

## *In vitro* antimicrobial activity of olive leaves

### Antimikrobielle Wirksamkeit von Olivenblättern *in vitro*

D. Markin, L. Duek and I. Berdicevsky

Department of Microbiology, Rappaport Faculty of Medicine, Technion – Institute of Technology, Haifa, Israel

#### Summary

We investigated the antimicrobial effect of olive leaves against bacteria and fungi. The microorganisms tested were inoculated in various concentrations of olive leaf water extract. Olive leaf 0.6% (w/v) water extract killed almost all bacteria tested, within 3 h. Dermatophytes were inhibited by 1.25% (w/v) plant extract following a 3-day exposure whereas *Candida albicans* was killed following a 24 h incubation in the presence of 15% (w/v) plant extract. Olive leaf extract fractions, obtained by dialysis, that showed antimicrobial activity consisted of particles smaller than 1000 molecular weight cutoffs. Scanning electron microscopic observations of *C. albicans*, exposed to 40% (w/v) olive leaf extract, showed invaginated and amorphous cells. *Escherichia coli* cells, subjected to a similar treatment but exposed to only 0.6% (w/v) olive leaf extract showed complete destruction. These findings suggest an antimicrobial potential for olive leaves.

#### Zusammenfassung

Wir untersuchten die antimikrobielle Wirkung eines wässrigen Extraktes aus Olivenblättern gegen Bakterien und Pilze. Eine Verdünnung von 0.6% tötete fast alle Bakterien innerhalb von 3 h ab. Dermatophyten wurden nach einer 3 h langen Exposition gegen eine Verdünnung von 1.25% im Wachstum gehemmt. *Candida albicans* war nach 24 h Inkubation in einem 15 %igen Extrakt abgetötet. Durch Dialyse erhaltene Extraktfraktionen mit antimikrobieller Aktivität bestanden aus Partikeln < 1000 Mr. Im Rasterelektronenmikroskop zeigte extraktgeschädigte *C. albicans* invaginierte, deformierte Zellen. *Escherichia coli* war schon durch 0.6 %igen Extrakt komplett zerstört.

**Key words:** Olive leaves, antifungal activity.

**Schlüsselwörter:** Olivenblätter, antimyketische Wirksamkeit.

#### Introduction

Olive tree, a plant which can survive for hundreds of years, is known to naturally possess strong resistance to microbial attack. The antimicrobial properties of the leaves have been utilized in traditional medicine to fight fever and overcome infections. These medicinal appli-

cations, however, have not yet been validated by research. Research to date has focused solely on the antimicrobial properties of the olive fruit. Phenolic compounds isolated from olive fruit have been shown to inhibit the growth of *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus*.<sup>1,2</sup> Oleuropein, a bitter iridoid glycoside, isolated from the olive fruit, has been shown to inhibit sporulation of *Bacillus cereus*.<sup>3</sup> Hydroxytyrosol, a metabolite of oleuropein, was recently proved effective against clinical human pathogenic strains of *Haemophilus influenzae*, *Moraxella catarrhalis*, *Salmonella typhi*, *Vibrio parahaemolyticus* and *S. aureus*.<sup>4</sup> Increasing resistance to antibiotics, wide-spread use of immunosuppressing drugs and a rise

Correspondence: Professor Dr Israela Berdicevsky, Department of Microbiology, Technion – Faculty of Medicine, PO Box 9649, Haifa 31096, Israel. Tel.: 972 4 8295293. Fax: 972 4 8295225. E-mail: israelab@technix.technion.ac.il

Accepted for publication 16 January 2002

in fungal infections all emphasize the necessity to find and develop new antimicrobial agents. In our study, we aimed to determine and characterize the antimicrobial activity of olive leaves against a wide spectrum of human pathogenic bacteria and fungi.

## Materials and methods

### Plant material

Leaves of *Olea europaea* var *manzanillo* and *barnea* (Oleaceae) were collected in November in Lower Galilee, Israel. The leaves were dried in open air, shady conditions until completely dried and then ground to a powder.

### Extraction of the plant

Ground powdered leaves were extracted in water at a 20% (w/v) concentration (200-g dried leaves per liter water) and autoclaved at 121 °C for 20 min. Leaves were then strained and the resulting extract was autoclaved again. The extract was stored at 4 °C up to 8 weeks.

