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ORIGINAL PAPER

Actinomycetes from Moroccan habitats: isolation and screening for cytotoxic activities

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Abstract In our screening for actinomycetes showing cytotoxic activities, 8 samples were collected from various Moroccan habitats, 136 isolates were tested for their capacity to produce antibacterial compounds against gram positive bacteria. Thirty-seven strains of these isolates were active against Gram-positive bacteria. Using the following steps of primary screening: antibacterial activity, confrontation between the isolates and toxicity to *Artemia salina*; fifteen different isolates were used for further investigation. The aqueous extracts of *Streptomyces* sp. T5 and *Streptomyces* sp. AS8 were selected for their cytotoxic activity against Hep2, BSR and P815 cell lines, and two active compounds were observed on HPLC. The two isolates exhibited high activity against human cancer cell lines and were inactive on PBMC cell lines. Furthermore, the *Streptomyces* sp. T5 extract showed a proliferative activity.

Keywords Actinomycetes · Antibacterial · Cytotoxic activity · Moroccan habitat · Screening

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Introduction

Cancer still represents one of the most serious human health problems despite the great progress in understanding its biology and pharmacology. The usual therapeutic methods for cancer treatment are surgery, radiotherapy, immunotherapy and chemotherapy (Cocco et al. 2003). These techniques are individually useful in particular situations and when combined, they offer a more efficient treatment for tumors. An analysis of the number of chemotherapeutic drugs and their sources indicates that over 60% of approved drugs are derived from natural compounds (Cragg et al. 1997; Newman et al. 2003) and many have been extracted from actinomycetes (Mendez and Salas 2001).

Actinomycetes are an important source of new bioactive compounds such as antibiotics and enzymes (Vining 1992; Edwards 1993; Demain 1995; Xu et al. 2005) which have diverse clinical effects and are active against many kinds of organisms (bacteria, fungi, parasites etc.). In fact more than 50% of the known natural antibiotics produced are from actinomycetes (Miyadoh 1993).

Antitumor antibiotics produced by actinomycetes are among the most important cancer chemotherapeutic agents including members of the anthracycline, bleomycin, actinomycin, mitomycin and aureolic acid families (Rocha et al. 2001; Newman and Cragg 2004).

In screening for actinomycetes able to produce bioactive compounds, the exploration of new soils and habitats has been recommended (Nolan and Cross 1988; Takahashi and Omura 2003). In this context, Moroccan habitats, particularly the rhizosphere of endemic plants, might be a rich source of actinomycetes species producing antibacterial and antifungal compounds (Ouhdouch et al. 2001; Barakate et al. 2002). However, the cytotoxic activity of actinomycetes from this habitat has not been investigated.

In the present work, actinomycete strains isolated from Moroccan habitats were selected and tested for their capacity to produce compounds active against gram-positive bacteria and substances cytotoxic against Hep2, BSR, P815 tumor cell lines as well as normal human peripheral blood mononuclear (PBMC) cells.

Materials and methods

Sampling

Samples were collected using the Pochon and Tardieux method (1962) from various Moroccan habitats: rhizospheric soil of *Stipa tenacissima* (ST); *Argania spinosa* (AS); *Vitis vinifera* (S); soil of Tensift (T), arid soil of Tinjdad (TD), camel's dung (CD), soil contaminated with olive oil mill wastewaters (OM) and soil of Rachidia (R).

The samples from each of the rhizospheric soils were taken with an auger (up to 10 cm depth) after removing approximately 3 cm of the soil surface. Samples were placed in sterile polyethylene bags, closed tightly and stored in the refrigerator at 4°C until use.

Isolation of actinomycetes

Samples of each soil were first mixed, suspended in sterile distilled water (1 g in 100 ml) homogenized by vortexing and finally treated 10–15 min by sonication according to Ouhdouch et al. (2001). All treated samples were serially diluted up to 10^{-6} and spread (0.1 ml) over the surface of soil extract agar (Barakate et al. 2002) and Actinomycetes Isolation Agar (Olson's medium) (Olson 1968) (5% glycerol, 0.2% sodium caseinate, 0.01% L-asparagin, 0.4% sodium propionate, 0.05% K_2HPO_4, 0.0001% $FeSO_4$ and 1.5% agar Difco). The two media were supplemented with 40 µg/ml actidione to inhibit the development of fungi (Olson 1968), and 10 µg/ml nalidixic acid to inhibit the bacteria capable of swarming, without affecting the growth of actinomycetes (Nonomura and Hayakawa 1988; Bulina et al. 1997).

