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Endophytic Actinomycetes of Some Medicinal Plants in Saint Katherine Area, Egypt

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ABSTRACT

A number of 41 morphologically different endophytic actinomycete isolates were recovered from surface-sterilized medicinal plants; *Artemisia herba-alba*, *Echinops spinosus*, *Mentha longifolia* and *Ballota undulate*. A simplified method for selective recovery of actinomycetes from internal plant tissues is described. Successful recovery was achieved on the media: starch-casein agar, 1/10 diluted starch casein agar, tap water-yeast extract agar and MG-plant extract agar. Preliminary description of the isolates, according to microscopic examination and amino acid composition of the cell wall hydrolystates, indicated prevalence of genus *Streptomyces*. The highest number of endophytic actinomycetes was recovered from *Mentha longifolia* and represented 34% of the isolates. Organic extracts of the growth metabolites from the cultures were tested for cytotoxicity against *Artemia salina* as well as for antimicrobial activities against 4 reference bacterial strains, 6 clinical bacterial cultures and two clinical fungal cultures. Variable activities were obtained with different actinomycete isolates; the highest activity could be detected against *Gardnerella vaginitis* and *Shigella boydii* strain ATCC 9207; being represented in 29% of the isolates in both cases. For the first time, the isolation of endophytic actinomycetes from Saint Katherine wild plants is reported and their potential use as novel source of bioactive compounds is discussed.

Key words: Antimicrobial activity, cytotoxicity, endophytic actinomycetes, medicinal plants, Saint Katherine.

INTRODUCTION

Actinomycetes are a diverse group of filamentous Gram-positive bacteria well known for their production of an extensive array of chemically diverse and medically important secondary metabolites. Only a few recent studies have highlighted the bioactive importance of endophytic actinomycetes, including biocontrol of fungal plant pathogens (El-Tarabily, 2003; Coombs *et al.*, 2004), production of antimalarial and antibimicrobial agents (Castillo *et al.*, 2002), production of anticancer compounds (Caruso *et al.*, 2000), production of plant growth regulators (Igarashi, 2004) and production of enzymes (Stamford *et al.*, 2002).

Plants growing in areas of great biodivesity usually have the prospect of harbouring endophytes with great biodiversity (Strobel and Daisy, 2003). The Saint Katherine World Heritage Site (WHS No. 954, UNESCO) of Sinai, Egypt, is one of the world's most biodiverse area; it is characterized by high percent of endimism in flora, fauna and related microbiota merging from the existing altitude gradient. However, to the best of our knowledge, microbiological studies on the endophytes residing in their plants were not considered yet. Thus, this habitat is one that deserves close examination for novel microbes that produce compounds with desired bioactivities. The present study describes the isolation of actinomycetes from internal tissues of some medicinal plants in the WHS of St. Katherine and evaluates metabolites for antimicrobial and cytotoxic activities.

MATERIALS AND METHODS

Site description and plant sampling

Plants were collected during the early summer of 2005 from six sites with characteristic granitic country rocks, located in the ring dyke of the WHS. These were: Wadi Shreiag (29.8° 33' 57" E, 51.9° 28' 33" N and 1545.3 m Altitude), Wadi El Arba'een (18.2° 33' 59" E, 24.7° 28' 40" N and 1306.1 m Altitude), Al-Kharazen (53.2° 33' 57" E, 12.2° 28' 43" N and 1627.6 m Altitude), El-Talaa (55.8° 33' 55" E, 00.6° 28' 34" N and 1627.6 m Altitude), Wadi El-Sheikh (08° 33' 59" E, 35.6° 28' 40" N and 1281.7 m Altitude) and Wadi El-Raha (14.6° 33' 57" E, 50.2° 28' 33" N and 1544.7 m Altitude). Four plant species were collected for investigation: *Artemisia herba-alba* (Shieh) and *Echinops spinosus* (Khasheer), belonging to the family Compositae; *Mentha longifolia* (Habak) and *Ballota undulate* (Ghassa), belonging to the family Labiatae. These four plant species were selected for there medicinal value due to their high content of functional flavonoids, sesquiterpene, lactones and other essential oils (Rimbau *et al.*, 1999). Healthy, green aerial parts were cut from these plants and placed in sealed plastic bags. Collected plant samples were dried at room temperature for 2 days and stored at 4°C for further processing.