Before use extract was lyophilized in a lyophilizer (CHRIST Model ALPHA 1-4; Martin-Christ, Osterode, Germany) to a dry powder. Saline was then added at different volumes to achieve desired concentrations, and solutions were autoclaved again.

### Strains, media and growth conditions

Clinical isolates of the following microorganisms were used:

Bacteria – *E. coli*, *Pseudomonas aeruginosa*, *S. aureus*, *B. subtilis* and *K. pneumoniae*.

Dermatophytes – *Trichophyton mentagrophytes*, *Microsporum canis* and *T. rubrum*.

Yeast – *Candida albicans*.

Bacteria were cultured on Trypticasein soya agar (TSA), and yeasts on yeast extract agar. Dermatophytes were cultured on Sabouraud glucose agar (SDA). Their response to the plant extract was assessed in Sabouraud glucose broth (SDB). All media constituents were purchased from the Difco laboratories (MI, Detroit, USA). All stock strains were kept at 4 °C.

### Test system

Bacteria  $10^6$  ml<sup>-1</sup>, yeasts  $10^5$  ml<sup>-1</sup> or dermatophyte spores  $10^5$  ml<sup>-1</sup> were inoculated in various concentrations of olive leaf water extracts, for different time periods. In all cases, the tests included a drug-free control. Cultures were then assessed for viable counts.

### Determination of minimal bactericidal concentration

Bacteria were added to test tubes containing 1 ml olive leaf extract at the following concentrations: 0.15% (w/v), 0.3% (w/v), 0.6% (w/v), 1.25% (w/v). Following a 1, 2, 3 and 4 h of incubation at 37 °C, bacteria were cultured on TSA and incubated at 37 °C for 24 h. Living colonies were then counted in order to determine amount of colony-forming units (CFU) per milliliter.

### Determining minimal fungicidal concentration

#### Yeasts

Yeasts were added to test tubes containing 1 ml of olive leaf extract at 10, 15, 20 and 30% (w/v) concentrations. Following a 24-h incubation period, samples were cultured on yeast extract agar and left for a 48-h incubation period at 30 °C. Colonies were then counted.

#### Dermatophytes

Dermatophyte spores were added to 1 ml olive leaf extracts at 0.15, 0.3, 0.6, 1.25, 2.5, 5 and 10% (w/v) concentrations. Test tubes were left for a 3-day incubation period at 30 °C. Solutions were then cultured on SDA and incubated at 30 °C for 10 days. Dermatophyte growth was determined as existent (+) or non-existent (0).

### Dialysis

Olive leaf water extract was subjected to dialysis by pouring the solution into dialysis bags of different pore sizes: 1000, 3500 and 7000 molecular rate cutoffs (Mr). Fifteen milliliter of olive leaf water extract was poured into each dialysis bag. The bag was immersed overnight in 1 l distilled water and stirred by a magnetic stirrer. Two fractions were then obtained: 'in fraction' which contained particles larger than the assessed molecular size and 'out fraction', containing the smaller particles. Prior to use, the 'out fraction' was lyophilized to a dry powder. Fifteen milliliter saline were then added to the powder in order to obtain the original concentration.

### Centrifugation

Olive leaf extracts [20% (w/v)] were subjected to centrifugation at the following speed: 765 **g**, 1400 **g**, 2125 **g**, 3060 **g**, 4160 **g** and 5440 **g** for 5 min. The suspension and sediment were separated. Saline was added to the sediment to re-achieve the original concentration. Both fractions were assessed for antimicrobial activity.

**Scanning electron microscopy**

*Candida albicans* and *E. coli* ( $10^7$  cells per ml) were inoculated in olive leaf water extract [40% (w/v) and 0.6% (w/v), respectively]. Following a 6- and 24-h incubation period, solutions underwent centrifugation in order to concentrate and isolate the cells. Cells were washed in phosphate-buffered saline (PBS) and fixed in 2.5% glutaraldehyde for 24 h. After washing in PBS, cells were fixed further in 2% osmium tetroxide for 2 h, followed by successive dehydration steps in ethanol solutions (25–100%). Cells were coated with gold (10–12 nm) and observed using scanning electron microscopy (SEM) JEOL-JSM-840 (high resolution) (JEOL, Tokyo, Japan).

