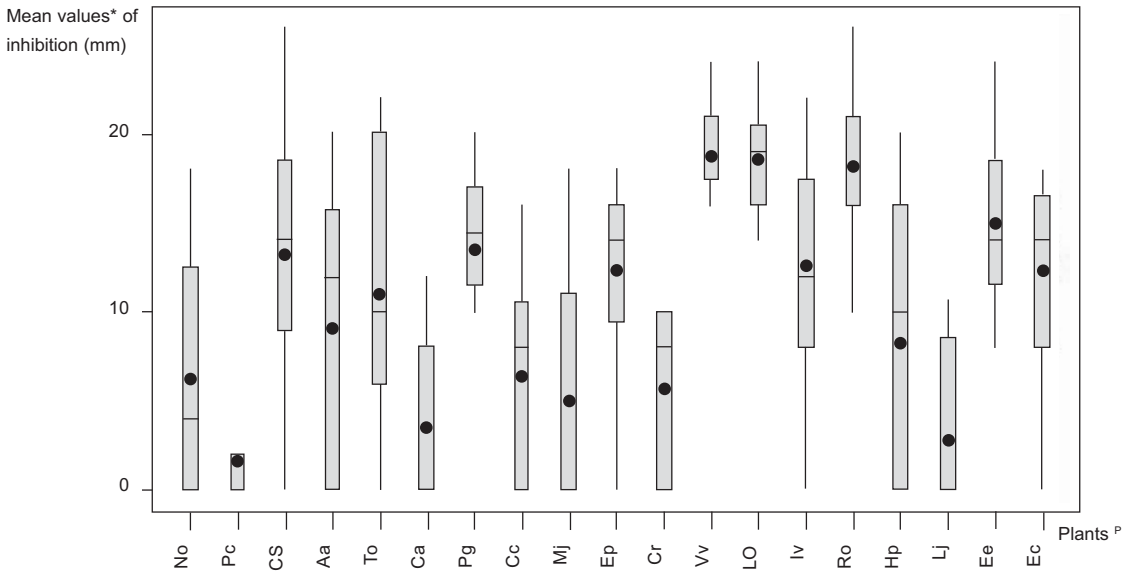

Figure 2. Mean values of antibacterial activity of plants against all tested clinical bacteria
* means are indicated by solid circles
[P] Plants; No: *N. oleander*, Pc: *P. coccinea*, Cs: *C. sanguinea*, Aa: *A. arborescens*, To: *T. orientalis*, Ca: *C. acinaciformis*, Pg: *P. granatum*, Cc, *C. canadensis*; Mj: *M. jalapa*, Ep: *E. peplus*, Cr: *C. reticulate*, Vv: *V. vinifera*, Lo: *L. orientalis*, Iv: *I. viscose*, Ro: *R. officinalis*, Hp: *H. perforatum*, Lj: *L. jJaponica*, Ee: *E. elaterium*, Ec: *E. camaldulensis*

plants used against multidrug-resistant bacteria and their toxicity have to be determined by additional studies.

In conclusion, all of the plant extracts tested in this study had potential antibacterial activities against the reference strains. Our results support the use of these plants in traditional medicine and suggest that some of the plant extracts possess compounds with good antibacterial properties that can be used as antimicrobial agents in the search for new drugs.

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Table 3. MIC values of selected plant ethanol extracts.

Plants	MIC (mg/ml)					
	Sa	***Ec***	***Pa***	***Sp***	**RB**	
					EsC	***SARL***
Cornus sanguinea L.	10.2	NA[a]	14.2	13.4	NA	9.4
Punica granatum L.	11.8	ND	9.4	ND	NA	ND
Vitis vinifera L.	9.4	ND	8.0	11.8	14.2	10.2
Euphorbia peplus L.	ND	13.4	10.2	ND	NA	ND
Liquidambar orientalis Mill.	10.2	13.4	9.4	9.4	14.2	12.6
Rosmarinus officinalis L.	8.6	12.6	ND	ND	13.4	13.4
Inula viscosa (L.) Aiton	ND	NA	8.0	ND	ND	ND
Ampicillin (µg/ml)	16	ND	ND	32	ND	8.0
Gentamicin (µg/ml)	4.0	6.0	ND	ND	4.0	2.0

Sa, methicillin resistant *Staphylococcus aureus*; *Ec*, *Escherichia coli*; *Pa*, *Pseudomonas aeruginosa*; *Sp*, *Streptococcus pneumoniae*; RB: Reference Bacteria, *EsC*: *Escherichia coli* ATCC 11229, *SARL*: *Sarcina lutea* ATCC 9341NA. MIC, minimum inhibitory concentration.
[a] ND, not determined; NA, not applicable.

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