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Antimicrobial and Cytotoxic Activity of *Marrubium alysson* and *Retama raetam* Grown in Tunisia

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Abstract: Antibacterial and antifungal activities of extracts obtained from *M. alysson*, *R. raetam* were tested using a solid medium technique. We showed that the petroleum ether extract of *M. alysson* had a Minimum Inhibitory Concentration (MIC) varied from 128 to 2000 μg mL^{-1} against different *Enterobacteriaceae* and antifungal activity against *Candida glabrata*, *Candida albicans*, *Candida parapsilosis* and *Candida kreusei* with a MIC of 256 μg mL^{-1}. The ethyl acetate extract of *R. raetam* showed the best activity against Gram positive organisms with MICs of 128 to 256 μg mL^{-1} against methicillin resistant *Staphylococcus aureus* but low activity against the different Candida species.

Key words: *Marrubium alysson*, *Retama raetam*, antibacterial, antifungal, activity

INTRODUCTION

Medicinal plants which contain many active principles of therapeutic values have been used for centuries as treatments for many human diseases. *M. alysson* is a perinial herb of the *Lamiaceae* family which is commonly distributed in Europe, Mediterranean, Asia (Mabberly, 1997) and in Tuinisia. Its flowered aerial parts as well as their aqueous and hydroalcoholic extracts are used in traditional medicine for treating cough but also as laxative during digestive and biliary complaints (Wichtel and Anton, 1999). *Retama raetam*, locally named as 'R'tm', is a wild plant belonging to the Fabaceae family. It is common to North, East Mediterranean regions (Mittler *et al*., 2001; Elbahri *et al*., 1999) and in Tunisia. It is used for traditional treatment of some renal diseases. Since it was reported to show significant diuretic activity (Caceres *et al*., 1987) it could be also be useful for treatment of hypertension (Archer and Pyke, 1991; Izhaki and Neeman, 1997; Kassem *et al*., 2000; Taylor, 1981).

The aim of this study was to investigate the antimicrobial and antifungal activity of different extracts of *M. alysson* and of *R. raetam*.

MATERIALS AND METHODS

Plant materials: Plants materials were collected in 2004 in kerker region (Tunisia).

Plant extraction: The aerial part of the dried powdered of *M. alysson* and *R. raetam* (75 g) was extracted for 5 h with 1 L of petroleum ether, ethyl acetate and methanol successively by Soxhlet extraction. Extracts of each solvent were evaporated under reduced pressure and the final residues were used for the bioassays.

Microorganisms: The micro-organism strains employed in the biological assays are listed in Table 1. Different American Type Cell Culture (ATCC) reference bacteria and fungi were used as well as clinical isolates including methicillin susceptible (MSSA) and methicillin resistant *S. aureus* (MRSA) strains.

Determination of antimicrobial and antifungal activity: Various concentrations (1 μg mL^{-1} up to 10 mg mL^{-1}) of *M. alysson* and *R. raetam* extracts were used to determine the antimicrobial activity. Overnight broth cultures were adjusted to yield approximately 1×10^{6} CFU mL^{-1} of

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bacteria or yeast. The Minimal Inhibitory Concentrations (MICs) were determined on Brain Heart Infusion (BHI) agar plates (Bio-Rad, Marne la Coquette, France) by a standard method (NCCLS, 1997) with a Steers-type replicator device that delivered ca. 10^4 bacteria per spot. One milliliter of each of extracts previously dissolved in 10% Dimethyl sulfoxide (DMSO) were mixed for each concentration with 19 mL of BHI agar at 40°C and poured over Petri dishes. The resulting DMSO concentration was at most 0.5%. Plates containing only medium or medium with 0.5% DMSO were used as controls to ensure that DMSO did not affect growth, a standard antibiotic (oxacilin, amoxicillin, ticarcillin and cefotaxim) used in order to control the sensitivity of the tested microorganism. After 18 h of incubation at 37°C, the Minimum Inhibitory Concentration (MIC) was defined as the lowest concentration of the extract inhibiting the visible growth of each microorganism. Each test was carried out in triplicate. The microorganisms tested in this study were provided from European Hospital of George Pompidou (HEGP) (France).