Isolation and characterization of endophytic actinomycetes

Plant materials were processed with modification of the method described by Demain and Davies (1999) and plated into a battery of ten media: Tap Water Yeast Extract (TWYE) agar, Starch-Casein (SC) agar, 1/10 Starch-Casein agar, Tryptone Soya (TS) agar, MGA-PE medium, Potato-Dextrose (PD) agar, CYC agar, Arginine-Glycerol Salts (AGS) agar, Starch Nitrate (SN) agar and Actinomycetes Isolation (AI) agar (Basil *et al.*, 2004; Demain and Davies 1999). Plants were aseptically cut into 1 g pieces (*ca*. 3-5 cm in length) and

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immersed in 70% ethanol for 5 minutes for surface sterilization, followed by rinsing in sterile distillated water and air drying under a laminar flow hood. Surface sterility check and processing of plant material for endophytes isolation were performed as illustrated in flow chart (Fig. 1). All plants were processed in triplicates and all media were supplemented with 50µg/ml cycloheximide (antifungus) after sterilization. Plates were incubated at 28°C with regular monitoring; counts were recorded after 4 weeks.

A total of 41 morphologically different actinomycete isolates were purified from plates using starch-casein agar medium and maintained as spore suspensions, in 20% glycerol, at 20°C (Hopwood *et al.*, 1985). Isolates were characterized for their micromorphology and diaminopimelic acid (DAP) isomer in whole cell hydrolysate. Microscopic examinations of sporulated mycelia were performed on cultures grown on ISP4 medium (Shirling and Gottlieb, 1966). The determination of the DAP isomer in whole cell hydrolysate was performed as described by Schaal (1985) using dried mycelia obtained from cultures grown in TS broth in baffled flasks on shaking incubator (100 rpm), at 28 °C for 4-7 days.

Enumeration of soil actinomycete

Soil samples (10-15 cm depth) were collected aseptically from the target localities and kept in sterile polyethylene bags at 4 °C until isolation. Actinomycetes were isolated after serial dilution in phosphate buffer on SC agar supplemented with cycloheximide (50 µg/ml) using spread plate technique. Counts were recorded after incubation for 2-3 weeks at 28 °C.

Fermentation procedure

Preserved spore suspensions of actinomycete isolates were inoculated into 30 ml of SC liquid medium and incubated in a shaker incubator (100 rpm), for 5-7 days at 28 °C. Cultures were extracted using equal concentrations of ethyl acetate, for three successive times with vigorous shaking to thirty minutes. This allowed any organic molecules to be suspended in the less polar solvent. Ethyl acetate fractions were evaporated under vacuum into a preweighed vial, and then redissolved in ethyl acetate giving a final concentration of 10 mg/ml.

Antimicrobial screening

The raw extracts were tested by disk diffusion using 0.1 mg per disk against a representative panel of human pathogenic microorganisms as described by Castillo *et al.* (2002). This panel included 4 reference bacterial strains *Salmonella typhimurium* (NCMB 74), *Escherichia coli* (NCMB 11943), *Shigella boydii* ATCC 9207 and *Pseudomonas aeruginosa* NCMB 8295; 6 clinical bacterial cultures *Vibrio* sp., *Proteus vulgaris, Klebsiella pneumonia, Gardnerella vaginitis, Staphylococcus aureus*, and *Streptococcus* sp.; and two fungal cultures *Candida albicans* and *Candida* sp.

Brine shrimp cytotoxicity assay

The brine shrimp eggs were hatched in seawater (40 mg/l) as described by Svoboda and Hampson (1999). Hatched eggs were supplemented with 6 mg/l dried yeast and oxygenated for 48 hours at room temperature. Crude organic extracts (1 mg in ethyl acetate) were transferred into sterile microtubes and evaporated under

