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Extraction methods and bioautography for evaluation of medicinal plant antimicrobial activity

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A. NOSTRO, M.P. GERMANÒ, V. D'ANGELO, A. MARINO AND M.A. CANNATELLI. 2000. A comparative study on the antimicrobial properties of extracts from medicinal plants obtained by two different methods was carried out. The screening of the antimicrobial activity of extracts from six plants was conducted by a disc diffusion test against Gram-positive, -negative and fungal organisms. The most active extracts (inhibition diameter $\geq$ 12 mm) were assayed for the minimum inhibitory concentration and submitted to phytochemical screening by thin-layer chromatography and bioautography. The results obtained indicate that the diethyl ether extracts were the most efficient antimicrobial compounds. The activity was more pronounced against Gram-positive and fungal organisms than against Gram-negative bacteria. Bioautography showed that the antimicrobial activity was probably due to flavonoids and terpenes.

INTRODUCTION

Medicinal plants have been used for centuries as remedies for human diseases because they contain components of therapeutic value. Recently, the acceptance of traditional medicine as an alternative form of health care and the development of microbial resistance to the available antibiotics has led authors to investigate the antimicrobial activity of medicinal plants (Bisignano *et al.* 1996; Lis-Balchin and Deans 1996; Maoz and Neeman 1998; Hammer *et al.* 1999). Moreover, the increasing use of plant extracts in the food, cosmetic and pharmaceutical industries suggests that, in order to find active compounds, a systematic study of medicinal plants is very important.

The aim of this study was to investigate the antimicrobial activity of extracts from medicinal plants used in folk medicine. We here report a comparative study on the antimicrobial properties of extracts obtained by two different methods in order to choose that which gives the most efficient antimicrobial compounds.

MATERIALS AND METHODS

Plant extract preparation

The plants used in this study were obtained commercially (A. Minardi, Ravenna, Italy) and were as follows: *Helichrysum italicum* G. Don (flowers), *Hieracium pilosella* L. (leaves), *Lonicera caprifolium* L. (plant), *Nepeta cataria* L. (plant), *Phytolacca dodecandra* L. (leaves) and *Plantago lanceolata* L. (leaves) (Table 1).

Two methods of extraction were employed. In method A, a known amount of powdered drug (50 g) was sequentially extracted at room temperature with petroleum ether (30°, 50 °C) (1), dichloromethane (2), dichloromethane : methanol (9 : 1) (3) and methanol (4). The four extracts were concentrated to dryness using a rotary evaporator at 40 °C. In method B, a known amount of powdered drug (50 g) was extracted as reported by Al-Sarraj *et al.* (1985). The drug was suspended in enough distilled water to form a homogeneous suspension. The mixture was adjusted to pH 2·0 with HCl (1 N) and then incubated at 37 °C for 30 min in a shaking water-bath. The mixture was then neutralized with NaOH (1 N) to pH 7·0, filtered, extracted with diethyl ether and concentrated to dryness (1). The remaining aqueous extract (2) was lyophilized. All solvents were purchased from Merck (Milan, Italy).

Organisms and media

The test organisms used in this study were as follows: *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883), *Proteus vulgaris* (ATCC 13315), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella enteritidis* (clinical isolate), *Serratia marcescens* (ATCC 19980), *Bacillus subtilis* (ATCC 6633), *Listeria monocytogenes* (ATCC 7644), *Staphylococcus aureus* (ATCC 6538P), *Clostridium sporogenes* (ATCC 10404), *Propionibacterium acnes* (ATCC 6919), *Candida albicans* (ATCC 10231), *C. tropicalis* (clinical isolate), *Aspergillus fumigatus* (wild type), *A. niger* (ATCC 16404), *Fusarium oxysporum*