Cytotoxicity assay: The cytotoxicity were evaluated on VERO cells (a line of green monkey kidney cells from ICN-Flow), grown on Dulbecco's modified MEM with 2% foetal calf serum (both from Gibco). The assays were performed in duplicate in 24- well plates with about 5 10^4 cell per well. Cytotoxicity was read after 48 h of incubation in an atmosphere of 5% CO_2 at 37°C, as the inhibition of cell multiplication in the presence of decreasing amounts of the compounds under study, with the use of a light microscope. The Minimal Toxic Dose (MTD) was considered to be the dose of the compounds, which reduced cell multiplication by at least 50%, as compared to control (Fioravantin *et al*., 1997).

Phytochemical screening: The screening of the chemical composition was carried out with the methanol extract using chemical methods (Allen, 1974; Harbone, 1976)

RESULTS

Antibacterial activities were tested with the petroleum ether, ethyl acetate and methanol extracts of *M. alysson* and *R. raetam*. Only active plant extracts are given in Table 1 witch summarized the in vitro inhibitory activity of the aerial part extracts of *M. alysson* and *R. raetam*. The aerial part extracts of *M. alysson* and *R. raetam* were active against Gram positive and Gram negative bacteria. Among the different extracts, the petroleum ether extract of *M. alysson* showed the most significant antimicrobial activity against *E. cloacae*, *K. pneumoniae* and *S. marcescens* with MICs of 0.128-0.256 mg mL^{-1}. It also showed the best activity against the different *Candida*

Table 1: Antibacterial activity of *Marrubium alyson* and *Retama raetam* against bacteria

		MIC range of plant extract (mg mL^{-1})			MIC of antibiotic (μg mL^{-1})[a]				
Strains	No. of strains	*M. alysson* Ep[b]	*M. alysson* Ea[c]	*R. raetam* EA[d]	OXA	AMX	TIC	CTX	AB
Gram negative bacteria									
Escherichia coli ATCC25922	1	2	8	>8	ND[e)]	8	ND	0.094	ND
Escherichia coli HEGP402, HEGP3815	2	2	8	>8	ND	[2;>256]	ND	0.094	ND
Klebsiella pneumoniae HEGP8326, HEGP2143, HEGP3142	3	0.256	[0.512-4]	[1-2]	ND	>256	ND	0.064	ND
Enterobacter cloacae HEGP4102, HEGP1001, HEGP7830	3	0.128	[0.512-1]	2	ND	>256	ND	[0.125;0.32]	ND
Serratia marcescens HEGP1002	1	0.128	0.51	2	ND	>256	ND	>256	ND
Acinetobacter baumannii HEGP1003	1	2	8	>8	ND	ND	1	ND	ND
Gram positive bacteria									
Bacillus subtilus ATCC6633	1	0.128	0.512	0.256	ND	0.064	ND	3	ND
Staphylococcus aureus ATCC25923	1	2	8	0.512	0.19	ND	ND	ND	ND
Staphylococcus aureus ATCC29213	1	2	8	0.512	0.256	ND	ND	ND	ND
Staphylococcus aureus MSSA[f] HEGP8064, HEGP8213, HEGP3167	3	2	8	0.128	[0.38-0.75]	ND	ND	ND	ND
Staphylococcus aureus MRSA[g] HEGP4945, HEGP4360, HEGP3828, HEGP3807, HEGP1008	5	[1-2]	[1-8]	[0.128-0.256]	[8-16]	ND	ND	ND	ND
Streptococcus pyogenes HEGP1004	1	0.128	0.512	0.256	ND	ND	ND	ND	ND
Streptococcus agalactiae HEGP1005, HEGP4249, HEGP5839	3	0.256	1	0.256	ND	0.094	ND	[0.032;0.064]	ND
Enterococcus faecalis ATCC29212	1	>8	8	2	ND	1	ND	ND	ND
Enterococcus faecalis HEGP7980, HEGP7476	2	>8	8	2	ND	1	ND	ND	ND
Enterococcus faecium HEGP1007, HEGP3044	2	>8	8	2	ND	[32>256]	ND	ND	ND
Corynebacterium sp. HEGP1006	1	1	1	0.064	ND	>256	ND	ND	ND

[a]The most appropriate reference antibiotic was chosen. OXA, oxacillin; AMX, amoxicillin; TIC, ticarcillin; CTX, cefotaxim, AB; amphotericin B; [b]*Marrubium alyson* petroleum ether extract; [c]*Marrubium alyson* ethyl acetate extract; [d]*Retama raetam* ethyl acetate extract; [e]Not done; [f]Strain, sensitive to methicillin; [g]Strain resistant to methicillin