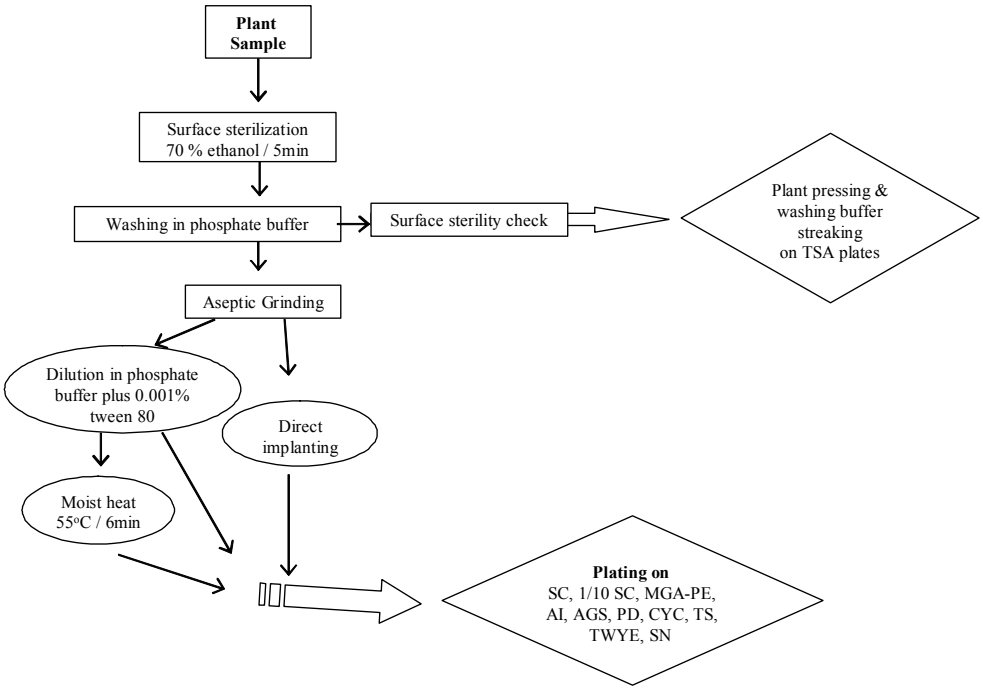

Figure (1): Proposed strategy for selective isolation of endophytic actinomycetes from internal plant tissues.

vacuum,to which 10-20 *A. salina* larvae in 1 ml of seawater were added. Control blind sample was accompanied by adding ethyl acetate and duplication was performed. Cytotoxiciy was scored at intervals up to 84 hours compared to the blind samples.

Statistical analysis

Significant differences among different cultivation media and for different plant origins were studied by one-way analysis of variance. The means were separated at significant difference ($p = 0.05$).

RESULTS

Culturable endophytic actinomycetes

The endophytic actinomycete populations from the investigated plants averaged between 10^2-10^3 CFU/g dry plants. It was noticed that, recovery of actinomycetes was only successful using MGA-PE, SC, 1/10 SC and TWYE agar media, while the other media plates were over-grown with fungi and unicellular bacteria. Statistical analysis showed significant differences ($p = 0.049$) for actinomycete counts using the above mentioned four media. As shown in Figure (2), higher numbers were recovered on 1/10 SC and TWYE media compared to the other two media. The maximum population recorded was from *Ballota undulata* on TWYE agar.

Direct implanting of grinded plant samples has recovered endophytic actinomycetes (data not shown); however, better separation of individual colonies was achieved by dilution and spread plate technique. In addition, the heat treatment did not enhance the recovery of actinomycetes from internal plant tissue as shown in Figure (3), and difference in actinomycetes counts between heat treated and untreated samples were not significant ($p = 0.072$).

Tentative identification by microscopic examination and analysis of amino acid composition of the cell wall (Holt *et al.*, 1994), indicated prevalence of genus *Streptomyces*, representing 41.5% of the isolated endophytic actinomycetes, in this study. Grouping of the isolates, based on DAP analysis of the whole cell hydrolysate revealed a number of 19 L-DAP containing isolates and 22 m-DAP containing isolates.

As shown in Figure (4), high significant difference could be noticed between soil actinomycete population in the three investigated localities ($p = 2.85$ E-06). In contrast, endophytic actinomycete counts from *Mentha longifolia* collected from these localities were not significantly different ($p > 0.05$).

Bioassay of antimicrobial and cytototoxic activities

Results indicated bioactivity of 81% of the isolates (Fig. 5). Of the 41 endophytic actinomycete isolates, 30 exhibited microbial inhibitory effect. The highest activity was against *Gardnerella vaginitis* and *Shigella boydii* ATCC 9207, being represented in 29 % of the isolates in both cases. All the isolates failed to give activity against *E. coli*, *P. Aeruginosa* as well as the clinical cultures *Proteus vulgaris* and *Candida* sp. Cytotoxic effect against *Artemia salina* was positive for 27 endophytic actinomycetes. Of these, nine isolates exhibited high mortality rate reaching to 100% death after 12 hours. Those were mainly *A. herba alba* and *E. spinosus* isolates (Table 1).

