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*Full Length Research Paper*

# Antimicrobial activity of *Rhaponticum acaule* and *Scorzonera undulata* growing wild in Tunisia

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**This study examined the *in vitro* antibacterial and antifungal activities of the extracts (butanolic, ethyl acetate, petroleum ether and the product H2) of 2 plants belonging to the Asteraceae family: *Rhaponticum acaule* L. and *Scorzonera undulata* L. Butanolic and ethyl acetate extracts of the *Rhaponticum acaule* plant showed a moderate antibacterial activity against 3 of the tested strains; *Staphylococcus aureus*, *Escherichia coli* and *Enterococcus fecalis* while *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Citrobacter freundeï* were resistant to the extracts. The product H2 showed an antibacterial activity against *S. aureus*, *C. freundeï* and *E. fecalis*. From the results of the antifungal activity, we observed that butanolic and ethyl acetate extracts of *R. acaule* showed a strong inhibition against *Trichophyton rubrum* with inhibition percentage of 56.25 and 78.75%, respectively. Butanolic extract showed a moderate inhibition of *Microsporum canis*, *Scopulariopsis brevicaulis* and *Aspergillus fumigatus* while ethyl acetate extract showed low inhibition. The aerial part ethyl acetate extract of *S. undulata* seemed to be more active than the petroleum one. It showed an antibacterial activity against all bacteria strains tested except for *E. coli.* For antifungal activity, the petroleum ether and ethyl acetate extracts of the aerial part inhibited strongly all tested fungi. The roots extracts from *S.undulata* show weak antimicrobial activities compared with the aerial parts extracts of the same plant.**

**Key words:** *Rhaponticum acaule* L, *Scorzonera undulata* L, Asteraceae, antibacterial activity, antifungal activity, Tunisia.

## INTRODUCTION

This present study is another contribution to the valorization of Tunisian National Patrimony (Boussaada et al., 2008; Braham et al., 2001; Boukamcha et al., 2004; Bel haj salah et al., 2006; Oueslati et al., 2004; Hichri et al., 2003; Nacef et al., 2003; Hichri et al., 2005). *Rhaponticum acaule* also known as *Lenzea acaulis* L. or *Centraurea chamaerhaponticum* Ball., is one of the most conspicuous aromatic plants of early spring flowering from March to May . It grows wild in rosette on the slopes of hills, fields and in sandy pastures. It is a mono specific genus belonging to the Asteracea family. It is a North African endemic species distributed in the north and central area of Tunisia (Pottier - Alapetite, 1981). *R. acaule* is a fragrant and perennial herb with yellow flower, large and pinnatisect leaves. The capitula are big with fleshy and hairy receptacles.

Le Floc'h (1983) did not report about its possible use in popular medicine in Tunisia. However, many species of the genus *Rhapanticum* have long been used in traditional medicine. Indeed, the root of *Rhaponticum*

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*uniflorum* has been used against intoxication and for the treatment of fever. It has been demonstrated that this species inhibits peroxydation of membrane lipids and possesses antiatherotic activity (Zhang et al., 2002).

Chemical composition and antimicrobial activity of volatile components from capitula and aerial parts of *R. acaule* has been described in one study of Mighri et al. (2008). From the 57 identified constituents, representing 95.5 and 96.3% of two oils, respectively, methyl eugenol, epi-13 manool, b- ionone, b-bisabolol, 1-octadecanol, phytol and farnesyl acetate were found to be the main components. Furthermore, the oils were tested against 6 gram-positive and gram-negative bacteria and four phytopathogenic fungi. It was found that oils from both parts of *R. acaule*, and especially that of capitula, exhibited interesting antibacterial activity, but no anti-fungal activity was observed (Boussaada et al ., 2008) .

*S. undulata* is another perennial asteraceae species (D'amato, 2000). It is a diploid and a very polymorphic plant; it grows in the pastures, hills and sandy clay alluvium (Pottier- Alapetite, 1981).

*S. undulata* is mainly used as food. In Tunisia, the roots are appreciated for their sweetness; they are either eaten raw or cooked in water (Le Floc'h, 1983). They are also used to prepare a decoction for its benefits as depurative. The ashes of burned roots would be effective in the treatment of burns (Boukef, 1986).