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Table 1 Disc diffusion test

Organisms	Plant																													
	Helichrysum italicum G. Don (Compositae) flowers					*Hieracium pilosella* L. (Compositae) leaves					*Lonicera caprifolium* L. (Caprifogliaceae) plant					*Nepeta cataria* L. (Labiatae) plant					*Phytolacca dodecandra* L. (Phytolaccaceae) leaves					*Plantago lanceolata* L. (Plantaginaceae) leaves				
	Methods of extraction																													
	A				B	A				B	A				B	A				B	A				B	A				B
	Inhibition zone diameters (mm)																													
	1	2	3	4	1	1	2	3	4	1	1	2	3	4	1	1	2	3	4	1	1	2	3	4	1	1	2	3	4	1
E. coli	-*	-	-	-	14	-	-	-	-	-	-	-	-	-	-	-	8	9	-	16	-	-	-	-	14	-	-	-	-	12
Kl. pneumoniae	-	-	-	-	15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	14	-	-	-	-	18	-	-	-	-	-
Pr. vulgaris	7	-	-	-	14	-	-	-	-	8	-	-	-	-	-	-	10	10	-	14	-	-	-	-	18	-	-	-	-	-
Ps. aeruginosa	-	-	-	-	14	-	-	-	-	-	-	-	-	-	-	-	8	9	-	16	-	-	-	-	17	-	-	-	-	-
Salm. enteritidis	-	-	-	-	12	-	-	-	-	-	-	-	-	-	-	-	9	9	-	14	-	-	-	-	18	-	-	-	-	10
Ser. marcescens	-	-	-	-	13	-	-	-	-	-	-	-	-	-	-	-	10	9	-	16	-	-	-	-	15	-	-	-	-	-
B. subtilis	16	9	9	-	20	10	9	9	-	16	-	-	-	-	8	10	10	10	-	20	14	10	10	-	16	8	8	8	-	15
L. monocytogenes	20	7	7	-	16	-	-	-	-	10	8	8	10	-	14	8	7	10	-	19	12	-	-	-	15	-	-	-	-	16
Staph. aureus	15	7	7	-	20	9	7	7	-	20	-	-	-	-	16	10	10	10	-	19	15	8	10	-	15	8	7	7	-	16
Cl. sporogenes	10	-	-	-	10	16	-	9	10	11	10	10	10	14	8	22	20	18	16	21	20	10	17	11	22	8	-	-	-	8
P. acnes	30	30	12	16	30	12	10	14	15	28	12	16	18	10	22	15	18	22	18	16	15	12	11	15	19	11	15	20	11	26
C. albicans	-	-	-	-	12	-	-	-	-	16	-	-	-	-	11	-	-	-	-	16	-	-	-	-	20	-	-	-	-	13
C. tropicalis	-	-	-	-	13	-	-	-	-	12	-	-	-	-	10	-	-	-	-	26	-	-	-	-	25	-	-	-	-	12
A. fumigatus	-	-	-	-	15	-	-	-	-	12	-	-	-	-	-	-	-	-	-	14	-	-	-	-	24	-	-	-	-	10
A. niger	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	26	-	-	-	-	22	-	-	-	-	-
F. oxysporum	-	-	-	-	14	-	-	-	-	7	-	-	-	-	-	-	-	-	-	26	-	-	-	-	26	-	-	-	-	9
Penicillium sp.	-	-	-	-	7	-	-	-	-	10	-	-	-	-	-	-	-	-	-	20	-	-	-	-	20	-	-	-	-	7

A, Method A: 1, petroleum ether extract; 2, dichloromethane extract; 3, dichloromethane:methanol (9:1) extract; 4 methanol extract; B, method B: 1, diethyl ether extract. *, inhibition zone was no greater than 6 mm. Mean of inhibition zone diameters (including disc diameter of 6 mm).

(wild type), *Penicillium* sp. (wild type), *Microsporum canis*, *M. gypseum* and *Tricophyton mentagrophytes* (clinical isolates).

The strains were maintained and tested on Tryptone Soya agar (bacteria) and Sabouraud Dextrose agar (mycetes). For the antimicrobial tests, cells were grown overnight in Tryptone Soya broth (bacteria) and Sabouraud Liquid Medium (yeasts). Strains of *P. acnes* and *Cl. sporogenes* were grown for 48 h on Tryptone Soya broth in an anaerobic chamber at 37 °C under an atmosphere consisting of 10% CO_2, 10% H_2 and 80% N_2. The moulds were cultivated on Sabouraud Dextrose agar in order to harvest the mature spores. All media were purchased from Oxoid (Milan, Italy).

Disc diffusion test

For antimicrobial testing, a 20% (w/v) stock solution of each dry extract (methods A and B) was prepared in pure dimethylsulfoxide (DMSO; BDH, Milan, Italy).