The plates were incubated at 28°C for three weeks. Actinomycetes were recognized on the basis of morphological features following directions given by International *Streptomyces* Project (ISP) (Shirling and Gottlieb 1966). All observed colonies were isolated, purified and conserved in 20% Glycerol at −20°C.

Antibacterial activity on solid media

The antibacterial activity against *Bacillus subtilis* ATCC 9524, *Bacillus cereus* ATCC 14579, *Micrococcus luteus* ATCC 10240 was determined by the plate diffusion method (Bauer et al. 1966). The isolates were grown on three different media: Bennett medium (B) (beef extract (Merck,Germany) 1 g/l; glucose (Merck) 10 g/l; peptone (Merck) 2 g/l; yeast extract (Merck) 1 g/l and agar (Difco) 15 g/l), nutrient agar (NA) (Difco) and GBAm (glycerol (Sigma) 20 g/l; starch (Riedel de Haën) 20 g/l; beef extract (Biokar Diagnostics) 5 g/l; $CaCO_3$ 3 g/l; peptone (Merck) 10 g/l and agar (Difco) 15 g/l). After 14 days, three discs (10 mm in diameter) were cut and placed on nutrient agar that was seeded with the test organism. Plates were first kept in a refrigerator (4°C) for at least 2 h to allow the diffusion of the produced antibiotics, and then incubated at 30°C. Inhibition zones were determined after 24 h.

Confrontation between the isolates of actinomycetes

The diversity of the active isolates was tested by the confrontation test. Isolates were suspended in distilled water and inoculated in the surface of nutrient agar or Bennett agar by streaking the tested isolate. After 24 h of incubation at 28°C, the other isolates of actinomycetes were inoculated in perpendicular directions of the tested isolate and incubated at 28°C for 7 days (Highley and Ricard 1988). The diversity in producing antibiotics is expressed by the inhibition zones between the different isolates.

Fermentation, extraction and antibacterial activity

Active isolates of actinomycetes were inoculated into an Erlenmeyer flask containing 200 *M. luteus* of Bennett or nutrient broths. The cultures were incubated for 7 days at 28°C on a rotary shaker. The active supernatants were later extracted with ethyl acetate (v/v) and *n*-butanol (v/v). The aqueous extract phase was concentrated in rotary evaporator to 5 *M. luteus* and the organic extracts were evaporated to dryness and suspended in DMSO 1% (5 ml). The antibacterial activity was tested against *Micrococcus luteus* (ML). The aqueous extracts activities were determined using the agar well diffusion method (Parente et al. 1994); the organic extracts activities were determined using disc diffusion method (Amade et al. 1994).

Toxicity to *Artemia salina*

Larvae of *Artemia salina* (24 h after egg rupture) were obtained as described by Harwig and Scott (1971) and Eppley (1974). For the toxicity test, different dilutions in seawater of organic and aqueous extract were transferred to 24-well cell culture plates. Second instar larvae of *A. salina* were then added. After incubation at 25°C for 24 h, the survivors were counted in each well and the total number of *A. salina* was counted after killing the surviving by the chloroform. Controls with and without DMSO 1% were run simultaneously and the number of live larvae was calculated by subtraction. These experiments were carried out in three replicates.

Cell lines and culture

BSR cells (kidney carcinoma of hamster), Hep2 (human laryngeal carcinoma) and P815 (murine mastocytoma) come from the stock of the Laboratory of Immunology, Biochemistry and Molecular Biology of the Faculty of Sciences and Technologies, Beni Mellal, Cadi Ayyad University, Morocco. These cells were cultured at 37°C in humidified atmosphere with 5% CO2 in complete culture medium (Dulbecco' s Modified Eagles Medium (D-MEM) supplemented with 5% of foetal calf serum, and 100 UI/ml of penicillin and 100 µg/ml streptomycin, 0.2% sodium bicarbonate).