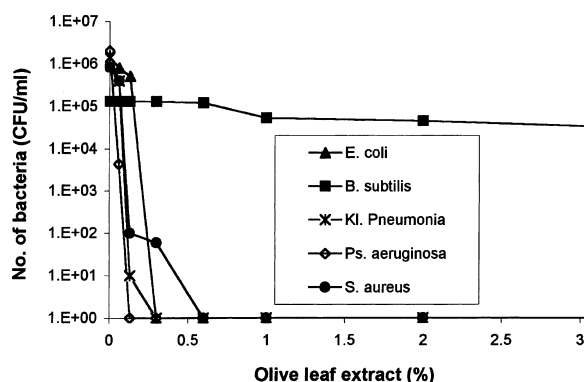
**Results**

**Minimal bactericidal concentration**

All bacteria tested, except *B. subtilis* were killed following a 3-h exposure to 0.6% (w/v) olive leaf extract (Fig. 1). *Bacillus subtilis* was inhibited only when subjected to a 20% (w/v) olive leaf extract for 24 h. This bacterial relative resistance may be due to its ability to form spores. Minimal bactericidal concentration (MBC) varied slightly among the rest of the tested bacteria (Fig. 2): *P. aeruginosa* – 0.13% (w/v), *K. pneumoniae* – 0.3% (w/v), *E. coli* – 0.3% (w/v), and *S. aureus* – 0.6% (w/v).

**Minimal fungicidal concentrations**

*Candida albicans* cells were killed following a 24 h incubation in a 15% (w/v) olive leaf extract (Fig. 3). Minimal fungicidal concentrations (MFC) for the

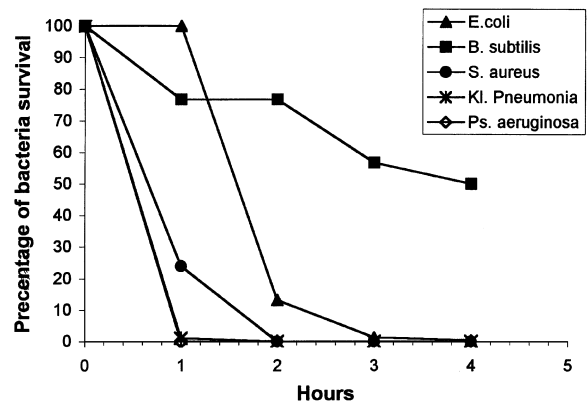


**Figure 1** The effect of different concentrations of olive leaf extract on viability of different bacteria (minimal bactericidal concentration, MBC), following 24-h exposure.

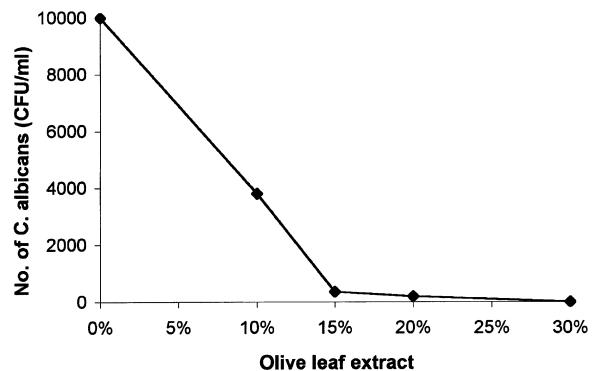
three dermatophytes, *M. canis*, *T. mentagrophytes* and *T. rubrum*, was 1.25% (w/v) (Table 1).

