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Antibacterial, antifungal, and antiviral activities of the lipophylic extracts of *Pistacia vera*

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Plant extract

Summary

In the present study, antibacterial, antifungal, and antiviral properties of 15 lipohylic extracts obtained from different parts (leaf, branch, stem, kernel, shell skins, seeds) of *Pistacia vera* were screened against both standard and the isolated strains of *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Candida albicans* and *C. parapsilosis* by microdilution method. Both *Herpes simplex* (DNA) and *Parainfluenza* viruses (RNA) were used for the determination of antiviral activity of the *P. vera* extracts by using Vero cell line. Ampicilline, ofloxocine, ketoconazole, fluconazole, acyclovir and oseltamivir were used as the control agents. The extracts showed little antibacterial activity between the range of 128–256 μg/ml concentrations whereas they had noticeable antifungal activity at the same concentrations. Kernel and seed extracts showed significant antiviral activity compared to the rest of the extracts as well as the controls.
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Introduction

Traditional medicines based mostly on medicinal plants have been used for the treatment of various diseases by mankind for centuries. Plants are also well-known to be the rich sources of biologically active compounds. Therefore, one approach that has been used for the discovery of antimicrobial agents from natural sources is based on the evaluation of traditional plant extracts. Also, it is important that acquired agents resistant to microorganisms can cause serious infections especially in immunosuppressed patients that withstand with usages of antimicrobial agents.

Pistacia vera L., a member of Anacardiaceae family, is native to Asia minor and widely distributed in the Mediterranean region as well as USA. Among the 11 species of *Pistacia* genus, *P. vera* is by far the most

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economically important one. In addition to its economical value, kernels of *P. vera* are remarkably rich in linoleic and linolenic acids, the fatty acids vital for human health (Garcia et al., 1992; Maskan and Karatas, 1998; Aslan et al., 2002; Satil et al., 2003). On the other hand, *Pistacia* species were reported to have various biological activities such as anti-atherogenic, hypoglycemic, antioxidant, anti-inflammatory, and anti-insect activities (Demo et al., 1998; Pascual-Villalobos and Robledo, 1998; Giner-Larza et al., 2000; Dedoussis et al., 2004; Hamdan and Afifi, 2004). Antifungal activity of the essential oils and the leaf extracts of three *Pistacia* species including *P. vera* were also studied (Duru et al., 2003; Kordali et al., 2003). Besides, the mastic obtained from *P. terebinthus* is also used as urinary and respiratory antiseptic in Turkish folk medicine (Baytop, 1999). On the other hand, antibacterial, antifungal and antiviral activities of the other parts of *P. vera* have not been studied so far. In this study, we aimed to investigate the antimicrobial, and antiviral properties of edible and non-edible parts of *P. vera*. On this purpose, 16 lipophylic extracts of the plant were tested against *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Candida albicans*, *C. parapsilosis*, *Herpes simplex virus* (HSV), *Parainfluenza virus* (PIV) (Jawetz et al., 1991).

Materials and methods

Plant material

Plant material (*P. vera*) was collected from Nizip, Gaziantep, Turkey in September 2003 and the leaves, branches, stem, shell skins and kernels were separated individually.

The plant parts used in this study were classified as follows: fresh leaves (PV-FL), stem (PV-ST), branches (PV-BR), fresh skin of natural-woody shell (non-processed) (PV-FSN), dried skin of natural-woody shell (non-processed) (PV-DSN), fresh kernel (PV-FK) and skin of processed-woody shell (PV-SPS). The seeds used were of three types, collected two localities in Turkey (Siirt & Gaziantep) and Iran in three stages including unripe, ripe and processed and were coded as follows: Siirt sample-ripe (PV-SR), Siirt sample-processed (PV-SP), Gaziantep sample-unripe (PV-GU), Gaziantep sample-ripe (PV-GR), Gaziantep sample-processed (PV-GP), Iran sample-unripe (PV-IU), Iran sample-ripe (PV-IR), Iran sample-processed (PV-IP).

Preparation of the extracts

The parts of *P. vera* mentioned above were chopped and extraction of the samples was carried out with *n*-hexane during 8 h continuously for each part using a Soxhlet apparatus in the presence of anhydrous Na_2SO_4. After filtrating, the *n*-hexane phases evaporated under vacuum at 40 °C to dryness and the lipophylic extracts were obtained as described in our previous study (Aslan et al., 2002).