Table 2: Antifungal activity of *Marrubium alyson* and *Retama raetam*

	MIC of plant extract (mg mL^{-1})				MIC of antibiotic (μg mL^{-1})
Yeast	No. of strains	*M. alysson* EP[a]	*M. alysson* EA[b]	*R. raetam* EA[c]	AB
Candida glabrata ATCC90030	1	0.256	0.512	2	0.5
Candida albicans ATCC90028	1	0.256	2	2	0.5
Candida parapsilosis ATCC 22019	1	0.128	1	4	0.5
Candida kreusei ATCC6258	1	0.256	1	4	0.5

AB: amphotericin B; [a]*Marrubium alyson* petroleum ether extract; [b]*Marrubium alyson* ethyl acetate extract; [c]*Retama raetam* ethyl acetate extract

Table 3: Cytotoxic activity of plants extracts

Extracts	MTD 50 (mg mL^{-1})
M. alysson EP[a]	5
M. alysson EA[b]	10
R. raetam EA[c]	1.25

MTD5O: Minimal Toxic dose which inhibit 50 cellular growth; [a]*Marrubium alyson* petroleum ether extract; [b]*Marrubium alyson* ethyl acetate extract; [c]*Retama raetam* ethyl acetate extract

Table 4: Chemical screening of methanolique plants extracts

Extracts	Chemical constituents
M. alysson	Alcaloids, diterpenoids and saponosids
R. raetam	Flavonoids, tannins and alcaloids

species. The ethyl acetate extract of *R. raetam* was the most active against Gram positive bacteria and in particular *S. pyogenes*, *S. agalactiae*, MSSA and MRSA with MICs being generally less than 1 mg mL^{-1}. We observed that the ethyl acetate extract of *M. alysson* had a moderate activity in candida species with MIC ranges from 0.512 to 1 mg mL^{-1}. The *Candida* species were resistant to the ethyl acetate extract of *R. raetam* with MIC ranges from 2 to 4 mg mL^{-1} (Table 2). These extracts have a good antibacterial activity because they aren't cytotoxic (Table 3). The chemical screening show that *M. alysson* contained alkaloids, diterpenoids and saponosids, but *R. raeatm* is rich with flavonoids, tannins and alcaloids (Table 4).

DISCUSSION

The results obtained could indicate the existence of antibacterial compounds in the aerial part extracts of *M. alysson* and *R. raetam*. Curiously, only a low activity was recorded against *E. coli* the most prevalent *Enterobacteriaceae*. These results give evidence to the ethnotherapeutic claims for treating skin diseases (Venkatesan *et al*., 2005) with such plants since *S. aureus* and *S. pyogenes* are the primary causative agents of skin and soft tissues infections. The activities observed for some of the different extracts studied in this study is in support for further isolation of the compounds responsible for the observed antimicrobial activity which includes that against *S. aureus*, streptococci and *Candida* sp. In particular the activity of the petroleum ether extract of *M. alysson* against some *Enterobacteriacae* could provide future active principles for the eradication of some pathogens responsible for urinary tract and nosocomial infections (Prescot *et al*., 1999; Stanier *et al*., 1986). It is interesting to note that these extracts were able to inhibit some of the bacteria and yeast at concentration much lower than the toxic dose. The screening of the chemical groups in the extracts of *M. alysson* revealed the presence of tannins, alkaloids and flavonoids. Tsuchia *et al*. (1999) linked the antimicrobial effects of flavonoids to their capacity to form complexes with extracellular and soluble proteins and with the cell wall; we suggest that presence of such compounds in *Marrubium alysson* extracts and *Retama raetam* extract may play a role in their observed antifungal and antibacterial activities. However, Cown (1999) showed that tannins are able to inhibit the growth of the hypes of many fungi; these molecules are used as antimicrobial agents (Scalbert, 1991).

CONCLUSIONS

In conclusion, our biological study on *M. alysson* and *R. raetam* plants showed that the ethyl acetate extract of *R. raetam* showed the best activity against Gram positive organism especially against methicillin resistant *Staphylococcus aureus* but it showed low antifungal activity, compared with the petroleum ether extract of *M. alysson* witch had an interesting antibacterial activity with MIC varied from 128 to 2000 μg mL^{-1} against different enterobacteriaceae and had good antifungal activity with MIC between 128 and 256 μg mL^{-1}.

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