**Antimicrobial action of different dialysis fractions**

Antibacterial activity of dialysed fractions against *E. coli*, was observed almost only by 'out fractions' (result not



**Figure 2** Bacterial exposure to olive leaf extract [0.6% (w/v)] for various time periods.

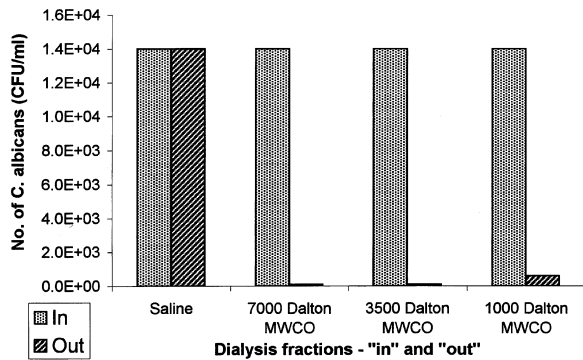


**Figure 3** Effect of different concentrations of olive leaf extract on viability of *Candida albicans* cells (minimal fungicidal concentrations, MFC) following 24-h exposure.

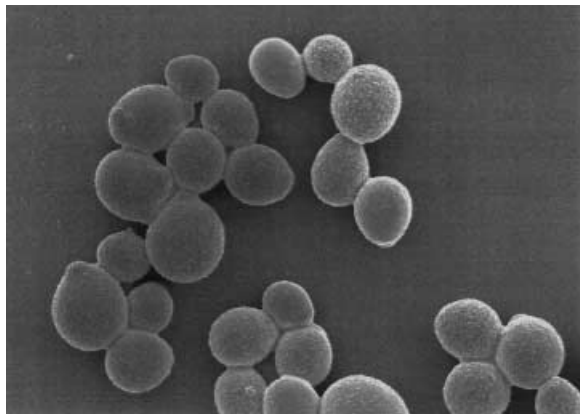
**Table 1** Inhibition of dermatophytes by different concentrations of olive leaf extract in liquid medium, following a 3 day incubation period.

Microorganism	Concentration of Olive leaf extract (%)						
	0.15	0.3	0.6	1.25	2.5	5	10
<i>Microsporium canis</i>	+	+	+	0	0	0	0
<i>Trichophyton mentagrophytes</i>	+	+	+	0	0	0	0
<i>Trichophyton rubrum</i>	+	+	+	0	0	0	0

+, Growth observed; 0, no growth observed.



**Figure 4** Antifungal activity of different dialysis fractions of olive leaf extract [0.3% (w/v)], on *Candida albicans* cells, following a 12-h exposure.



**Figure 5** Scanning electron micrograph of *Candida albicans* – control – starting point –  $\times 5000$ .

shown). Similar results were obtained when assessing anti-*Candida* activity, but with a 20% (w/v) olive leaf extract as shown in Fig. 4. It was therefore concluded that microbiologically active fractions contain active constituents which are smaller in size than 1000 Da. When assessing antimicrobial activity of fractions obtained following centrifugation, the suspensions but not the sediment of the olive leaf extract killed *E. coli* and *C. albicans* (results not shown). These results further sustain the conclusion that the active constituents consist of the smaller and lighter molecules.

#### Scanning electron microscopy

**1** *Candida albicans* cells: the non-treated yeast cells showed oval or regularly spherical forms (Fig. 5). In contrast, modifications to yeasts which were subjected to a 20% (w/v) olive leaf extract appeared following 6-h incubation and were even more marked following 24-h



**Figure 6** Scanning electron micrograph of *Candida albicans* – following 24-h exposure to 20% (w/v) olive leaf extract that underwent dialysis –  $\times 7000$ .

incubation. Cells became amorphous and deformations of the cell wall were manifested as inward invaginations (Fig. 6).

**2** *Escherichia coli* cells: the non-treated cells appear as symmetrical rods. *Escherichia coli* cells which were treated with 0.3% (w/v) olive leaf extract for 6 h had begun to lose their symmetrical appearance and cell wall invaginations were noticed. Following 24-h incubation in 0.6% (w/v) olive leaf extract, *E. coli* cells exhibited complete destruction.

#### Discussion

Despite the stunning success of the pharmaceutical industries in creating new antibiotics finding new wide-spectrum antimicrobial agents is still a priority because of resistant bacterial infections.<sup>5</sup>

We found olive leaves to exhibit 'in vitro' antimicrobial activity against bacteria and fungi. This combined antimicrobial action is unusual and may prove useful in cases where prolonged use of antibiotics encourage development of opportunistic infections.<sup>6</sup> Furthermore, olive leaves proved especially effective against *Klebsiella* and *Pseudomonas*, two bacterial genera which pose a major resistance problem.<sup>5</sup> The antimicrobial active fractions consist of particles smaller than 1000 Da. The antimicrobial effect against *E. coli* and *C. albicans* was also demonstrated by using a SEM.