Microbiological studies

Test materials

The lipophylic extracts of *P. vera* were dissolved in ethanol:hexane (1:1) by using 1% Tween 80 solution at a final concentration of 1024, 512 and 256 μg/ml and sterilized by filtration using 0.22 μm Millipore (MA, USA) and used as the stock solutions.

Standard antibacterial powders of ampicillin (AMP, Faco), ofloxacin (OFX, Hoechst Marion Roussel) and standard antifungal powders of ketoconazole (KET, Bilim) and fluconazole (FLU, Pfizer), were obtained from their respective manufacturers and dissolved in phosphate buffer solution (ampicillin, pH: 8.0, 0.1 mol/l), dimethylsulphoxide (ketoconazole), in water (fluconazole, ofloxacin). The stock solutions of the agents were prepared in medium according to the NCCLS recommendations (National Committee, 1996).

Microorganisms

Standard and the isolated strains of the following bacteria, namely *E. coli* (ATCC 35218), *P. aeroginosa* (ATCC 10145), *E. faecalis* (ATCC 29212), and *S. aureus* (ATCC 25923) for the determination of antibacterial activity, and standard strains of *C. albicans* (ATCC 10231) and *C. parapsilosis* (ATCC 22019) for the determination of antifungal activity were used. Microorganisms were obtained from Department of Microbiology and Clinical Microbiology, Faculty of Medicine, Karadeniz Technical University (Trabzon-Turkey). The isolated strains of *E. coli*, *P. aeruginosa*, *E. faecalis* and *S. aureus* were obtained from Department of Medical Microbiology, Faculty of Medicine, Gazi University (Ankara-Turkey).

Inoculum preparation

Mueller–Hinton Broth (Difco) and Mueller–Hinton Agar (Oxoid) were applied for growing and diluting of the microorganism suspensions.

Before the test, all strains of fungi were cultured on Sabouraud dextrose agar (SDA) (Oxoid) and

passaged at least twice to ensure purity and viability at 35 °C for 24–48 h. Five colonies were used from each culture. The synthetic medium RPMI-1640 with L-glutamine was buffered pH: 7 with 3-[*N*-morpholino]-propansulfonic acid (MOPS) and culture suspensions were prepared according to the NCCLS M27-A. Also, standard and isolated strains of bacteria were grown to exponential phase in medium (MHB) at 37 °C overnight with aeration. Twenty microlitre of the culture was then plated on MHA. After overnight incubation at 37 °C with aeration, approximately five colonies of cultures were plated in MHB.

The bacterial suspensions used for inoculation were prepared at 10^5 cfu/ml by diluting fresh cultures at McFarland 0.5 density (10^8 cfu/ml). The fungi suspension was prepared by the spectrophotometric method of inoculum preparation at a final culture suspension of 2.5×10^3 cfu/ml (National Committee, 2002; Baran et al., 1994).

Antibacterial and antifungal tests

The microdilution method was employed for antibacterial and antifungal activity tests. Media were placed into each 96 wells of the microplates. Extract solutions at 256 and 512 μg/ml were added into first raws of microplates and two fold dilutions of the compounds (256–0.06 μg/ml) were made by dispensing the solutions to the remaining wells. Ten microlitre culture suspensions were inoculated into all the wells. The sealed microplates were incubated at 35 °C for 24 and 48 h in humid chamber. The lowest concentration of the extracts that completely inhibit macroscopic growth was determined and minimum inhibitory concentrations (MICs) were reported. Antimicrobial activities of the extracts were tested against two Gram-positive, two Gram-negative bacteria and isolated strains of these bacteria and a yeast-like fungus using ampicillin, ofloxacin, ketoconazole and fluconazole as the standards.

Cytotoxicity and antiviral tests

Cell line and growth condition

Vero cell line (African green monkey kidney) used in this study was obtained from Department of Virology, Faculty of Veterinary, Ankara University (Ankara-Turkey).