The chemical composition and antimicrobial activities of volatile components from capitula and aerial parts of *S. undulata* have been described (Mighri et al., 2008) . In fact 36 constituents were identified in the oils and the main components of them were methyl hexadecanoate (30.4%), methyl linolenate (23.9%) and heneicosane (12.2%). The *S. undulata sub sp. deliciosa* oil exhibited an interesting antibacterial activity against gram-positive and gram -negative bacteria but no antifungal activity was detected.

Other studies have been made on other species of the family Asteraceae. In fact Mighri et al. (2001) reported that essential oils from *Inula graveolens* had an antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. *Cardopatium amethystinum*, another species of the Asteraceae family, showed an antibacterial activity against *S. aureus*, *Escherichia coli* and *Salmonella typhimurium* (Boukamcha et al., 2004).

This study was focused on the antimicrobial activity of the extracts from the two species and the secondary metabolic compound isolated from *R. acaule*.

## MATERIALS AND METHODS

### Plant material and extraction

*R. acaule* was gathered at the flowering stage in May 2005 in the area of Chebba (North east Tunisia) and *S. undulata* in April 2008 in the area of Amira (North east Tunisia). A voucher specimen, Rhap 1170 and SCR 1171 were respectively deposited in the Laboratory of Natural substances chemistry and organic synthesis, Faculty of sciences, Monastir, Tunisia.

The plant samples were air -dried for several weeks. Powdered plant tissues were extracted 3 times by maceration with methanol, the resultant extract was concentrated under reduced pressure. The methanol extract was extracted successively with equal volume of 3 organic solvents of increasing polarity (petroleum ether, chloroform and ethyl acetate). Each fraction was taken to dryness under vacuum and stored at 4°C until tested.

### Antifungal activity

The extracts were individually tested against 5 strains of fungi that comprised two opportunist pathogenic yeasts (*Candida albicans* and *Cryptococcus neoformans*), two dermatophytes (*Trichophyton rubrum*, *Microsporum canis*) and one hyphamycet (*Scopulariopsis brevicaulis*). The micro-organisms were obtained from the laboratory of the transmissible diseases and biologically active substances, Faculty of Pharmacy Monastir.

Antifungal activity was assayed by the agar incorporation method (dilution in a solid medium) including a negative control, as described previously (Bel haj salah et al., 2006). Briefly, the test was performed in sterile Petri dishes (33 mm) that contained Sabouraud Glucose Agar (SGA) . Samples were mixed aseptically with SGA (100 ml) to give stocks with concentrations of 1000 µL/ml. Stocks were dissolved in 10% DMSO(Dimethyl sulfoxide), and this solvent was used as the negative control. After cooling and solidification, the medium was inoculated with a small amount (5 mm) of a 7 day old mycelium culture (for dermatophytes), a 3 day culture suspension adjusted to $10^5$ conidies/ml (*Aspergillus* and *Scopulariopsis*) or a 3 day culture suspended in sterile distilled water and adjusted to $10^5$ spores/ml (yeasts). The Petri dishes were then incubated for 7 days at 24°C for dermatophytes, 24 h at 37°C for *Candida* and *Aspergillus* and 48 h at 37°C for *Cryptococcus*. 3 replications were carried out for each concentration and for each micro-organism.

The antifungal activity of the extracts was evaluated using two methods: (a) by calculating the percentage inhibition (%I) from the diameters of the colonies in the control plate (dC) and the colonies in the treated plate (dE); %I= (dC-dE)/dC, according to the method of Singh et al. (1993); (b) by determining the minimal inhibitory concentration (MIC), defined as the lowest concentration which inhibits the visible growth of fungi during the defined incubation period for each species.

### Antibacterial activity

The antibacterial activity of extracts was evaluated by paper disc diffusion and dilution methods against 6 selected gram-positive and gram-negative species: *S. aureus* ATCC 25923, *E. fecalis* ATCC 29212, *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *C. freundeï* and *Proteus mirabilis*.