Methyl thiazole tetrazolium cell viability test (MTT)

This test was performed as previously described (Mosmann (1983). Briefly, tumor cells were trypsinized, when adherent, (0.15% trypsin, 0.1% EDTA) and 1–1.5 × 10^5cells/ml were incubated in flat-bottomed 96-well microtiter plates (Bioster, Bastia di Rovolon, Italy) in 100 µl of complete medium. Appropriate dilutions of each extract and adriamycin were carried out in culture medium before their addition to the cultured cells (final volume of 200 µl). After 48 h of incubation in humidified atmosphere at 37°C, and 5% CO2, 20 µl of MTT (5 mg/mlPBS) were added in each well. After 3 h incubation at 37°C and 5% CO2, 100 µl medium was carefully removed from each well and replaced with 100 µl HCl-Isopropanol. After 10 min incubation at 37°C, the solubilized formazan produced by metabolically active cells was measured by scanning the 96-well plates at dual-wavelength of 540–630 nm using a Multiskan apparatus (Labsystem, Helsinki, Finland). Using this colorimetric procedure, extracts and adriamycin, cytotoxic effects could be measured as compared to the viability of untreated cells, according to the following calculation:

$$\% \text{ cell killing} = 100 \times (OD - OD_o)/OD_o),$$

where OD_o and OD are the optical density obtained respectively for untreated and extract or adriamycin-treated cells.

Effect of extracts on human peripheral blood mononucluear cells (PBMC)

This test was realized in order to evaluate the effect of our extracts on normal human cells. To isolate the PBMC, blood samples were collected from two healthy donors in heparinized tubes, and peripheral blood mononuclear cells were isolated using standard Ficoll-hypaque density centrifugation. The interface lymphocytes were washed twice with phosphate-buffered saline (PBS). Extracts, phytohaemagglutinin (PHA) and adriamycin cytotoxic effect was measured by MTT test as detailed above.

Extraction and HPLC isolation of the compounds produced by selected isolates

Selected isolates were cultured in solid media at 28°C for 7 days. The solid media was extracted with organic solvents (ethyl acetate (EA) and methanol (M)). After filtration through filter paper, the extracts were evaporated under vacuum at 40°C to dryness and suspended in DMSO, centrifuged (14000 rev/min, 10 min) and the supernatant analysed by HPLC/ELS (the liquid chromatograph comprised a Water 600 controller, a Water in-line degasser AF, a Waters 717 plus autosampler, a Waters 2420 evaporative light scattering detector (ELS), with Empower 2 software to control the analytical system and data processing) using a preparative column (Sunfire TM C18 OBDTM 19 × 50 mm) eluted at 12 ml/min, respectively with water and acetonitrile, both solvent contained 0.1% formic acid. The injected volume was 1.5 ml (50 mg/ml).

Statistical analysis

The results are presented in the form of averages ± SEM. The comparison of the averages is made by ANOVA. The differences are considered significant at $P < 5\%$.

Results and discussion

Isolation and antibacterial activity

In our screening for actinomycetes showing cytotoxic activities, eight samples were collected from various Moroccan habitats, 136 isolates were isolated using Olson's medium and soil extract agar. This medium seems to be specific and sensitive for actinomycetes (Barakate et al. 2002).

Table 1 Origin of the samples and number of active isolates

Samples	Number of isolated isolates	Number of active isolates[a]	% of active isolates
ST	14	3	21.42
AS	27	8	29.62
T	11	4	36.36
TD	20	5	25
CD	13	4	30.76
OM	7	3	42.85
S	20	4	20
R	24	6	25
Total	136	37	27.2

[a] Active isolates against *M. luteus*

The antibacterial activity of the isolates was tested. Among all isolates, 37 (27%) produced active substances against Gram-positive bacteria (Table 1). This percentage is smaller than those described by Barakate et al. (2002) studying the activity of Moroccan soil actinomycetes. These results are also different from those of other authors showing 16% in soil of Turkey (Oskay et al. 2004) and 53–61% in Algerian soil (Sabaou et al. 1998).

In this study, all the 37 active isolates were retained for the screening for cytotoxic compound production.

Characterizations of active isolates

In the present investigation, the chemical diversity of the produced molecules by the 37 active isolates was evaluated using the antibacterial activity against three gram-positive bacteria: *Micrococcus luteus*, *Bacillus subtilis* and *B. cereus* and three different production media: nutrient agar, Bennett medium and GBAm. The results are shown in Table 2 and Fig. 1.

According to the results, Bennett medium seems to be the most favorable for the development and the antibiotic production of the 136 actinomycetes isolates. The same observation is obtained for the production of secondary metabolites by actinomycetes from Moroccan habitats (Ouhdouch et al. 2001).