The culture of the cells were grown in Eagle's Minimal Essential Medium (EMEM) enriched with 10% fetal calf serum (FCS) (Biochrom, Germany), 100 mg/ml of streptomycin and 100 IU/ml of penicillin in a humidified atmosphere of 5% CO_2 at 37 °C. The cells were harvested using trypsin solution (Bipco Life Technologies, UK).

Test viruses

In order to determine the antiviral activity of the extracts, HSV and PIV were used. The test viruses were obtained from Department of Virology, Faculty of Veterinary, Ankara University.

Antiviral activity

Media (EMEM) were placed into each 96 wells of the microplates (Greiner^R, Germany). Stock solutions of the extracts were added into first raws of microplates and two-fold dilutions of the extracts (512–0.00006 μg/ml) were made by dispensing the solutions to the remaining wells. Two-fold dilution of each material was obtained according to Log_2 on the microplates. Acyclovir (Biofarma) and oseltamivir (Roche) were used as the control agents.

Strains of HSV and PIV titers were calculated by the Frey and Liess method as $TCID_{50}$ (Frey and Liess, 1971). They were inoculated into all the wells. The sealed microplates were incubated in 5% CO_2 at 37 °C for 2 h to detect the possible antiviral activities of the samples. After incubation, 50 μl of the cell suspension of 300.000 cells/ml which were prepared in EMEM+5% fetal bovine serum was put in each well and the plates were incubated in 5% CO_2 at 37 °C for 48 h. After the end of this time, the cells were evaluated using cell culture microscope ($\times$ 400), comparing with treated–untreated control cultures and with acyclovir and oseltamivir as the control agents. Consequently, maximum CPE (Cytopathogen Effect) concentrations as the indicator of antiviral activities of the extracts were determined (Esquenazi et al., 2002).

Cytotoxicity

The maximum non-toxic concentration (MNTC) of each sample was determined by the method described previously by Uysal-Gökçe et al. based on cellular morphologic alteration. Several concentrations of each sample were placed in contact with confluent cell monolayers and incubated in 5% CO_2 at 37 °C for 48 h. MNTC were determined by comparing treated and controlling untreated cultures (Uysal-Gökçe et al., 2005).

Results and discussion

Fifteen lipophylic extracts of *P. vera* were screened for their antibacterial activities against both standard and the isolated strains of *E. coli*, *P. aeruginosa*, *E. faecalis* and *S. aureus*. The MICs of the extracts were shown in Table 1. The effect of extracts was compared to the control agents. According to the results obtained, no significant inhibition was observed with any of the extracts compared to the control agents. There was no inhibitory activity in the wells containing only ethanol:hexane (1:1**)** with 1% Tween 80 solution used as blank. However, all of the extracts greater potent antibacterial activity against Gram-positive bacteria than Gram-negative in this assay. In addition, several in vitro studies also demonstrated that the ethanolic extract of *P. terebinthus* and the essential oil of mastic gum of *P. lentiscus* var. *chia* displayed higher antibacterial activity against Gram-positive bacteria than Gram-negative bacteria (Magiatis et al., 1999).

As also shown in Table 1, all of the extracts displayed remarkable antifungal activity against *C. albicans* and *C. parapsilosis* at 16 μg/ml concentration which is close to the effective concentrations of the control agents.

The data obtained from antiviral activity screening showed that PV-SPS (16–<0.00006 μg/ml), PV-FK (8–<0.00006 μg/ml) and PV-GU (8–<0.00006 μg/ml) had significant activity against DNA virus same as the acyclovir. The other three extracts, namely PV-GR (32–0.0039 μg/ml), PV-SR (32–0.125 μg/ml) and PV-DSN (16–1 μg/ml), were less active than the extracts given above. The rest of the extracts did not possess any antiviral activity against DNA virus (Table 2). On the other hand, PV-GR (32–16 μg/ml), PV-GP (32–16 μg/ml), PV-IP (32–16 μg/ml) and PV-FSN (32–4 μg/ml) showed outstanding activity against RNA virus. Besides, PV-ST (16–8 μg/ml), PV-BR (16–8 μg/ml), PV-IU (8–8 μg/ml) and PV-SR (8–8 μg/ml) had also lower activity compared to the extracts above. Remaining eight extracts showed no activity.