#### *Agar diffusion method*

The qualitative antibacterial assay of the extracts of *R. acaule* and *S. undulata* was carried out by the disc diffusion method (Marmonier, 1987) . All the microorganisms mentioned above were incubated at 37°C for 24 h by inoculation into Mueller-Hinton (M-H) broth. The culture suspensions were prepared and adjusted to

**Table 1.** Antibacterial activity of *Rhaponticum acaule* L. and *Scorzonera undulata L*.

| | ***R. acaule* L.** | | | | | | ***S. undulata L.* aerial parts** | | | | ***S. undulata L.* root parts** | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Butanolic extract** | | **Ethyl acetate** | | **Compound H2** | | **Ethyl acetate** | | **Petroleum ether** | | **Ethyl acetate** | | **Petroleum ether** | |
| | **IZ** | **MIC** | **IZ** | **MIC** | **IZ** | **MIC** | **IZ** | **MIC** | **IZ** | **MIC** | **IZ** | **MIC** | **IZ** | **MIC** |
| Pa | - | nt | - | nt | - | nt | 9.5 | 1000 | 8.5 | 2000 | - | nt | - | nt |
| Sa | 8 | 2000 | 11 | 500 | 8 | 2000 | 10 | 1000 | 9 | 1000 | 8.6 | 2000 | 10.5 | 500 |
| Pm ([a]) | - | nt | - | nt | - | nt | 8 | 2000 | - | nt | - | nt | - | nt |
| Cf ([a]) | - | nt | - | nt | 8 | 2000 | 8 | 2000 | 9 | 1000 | 9 | 1000 | 10 | 1000 |
| Ef | 11 | 500 | 9 | 1000 | 9 | 1000 | 8 | 2000 | - | nt | 9 | 1000 | 8 | 2000 |
| Ec | 9 | 1000 | 8 | 2000 | - | nt | - | Nt | - | nt | - | Nt | - | nt |

Pa, *Pseudomonas aeruginosa* ATCC 27853; Sa, *Staphylococcus aureus* ATCC 25923; Ef, *Enterococcus fecalis* ATCC 29212; Cf, *Citrobacter freundeï* ([a]); Ec, *Escherichia coli* ATCC 25922; Pm, *Proteus mirabilis*([a]). (-), absence of antibacterial activity; IZ, inhibition zone (mm); nt, not tested; MIC, minimal inhibitory concentration (µg/mL) (ATCC), American type culture collection; ([a]): clinical laboratory strains of Bacteriology, University Hospital Fattouma Bourguiba, Monastir.

approximately $10^6$ CFU/ml of bacteria.

200 µl of the inoculums were spread over plates that contained sterile Mueller–Hinton agar (pH 7.2) and wattman disc paper (6 mm) inoculated with 10 µl of each fraction (petroleum ether and butanolic fractions) and was placed on the surface of the media. The plates were left for 30 min at room temperature to allow the diffusion of the extract and were incubated at 37°C for 18 h. At the end of that period, the inhibition zone around the disc was measured. 2 controls were also included in the test; a positive control without extracts and a reference control using one standard antibiotic (Gentamicin10 µg), to evaluate the susceptibility of the tested strains. The experiments were run in triplicate, and the developed inhibition zones were compared with those of reference discs.

***Micro-well dilution assay***

The minimal inhibition concentration (MIC) values were also studied for the microorganisms which were determined as sensitive in disc diffusion assay (diameter superior to 8 mm). The inocula of microorganisms were prepared from 12 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. The extracts of *R.acaule* and *S.undulata* dissolved in 10% dimethylsulfoxide (DMSO) were first diluted to the highest concentration (20 mg/ml) to be tested, and then serial two-fold dilutions were made in a concentration range from 2.5 to 20 mg/ml in 10 ml sterile test tubes with nutrient broth. MIC values were determined based on a micro-well dilution method (Sahin et al., 2002; Gulluce et al., 2004; Gulluce et al., 2004). The 96-well plates were prepared by dispensing into each well 95 µl of nutrient broth and 5 µl of the inoculum. 100 µl from extracts initially prepared at the concentration of 5 mg/ml were added into the first wells. Then, 100 µl from their serial dilutions was transferred into 6 consecutive wells. The last well with 195 µl of nutrient broth without compound and 5 µl of the inoculum on each strip was used as negative control. The final volume in each well was 200 µl. Gentamicin at a concentration range of 0.156-20 mg/ml was prepared in nutrient broth and used as standard drug for positive control. The plate was covered with a sterile plate sealer. Contents of each well were mixed on a plate shaker at 300 rpm for 20 s and then incubated at 37° C for 24 h.