The investigation of the activity against *M. luteus* on three different media allowed the possibility to divide the

Table 2 Percentage of active isolates obtained using several production media

Production media	Microorganisms tests		
	ML	BC	BS
Bennett	89.18	40.54	43.24
Nutrient agar	78.37	27.02	32.43
GBAm	78.37	40.54	40.54

Total number of active strains is 37

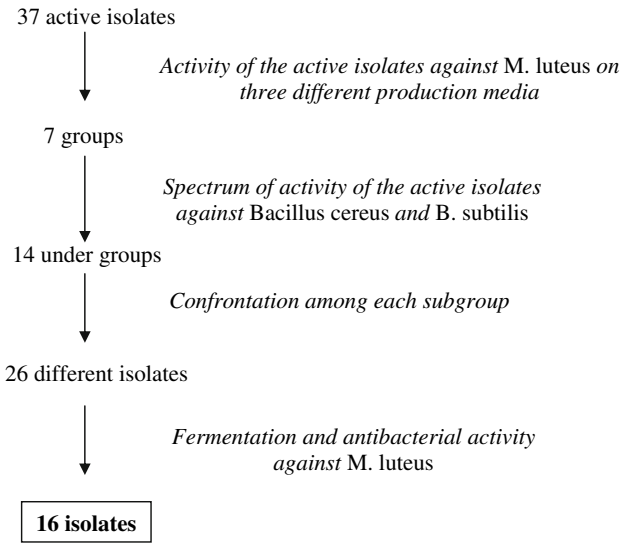

Fig. 1 Different screening steps of active isolates

37 active isolates into 7 groups. These 7 groups were divided into 14 different subgroups using their spectrum of activity against the three micro-organisms tested. The dual culture on agar allows the distinction between different isolates belonging to same subgroup. The inhibition zones between the different isolates express their diversity to produce different antibiotics (Raaijmakers et al. 2002). Using this strategy, hence, twenty-six different isolates were retained (Fig. 1). The morphological criteria, including characteristics of colonies on the plate, morphology of substrate, aerial hyphae, and produced pigments confirmed the taxonomic diversity of selected isolates.

The 26 isolates were fermented in two different liquid media: nutrient broth or/and Bennett medium. Only 16 isolates were able to grow in liquid media and present an antibacterial activity. For all selected isolates, the antibacterial activity was more significant in solid than in liquid media. It has been established that solid medium is more adequate to the development of the isolates and the production of antibiotics (Iwai and Omura 1982; Shomura et al. 1979; Badji et al. 2005). After extraction by the ethyl acetate and butanol, 17 organic and 4 aqueous extracts (ST_3, R_5, T_5 and AS_8) show the antibacterial activity against *M. luteus.* The aqueous extracts are interesting for the study of toxicity to *Artemia salina* and cytotoxicity against tumoral cells (Orsolic and Basic 2003).

Toxicity to *Artemia salina*

Since *Artemia salina* larvae have been used as a target to detect the bioactive compounds and their toxicity (McLaughlin 1991), it was used in our screening for cytotoxic molecules produced by the actinomycetes. Among the 21 extracts obtained, the butanol extract of the ST_9 presented