Overnight broth cultures, adjusted to yield approximately $1{\cdot}0 \times 10^8$ cfu ml^{-1} for bacteria and $1{\cdot}0 \times 10^7$ cfu ml^{-1} for yeasts, were streaked with a calibrated loop on plates containing appropriate solid medium. For moulds, suspensions of mature spores were obtained by gently washing the surface of solid media with a 0·05% (v/v) solution of Tween 80 (Sigma-Aldrich, Milan, Italy) and the resulting suspension was adjusted to 10^6 spores ml^{-1}. Filter paper discs (6 mm diameter; Oxoid) were placed on the inoculated agar surfaces and impregnated with 15 μl of stock solutions. Pure DMSO (15 μl) was used as a negative control while vancomycin discs (30 μg), amoxicillin discs (10 μg; Oxoid) and amphotericin B discs (100 μg; Pasteur, Milan, Italy) were used as positive controls.

The plates were observed after 18 h at 37 °C for bacteria and after 48 and 96 h at 25 °C for yeasts and moulds, respectively. Strains of *P. acnes* and *Cl. sporogenes* were incubated in an anaerobic chamber at 37 °C for 48 h.

All tests were performed in duplicate and the antibacterial activity was expressed as the mean of inhibition diameters (mm) produced by the plant extracts.

Minimum inhibitory concentration

The diethyl ether extracts (1) obtained by method B showed good activity in the agar diffusion test. Those extracts giving an inhibition zone $\geq$ 12 mm in diameter were chosen to assay the minimum inibitory concentration (MIC) with the agar dilution method.

A stock solution of each extract was serially diluted twofold in pure DMSO and 0·2 ml of each dilution were incorporated in 20 ml of the appropriate melted agar medium and poured into a Petri dish. The final concentrations of extracts in the medium ranged from 0·2 to 0·006% (w/v) and the resulting DMSO concentration was 1% (v/v).

Overnight culture dilutions (1 : 100) were streaked in radial patterns on the surface of the plates. Suspensions of moulds, obtained as before, were inoculated in the centre of the plates, while dermatophytes were inoculated on the surface of slants with a 2 × 2 mm block of a 3-week culture. The samples were incubated for up to 3 weeks at 30 °C. Slants and plates containing only medium or medium with 1% (v/v) DMSO were used as controls. The MIC was defined as the lowest concentration of the extract inhibiting the visible growth of each micro-organism.

Phytochemical screening

The diethyl ether extracts (1) obtained with method B were selected for preliminary phytochemical screening because of their good antimicrobial activity.

Identification by chemical test. Tests for alkaloids, coumarins, flavonoids, sterols and triterpenes were carried out according to the methods of Harborne (1973).

Identification by thin-layer chromatography. The diethyl ether extracts were submitted to thin-layer chromatography (TLC). Plates (GF 254 60; Merck) 250 μm thick were developed with $CHCl_3 : CH_3OH : H_2O$ (80 : 18 : 2), which separated components into a wide range of R_F values. The components were visualized under visible and u.v. light (254 and 366 nm) and sprayed with the following reagents in order to reveal spots of different groups: modified Dragendorff's reagent for alkaloids, methanolic potassium hydroxide for coumarins, aluminium chloride for flavonoids and anisaldehyde/sulphuric acid for steroids and terpenes (Krebs *et al.* 1969).

Bioautography. Bioautography was performed with a culture of *Staph. aureus* which showed a good sensitivity to the extracts. Developed TLC plates were carefully dried for complete removal of the solvents and overlaid by agar seeded with an overnight culture of *Staph. aureus*. The plate was incubated for 24 h at 37 °C and then sprayed with an aqueous solution of 2 mg ml^{-1} *p*-iodonitrotetrazolium violet (Sigma). The areas of inhibition, coloured yellow, were compared with the R_F of the related spots on the reference TLC plate.

RESULTS

Disc diffusion test

The results of the disc diffusion testing of plant extracts are listed in Table 1.

The DMSO negative control showed no inhibiting effect. The positive controls showed inhibition diameters ranging

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from 16 to 18 mm (vancomycin) against Gram-positive and from 17 to 21 mm (amoxicillin) against Gram-negative bacteria. The inhibition diameters of amphotericin B ranged from 12 to 15 against fungal organisms.