Previous studies have focused solely on the antimicrobial properties of olive fruit. These properties were investigated mainly for their potential application in the food industry<sup>7–10</sup> and in agriculture.<sup>2</sup> An antifungal effect against dermatophytes was not suggested. The use of olive leaves has been researched for their

hypoglycemic<sup>11</sup> and hypocholesterolemic<sup>12,13</sup> actions and olive leaves are already available in pharmacies and health stores for these purposes. An additional use for olive leaves as antimicrobial agents may be advantageous as these are widely available and demonstrate a wide spectrum of 'in vitro' antimicrobial action against human pathogenic microorganisms. Further investigation is needed to determine whether olive leaves consist of principles which differ from the phenolic compounds which were isolated from the olive fruit.<sup>1</sup> More research is also necessary to investigate modes of actions. Future 'in vivo' studies will determine whether this agent could be applicable clinically as a new antimicrobial drug.

## References

- 1 Aziz NH, Farag SE, Mousa LAA, Abo-Ziad, MA. Comparative antibacterial and antifungal effects of some phenolic compounds. *Microbios* 1998; **93**: 43–54.
- 2 Paster N, Juven BJ, Harshemesh H. Antimicrobial activity and inhibition of aflatoxin B<sub>1</sub> formation by olive plant tissue constituents. *J Appl Microbiol* 1988; **64**: 293–7.
- 3 Tassou CC, Nychas GJE. Effect of phenolic compounds and oleuropein on the germination of *Bacillus cereus* T spores. *Biotechnol Appl Biochem* 1991; **13**: 231–7.
- 4 Bisignano G, Tomaino A, LoCascio R, Crisafi G, Uccella N, Saija A. On the in-vitro activity of oleuropein and hydroxytyrosol. *J Pharm Pharmacol* 1999; **51**: 971–4.
- 5 Neu HC. The crisis in antibiotic resistance. *Science* 1992; **257**: 1064–73.
- 6 Verduyn-Lunel FM, Meis JF, Voss A. Nosocomial fungal infections: candidaemia. *Diagn Microbiol Infect Dis* 1999; **34**: 213–20.
- 7 Juven B, Henis Y. Studies on the antimicrobial activity of olive phenolic compounds. *J Appl Bacteriol* 1970; **33**: 721–32.
- 8 Fleming HP, Walter WM, Etchells JL. Antimicrobial properties of oleuropein and products of its hydrolysis from green olives. *Microbiology* 1973; **26**: 777–82.
- 9 Ruiz-Barba JL, Garrido-Fernandez A, Jimenez-Diaz R. Bactericidal action of oleuropein extracted from green olives against *Lactobacillus plantarum*. *Lett Appl Microbiol* 1990; **12**: 65–8.
- 10 Tranter HS, Tassou SC, Nychas GJ. The effect of the olive phenolic compound, oleuropein, on growth and enterotoxin B production by *Staphylococcus aureus*. *J Appl Bacteriol* 1993; **74**: 253–9.
- 11 Gonzalez M, Zarzuelo A, Gamez MJ, Utrilla MP, Jimenez J, Osuna I. Hypoglycemic activity of olive leaf. *Planta Med* 1992; **58**: 513–5.
- 12 Bennani-Kabchi N, Fdhil H, Cherrah Y, Kehel L, El Bouayadi F, Amarti A, Saidi M, Marquie G. Effects of *Olea europaea* var. *oleaster* leaves in hypercholesterolemic insulin-resistant sand rats. *Therapie* 1999; **54**: 717–23.
- 13 Bennani-Kabchi N, Fdhil H, Cherrah Y, El Bouayadi F, Kehel L, Marquie G. Therapeutic effect of *Olea europaea* var. *oleaster* leaves on carbohydrate and lipid metabolism in obese and prediabetic sand rats. *Ann Pharm Fr* 2000; **58**: 271–7.