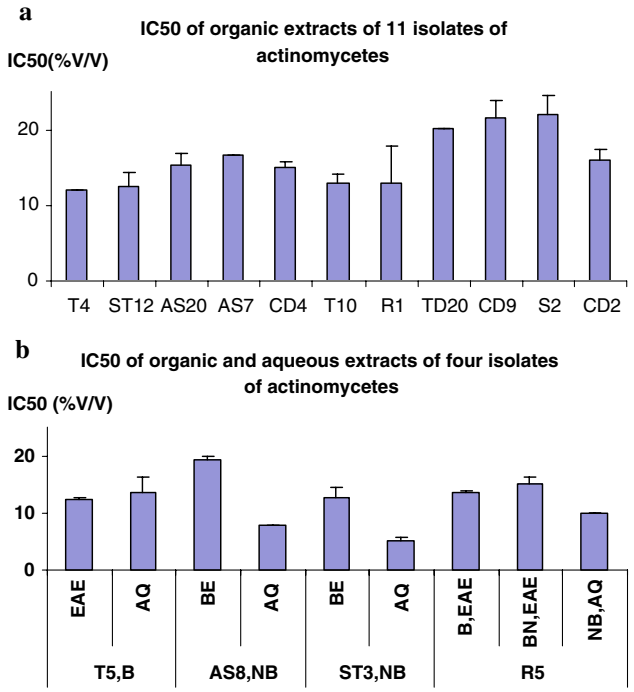

Fig. 2 Cytotoxic activity of organic and aqueous extracts against BSR cell lines ($n = 3$) (**a**): IC_{50} (%v/V) of organic extracts of 11 isolates. (**b**): IC_{50} (v/v) of organic and aqueous extracts of 4 active isolates (BE = butanolic extract; AQ = aqueous extract; EAE = ethyl acetate extract. Production media: B = Bennett medium; NB = Nutrient Broth)

toxic effects against *Artemia salina*, while the other organic and aqueous extracts did not show any toxicity.

Cytotoxic activity

In order to investigate the antitumor activity of the 16 organic and 4 aqueous extracts selected by the *Artemia salina* test, BSR tumor cell lines were cultured for 48 h in the presence of increasing concentrations of each extract. The cytotoxic activity was determined using the MTT test. The concentration of each extract leading to 50% cytotoxicity was then determined. The results (Fig. 2) shown that the aqueous extracts from the 4 isolates exhibited high cytotoxic activity (IC_{50} T_5 = 13.597(%v/V); IC_{50} R_5 = 10.052 (%v/V); IC_{50} AS_8 = 7.8221 (%v/V); IC_{50} ST_3 = 5.175 (%v/V)). The cytotoxic activity of these extracts was then studied against Hep2, P815 and BSR tumor cell lines. The obtained results are summarized in Table 3. It is shown in this table that the cytotoxic activity was variable from one extract to another and depended on the tumor cell lines used as target. Since the criteria of cytotoxicity activity for the crude extracts, as established by the American National Cancer Institute (NCI) is in IC_{50} <30 μg/ml, and for all standard antitumor agents, the IC_{50} value was less than 25 μg/ml (Zheng et al. 2000), the aqueous extracts of T5 and AS8 were cytotoxic against Hep2 tumor cell lines.

Table 3 In vitro cytotoxic activity of aqueous extracts of four actinomycetes determined by MTT assay ($n = 6$)

Extract of isolates and positive control	IC_{50} (mg/ml)		
	P815	BSR	Hep2
T5	$10^3 \pm 0.580$	664.99 ± 0.033	<7.81
AS8	471.6 ± 0.147	590.22 ± 0.287	<7.81
R5	$10^3 \pm 0.248$	802.68 ± 0.198	358.8 ± 0.552
ST3	464.77 ± 0.225	455.2 ± 0.041	278.54 ± 0.225
Adriamycin	1.48	0.31	0.97

In order to eliminate the possibility that the extracts were toxic against normal cells (PBMC), we tested the activity of the 4 extracts with two different donors using the MTT test. The 4 aqueous extracts did not show a significant toxic effect on the PBMC (Fig. 3a) in comparison with adriamycin as positive control antitumor product. Interestingly, the lowest concentration of the aqueous extract (15.6 μg/ml) of the isolate T_5 exhibited a proliferative and not a cytotoxic effect against the PBMC. Indeed, the viability of the PBMC score is 233.6% for the first donor and 321.47% for the second one. The proliferative effect of T_5 was confirmed by cell morphology evaluation by inverted light microscopy (Fig. 3b). These results are in agreement with those of Schubert et al. describing an induction of cell proliferation with extracts from actinomycetes (Schubert et al. 1996). The immunostimulant and cytotoxic activities showed by the compounds present in the T_5 aqueous extract could be explained by its chemical composition, either the same molecules having activities dose depending or several molecules.

The two strains T_5 and AS_8 were selected for their cytotoxic activity against cancer cell lines and were identified by the Paris Institut Pasteur as *Streptomyces parvus*. Although they belong to the same species using partial 16S rDNA, the isolates produced different active compounds expressed by their biological activity spectrum.