Among extracts obtained with method A, only those of *N. cataria* in dichloromethane (2) and dichloromethane : methanol (3) showed some activity (inhibition diameters of 8–10 mm) against Gram-negative bacteria (*E. coli, Pr. vulgaris, Ps. aeruginosa, Salm. enteritidis* and *Ser. marcescens*). The petroleum ether extract (1) from *H. italicum* and *P. dodecandra* was active against Gram-positive bacteria (inhibition diameters ranged from 12 to 20 mm) while dichloromethane (2) and dichloromethane : methanol extracts (3) from all plants were found to have a low antimicrobial activity. No activity was found with all the methanol extracts (4) against some Gram-positive (*B. subtilis, L. monocytogenes* and *Staph. aureus*) or Gram-negative bacteria.

Anaerobic bacteria were found to be sensitive to all extracts (inhibition diameters ranged from 10 to 30 mm), although *Cl. sporogenes* proved to be highly sensitive only to *L. caprifolium, N. cataria* and *P. dodecandra*.

All the extracts (from method A) were inactive against moulds and yeasts.

The diethyl ether extracts (1) obtained with method B showed a good activity against Gram-positive bacteria, whereas Gram-negative bacteria were inhibited only by extracts of *H. italicum, N. cataria* and *P. dodecandra*. *Propionibacterium acnes* showed a higher sensitivity than *Cl. sporogenes* to all plants (inhibition diameters ranged from 16 to 30 mm). All extracts obtained with method B were active against yeasts (inhibition diameters ranged from 10 to 26 mm) whereas only those from *N. cataria* and *P. dodecandra* were active against four strains of moulds (inhibition diameters ranged from 14 to 26 mm). The remaining aqueous extracts (2) of all plants were not active.

Minimum inhibitory concentration

The MIC values of diethyl ether extracts are given in Table 2.

The DMSO control showed no toxic effect at 1% (v/v). The higher sensitivity of Gram-positive and anaerobic bacteria was confirmed by the agar dilution method; the MIC values ranged from 0·0125 to 0·2% (w/v), with *H. italicum* showing the maximal activity on *B. subtilis* and *Staph. aureus*. The MIC values for Gram-negative bacteria were > 0·2% (w/v). All plant extracts, except *L. caprifolium*, were active

Table 2 Minimum inhibitory concentrations (MICs) of diethyl ether extracts (method B)

	MIC (% w/v)					
Organisms	*H. italicum*	*H. pilosella*	*L. caprifolium*	*N. cataria*	*P. dodecandra*	*P. lanceolata*
E. coli	> 0·2	–*	–	> 0·2	> 0·2	0·2
Kl. pneumoniae	> 0·2	–	–	> 0·2	> 0·2	–
Pr. vulgaris	> 0·2	–	–	> 0·2	> 0·2	–
Ps. aeruginosa	> 0·2	–	–	> 0·2	> 0·2	–
Salm. enteritidis	> 0·2	–	–	> 0·2	> 0·2	–
Ser. marcescens	> 0·2	–	–	> 0·2	> 0·2	–
B. subtilis	0·0125	0·1	–	0·2	> 0·2	0·1
L monocytogenes	0·2	–	0·1	0·2	> 0·2	0·1
Staph. aureus	0·0125	0·05	0·1	0·1	0·2	0·1
Cl. sporogenes	–	–	–	0·2	>0·2	–
P. acnes	0·1	0·1	0·1	0·2	> 0·2	0·1
C. albicans	0·1	0·1	–	0·1	0·2	0·2
C. tropicalis	0·1	0·1	–	0·1	0·2	0·2
A. fumigatus	> 0·2	0·2	–	0·1	0·1	–
A. niger	–	–	–	0·2	> 0·2	–
F. oxysporum	> 0·2	–	–	0·2	> 0·2	–
Penicillium sp.	–	–	–	0·2	> 0·2	–
M. canis	0·05	0·05	> 0·2	0·1	0·2	0·05
M. gypseum	0·1	0·1	> 0·2	0·1	0·2	0·2
T. mentagrophytes	0·1	0·1	> 0·2	0·1	0·2	0·05

*Minimum inhibitory concentration not determined because inhibition zone diameters by disc diffusion test were < 12 mm.

against yeasts, whereas only that of *N. cataria* showed MIC values 0·1–0·2% (w/v) with all the moulds.