In a previous study, essential oils obtained from the leaves of three *Pistacia* species were found to have antifungal activity against three species of plant pathogen fungi (Duru et al., 2003). In another study, the petroleum ether extract of *P. vera* leaves has also been found to be effective against plant pathogen fungi (Kordali et al., 2003). Some researchers reported that the mastic gum of *P. lentiscus* var. *chia* had antifungal activity against *C. albicans* (Iauk et al., 1996) which supports our findings.

As mentioned above, among the extracts, PV-SPS, PV-FK and PV-GU against HSV, PV-GR, PV-GP, PV-IP and PV-FSN against PIV showed the strong antiviral activity identical to acyclovir and oseltamivir, respectively. In our previous work, we had analyzed the lipophylic extracts of *P. vera* from the

Table 1. Antimicrobial activity as MICs (μg/ml) of the extracts and the standards against tested control and isolated strains

Microorganisms	A	A_1	B	B_1	C	C_1	D	D_1	E	F
Extracts										
PV-FL	128	256	128	256	32	64	32	64	16	16
PV-ST	128	256	128	256	64	64	32	64	16	16
PV-BR	128	256	128	256	32	64	32	64	16	16
PV-FSN	128	256	128	256	32	64	32	64	16	16
PV-DSN	128	256	128	256	32	64	16	64	16	16
PV-SPS	128	256	128	256	32	64	16	64	16	16
PV-FK	128	256	128	256	32	64	16	64	16	16
PV-SR	128	256	128	256	32	64	32	64	16	16
PV-SP	128	256	128	256	32	64	16	64	16	16
PV-GU	128	256	128	256	32	64	16	64	16	16
PV-GR	128	256	128	256	32	64	16	64	16	16
PV-GP	128	256	128	256	32	64	32	64	16	16
PV-IU	128	256	128	256	32	64	16	64	16	16
PV-IR	128	256	128	256	32	64	16	64	16	16
PV-IP	128	256	128	256	32	64	16	64	16	16
AMP	2	32	—	—	0.25	1	1	16	—	—
OFX	0.12	8	1	8	1	8	0.5	—	—	—
KET	—	—	—	—	—	—	—	—	2	2
FLU	—	—	—	—	—	—	—	—	4	4

A: *E. coli*, A_1: isolates strains, B: *P. aeruginosa*, B_1: isolates strains, C: *E. faecalis*, C_1: isolates strains, D: *S. aureus*, D_1: isolates strains, E: *C. albicans*, F: *C. parapsilosis*, OFX: Ofloxasine, AMP: Ampicilline, KET: Ketoconazole, FLU: Fluconazole.

Table 2. Cytotoxicity and antiviral activity results of the lipophylic extracts of *P. vera*

Vero cells (μg/ml)	MNTC (μg/ml)	CPE inhibitory concentration			
		HSV		PIV	
		Max.	Min.	Max.	Min.
Extracts					
PV-FL	8	—	—	16	0.5
PV-ST	32	—	—	16	8
PV-BR	16	—	—	16	8
PV-FSN	16	—	—	32	4
PV-DSN	16	16	1	8	—
PV-SPS	16	16	<0.00006	8	—
PV-FK	8	8	<0.00006	32	—
PV-SR	32	32	0.125	8	8
PV-SP	16	—	—	1	0.5
PV-GU	8	8	<0.00006	8	4
PV-GR	32	32	0.0039	32	16
PV-GP	16	—	—	32	16
PV-IU	16	—	—	8	8
PV-IR	—	—	64	—	32
PV-IP	16	—	—	32	16
Acyclovir	16	16	<0.00006	—	—
Oseltamivir	32	—	—	32	8

—: no activity observed; MNTC: maximum non-toxic concentration; CPE: cytopathogenic effect.

view point of the fatty acid compositions. Since these seven extracts contained palmitic and linoleic acids in common, it may be concluded that these fatty acids contribute to the activity of the extract. In another study by Patterson, palmitic acid was also found to have potent antiviral activity against HIV-1 and HIV-2 which support our findings. To our knowledge, our study is the first report about the antifungal and antiviral activities of *P. vera* against human pathogen fungi. Further research on the isolation and identification of the active principle(s) of these extracts is in progress in our laboratory in order to discover new and potent antiviral and antifungal compound(s).

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