Extraction and HPLC isolation of the compounds produced by selected isolates

Preparative HPLC/ELS was used for the separation of the different compounds present in *Streptomyces* sp. AS8 cytotoxic methanolic extract. The HPLC profile is shown in Fig. 4. Five different compounds (M1–M5) were separated. Samples were collected for cytotoxic activity and identification. The compounds M1 and M2 were also found to be active against the human cell lines Hep2. The determination

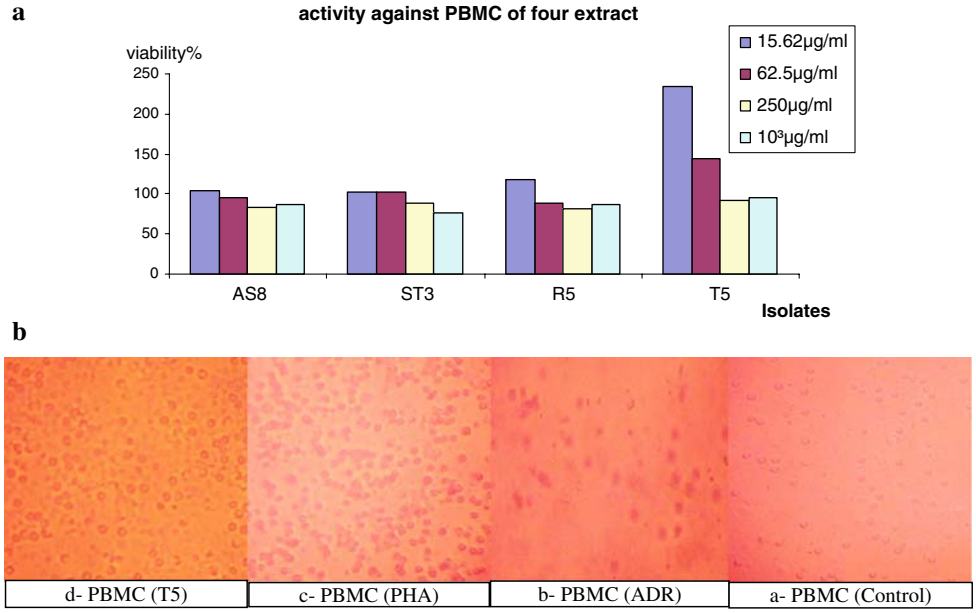

Fig. 3 Effect against Peripheral Blood Mononuclear Cells (PBMC) (**a**): Percentage of viability of four aqueous extracts of actinomycetes ($n = 2$). (**b**) Microscopic observation of Peripheral Blood Mononuclear Cells: a- Negative control; b- Effect of adriamycin as antitumor product; c- Effect of PHA as immunostimulant product; d- Effect of aqueous extract of T5 isolate

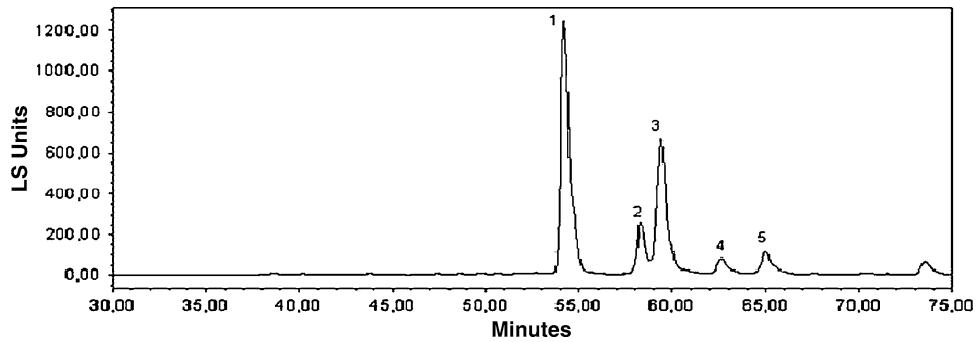

Fig. 4 Chromatogram (HPLC/ELS) of methanolic extract of *Streptomyces* sp. AS8

of chemical structure of all compounds by 1H NMR, 13C NMR and mass spectrum is being carried out.

In conclusion, Moroccan habitats might be a rich source of actinomycetes species producing the cytotoxic molecules. The isolates T5 and AS8 were selected for their cytotoxic activity against cancer cell lines and identified as *Streptomyces parvus* by 16SrDNA. Taxonomic characterization using DNA/DNA hybridization of *Streptomyces* sp.T5 and *Streptomyces* sp.AS8 selected during this study as well as purification and structural elucidation of the cytotoxic and immunostimulant compounds produced are under investigation.

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