More relevant results were obtained with dermatophytes with MIC values ≤ 0·1% (w/v) for *H. italicum, Hier. pilosella, N. cataria and Pl. lanceolata*.

Phytochemical screening

The results of the phytochemical screening of diethyl ether extracts are listed in Table 3. Both chemical tests and TLC analysis showed the presence of flavonoids and terpenes in all the extracts, whereas coumarins, alkaloids and steroids were detected in five, two and one of six plant extracts, respectively.

The bioautography revealed clear zones of bacterial growth inhibition for *N. cataria, H. italicum* and *P. dodecandra* while, for *Hier. pilosella, L. caprifolium* and *Pl. lanceolata*, zones of inhibition were less visible.

DISCUSSION

The results obtained in this study indicate a considerable difference in antimicrobial activity between extracts obtained with method A and method B, the diethyl ether extracts (method B) being more active than the other extracts (method A).

This activity was more pronounced against fungal organisms and Gram-positive than against Gram-negative bacteria. The antifungal compounds of the plants assayed are not well known; however, the presence of flavonoids and terpenes and a certain degree of lipophilicity might determine toxicity by the interactions with the membrane constituents and their arrangement (Tomas-Barberan *et al.* 1990). The reason for the different sensitivity between Gram-positive and -negative bacteria could be ascribed to the morphological differences between these micro-organisms, Gram-negative bacteria having an outer phospholipidic membrane carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to lipophilic solutes, while porins constitute a selective barrier to the hydrophilic solutes with an exclusion limit of about 600 Da (Nikaido and Vaara 1985). The Gram-positive bacteria should be more susceptible having only an outer peptidoglycan layer which is not an effective permeability barrier (Scherrer and Gerhardt 1971). In spite of this permeability difference, the diethyl ether extracts (method B), also exerting some inhibition on Gram-negative bacteria and fungal organisms, have a broader spectrum of inhibitory activity than the other extracts (method A).

In conclusion, method B proved to be more efficient in extracting the active compounds and this could be ascribed to the acidified aqueous environment which promotes an easy extraction. The best results were obtained with the *H. italicum* extract against *B. subtilis* and *Staph. aureus* and the *N. cataria* extract against moulds. From the bioautography results, *H. italicum* and *N. cataria* showed well-defined inhibition bands in correspondence with those of flavonoids and terpenes and *P. dodecandra* in correspondence with flavonoid bands, whereas *Hier. pilosella, L. caprifolium* and *Pl. lanceolata* manifested less visible inhibition bands, with pronounced marginal growth. Previous studies demonstrated the presence of flavonoid components (Maffei Facino *et al.* 1990) and triterpenoic substances (Mezzetti *et al.* 1970) in *H. italicum.* Tomas-Barberan *et al.* (1990) reported the antimicrobial activity of *H. italicum* phenolic compounds. Other authors showed the presence of terpenes and isomers of nepetalactone in *N. cataria* and their bacteriostatic and fungistatic properties (Bourrel *et al.* 1993). However, it is difficult to compare the data with the literature because several variables influence the results, such as the environmental and climatic conditions of the plant and the choice of the extrac-

Table 3 Phytochemical screening of diethyl ether extracts

	Components					
Plant	Alkaloids a	Coumarins b	Flavonoids c	Steroids d	Terpenes e	Inhibitors
Helichrysum italicum	–	+	+	+	+	c*, e*
Hieracium pilosella	–	+	+	–	+	b†, c†, e†
Lonicera caprifolium	–	+	+	–	+	b†, c†
Nepeta cataria	+	–	+	–	+	c*, e*
Phytolacca dodecandra	+	+	+	–	+	c*, e†
Plantago lanceolata	–	+	+	–	+	b†, c†, e†

*Well-defined inhibition bands.
†Inhibition bands less visible.

tion method and antimicrobial test. Moreover, standard criteria for the evaluation of the plant activity are lacking and therefore the results obtained by different authors are widely different (Recio *et al.* 1989; Vanden Berghe *et al.* 1991).

The results obtained from *H. italicum* and *N. cataria* might be considered sufficient for further studies aimed at isolating and identifying the single active principle(s) and evaluating possible synergism of antimicrobial activity among these extracts. Investigations are in progress to determine the degree of toxicity of these extracts. The applications could concern either the natural preservation in the cosmetic and food industries or an accessible and safe alternative to synthetic antimicrobial drugs.

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