Microbial growth was determined by absorbance at 600 nm and confirmed by plating 5 µl samples from clear wells on Muller Hinton Agar medium. The extract tested in this study was screened 2 times against each organism. The MIC was defined as the lowest concentration that inhibited the growth of microorganisms.

## RESULTS AND DISCUSSION

### Antibacterial activity

The antibacterial activity of *R. acaule* and *S. undulata* was tested *in vitro* by disc diffusion and liquid dilution methods.

The antibacterial MIC values of the ethyl acetate and butanolic extract of R*. acaule* are presented in Table 1.

Results presented in Table 1 shows that *S. aureus*, *E. coli* and *E. fecalis* were most sensitive to the ethyl acetate extract whereas the 3 bacteria, *P. mirabilis*, *P. aeruginosa* and *C. freundeï* strains were most resistant to the two extracts (ethyl acetate and butanolic extract) of the plant *R. acaule*. According to several authors, these gram-negative bacteria appeared to be the least sensitive to the action of many other plants extracts and tested compounds (Boukamcha et al., 2004). The high resistance of gram- negative bacteria could be due to the differences in the cell membrane of these bacterial groups. Indeed, the external membrane of gram-negative bacteria renders their surfaces highly hydrophilic (Smith-Palmer et al., 1998) whereas the lipophilic ends of the lipoteichoic acids of the cell membrane of gram positive bacteria may facilitate penetration of hydrophobic compounds (Boussaada et al., 2008).

**Table 2.** Antifungal activity of *Rhaponticum acaule* L. and *Scorzonera undulata* L.

| Sample | Extract concentration and MIC | | Asper(b) | M. canis(b) | T. rub(b) | Scop(b) | C. albi | C. neof |
|---|---|---|---|---|---|---|---|---|
| *Rhaponticum* | Butanolic | I% | 43.54 | 46.42 | 56.25 | 38.63 | 0 | 0 |
| *acaule* L. | extract | MIC | 800 | 700 | 700 | 850 | >1000 | >1000 |
| | Ethyl | I% | 11.95 | 10 | 78.75 | 2.27 | 0 | 0 |
| | acetate | MIC | 900 | >1000 | 500 | >1000 | >1000 | >1000 |
| *Scorzonera undulata* L. aerial parts | H2 | I% | 28.06 | 35.71 | 25.71 | 42.72 | 0 | 0 |
| | | MIC | 850 | 850 | 850 | 800 | >1000 | >1000 |
| | Ethyl | I% | 41.07 | 64.24 | 42.85 | 27.08 | 0 | 0 |
| | acetate | MIC | 800 | 700 | 800 | 850 | >1000 | >1000 |
| | Petroleum | I% | 48.21 | 78.57 | 58.92 | 53.57 | 0 | 0 |
| | ether | MIC | 750 | 500 | 700 | 700 | >1000 | >1000 |
| *Scorzonera* | Ethyl | I% | 3.57 | 10.71 | 10.7 | 21.42 | 0 | 0 |
| *undulata L.* | acetate | MIC | >1000 | >1000 | >1000 | 950 | >1000 | >1000 |
| root parts | Petroleum | I% | 17.85 | 46.42 | 46.42 | 21.42 | 0 | 0 |
| | ether | MIC | 900 | 800 | 800 | 950 | >1000 | >1000 |

***Asper*** , *Aspergillus fumigatus ;* ***M.canis,*** *Microsporum canis ;****T. rub,*** *Trychophyton rubrum ;* ***Scop,*** *Scopulariopsis brevicaulis;* ***C. albi,*** *Candida albicans;* ***C. neof,*** *Cryptococcus neoformans.* MIC, Minimum inhibitory concentration (µg/ml); I%, Percentage inhibition of microorganisms. Inhibitory power was interpreted as follows: 0-25%: no or little inhibition; 26-50%: average inhibition; 51-100%: strong inhibition; (b), microorganisms from Laboratory of the Transmissible Diseases and Biologically Active Substances, Faculty of Pharmacy Monastir.

In addition, the compound H2 showed an antibacterial activity against *S. aureus*, *C. freundeï* and *E. fecalis* at different levels.

Table 1 shows that ethyl acetate extract showed antibacterial activity against 3 tested strains with MIC of 500 µg/ml, 1000 µg/ml and 2000 g/ml against *S. aureus*, *E. fecalis* and *E. coli*, respectively while *E. fecalis, E. coli, S. aureus* were sensible to the butanolic extract with MIC of 500 g/ml, 1000 g/ml and 2000 g/ml, respectively. The product H2 had MIC of 1000 g/ml against *E. fecalis* and 2000 g/ml respectively for *S. aureus* and *C. freundeï* but no activity was detected against *P. aeruginosa, P. mirabilis* and *E. coli.* The MIC results confirmed those found by the method of disc (Table 1).

According to the results in Table 1, it is that the aerial part ethyl acetate extract of *S. undulata* has an antibacterial activity against five strains (*P. aeruginosa*, *S. aureus*, *E.* fecalis, *C. freundeï* and *P. mirabilis*) with MIC which varied from 1000 to 2000 g/ml. While the petroleum ether extract from the aerial part showed only antibacterial activity against three strains (*P. aeruginosa*, *S. aureus* and *C. freundeï*). These results confirm those found by the method of disc (Table 1).

The 2 extracts (ethyl acetate and petroleum ether) from the root parts of *S. undulata* presented an antibacterial activity against 3 strains (*S. aureus*, *E. fecalis* and *C. freundeï*). However, the petroleum ether extract exhibited antibacterial activity greater than the ethyl acetate extract with a MIC of 500 g/ml against *S. aureus*. Moreover, all fractions appeared very active against the tested gram-positive than gram-negative bacteria. This result was in agreement with many studies on other plant species belonging to Asteraceae family (Boukamcha et al., 2004; Oueslati et al., 2004).

## Antifungal activity

The antifungal activities of the extracts of *R. acaule* and *S. undulata* are presented in Table 2. Both extracts from *R. acaule* showed a strong inhibition against *T. rubrum* with a percentage inhibition of 56.25% (MIC 700 µg/ml) for the butanolic extract and 78.75% (MIC 500 µg/ml) for the ethyl acetate extract while the other fungi (*M. canis*, *S. brevicaulis* and *A. fumigatus*) tested with the butanolic extract showed a moderate inhibition which became low when tested with the ethyl acetate extract. The compound H2 had an average inhibition against *S. brevicaulis* (42.72%) and *M. canis* (35.71%) with MIC of 800 µg/ml. The MIC results reflect those obtained using the 1% method (Table 2).

Antifungal values of the ethyl acetate and petroleum ether of aerial and root parts of *S. undulata* are presented in Table 2.

The petroleum ether extract from aerial part strongly inhibited 3 fungi; *A. fumigatus* (48.21%), *T. rubrum* (58.92%), *S. brevicaulis* (53.57%) and showed a very good inhibition against *M. canis* (78.57%) with MIC values from 500 to750 µg/ml. *M. canis* was also strongly inhibited by the ethyl acetate extract of the aerial part with a percentage inhibition of 64.24% and MIC of 700 µg/ml.

Root ethyl acetate extract, showed a low inhibition (3.57%) of *A .fumigatus* while *M. canis* (46.42%) and *T. rubrum* (46.42%) were moderately inhibited by the root petroleum ether extract with MIC of 800 µg/ml but all the extracts of the 2 species have no activity against *C. albican* and *C. neoformans*

The antibacterial and antifungal results of the aerial part of the plant *S. undulata* are more significant than those of the root parts; this can be explained by the presence of chemical compounds that have antibacterial and antifungal activity in that part of the plant. Indeed, the first chemical composition analysis and antimicrobial activity report on the aerial part of *S. undulata* presented an antibacterial activity and no antifungal activity (Boussaada et al., 2008).

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