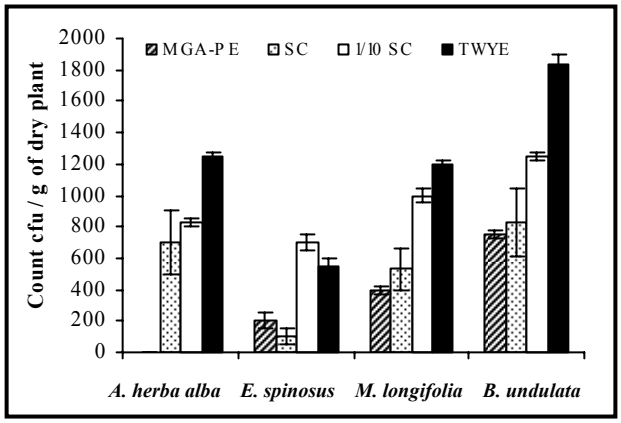

Figure (2): Enumeration of endophytic actinomycetes on four selected media. Counts are average of triplicates with bars indicating Standard Error of the mean.

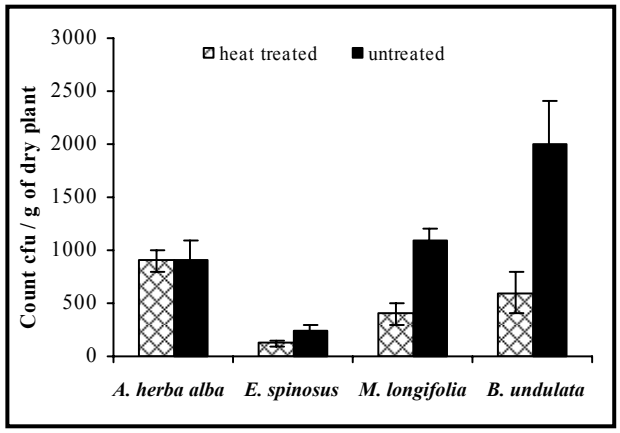

Figure (3): Effect of heat treatment on recovery of endophytic actinomycetes from four plant species on 1/10 SC media. Counts are average of triplicates with bars indicating Standard Error of the mean.

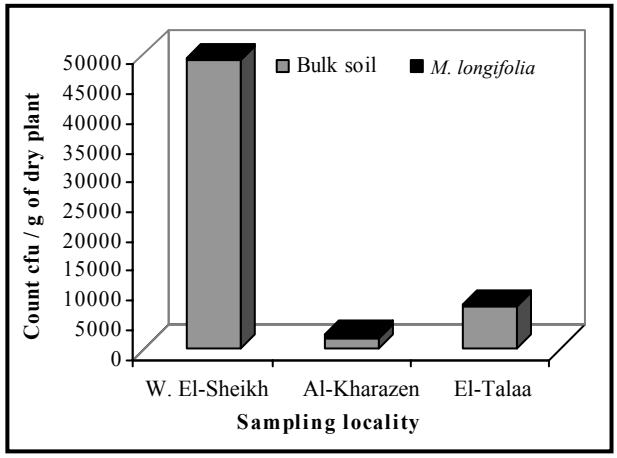

Figure (4): Actinomycetes population from *Mentha longifolia* and the surrounding bulk soil. Counts are average of three replications.

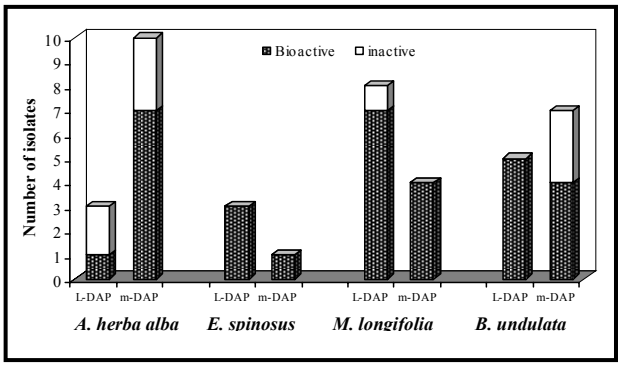

Figure (5): Total number of endophytic actinomycete isolates showing bioactivities. Isolates from the four investigated plant species are categorized into L-DAP and m-DAP containing groups.

DISCUSSION

It is recently apparent that plants can serve as a reserviore of endophytic microorganisms, meantime evidence thus far indicates that, metabolites from these sources hold pharmaceutical and agriculture promise (Castillo *et al.*, 2002; Kunoh, 2002). In the present study, endophytic actinomycetes isolation was significantly higher on the low nutrient-content media 1/10 SC and TWYE, compared to the high nutrient-content media which allowed fast growing fungi and unicellular bacteria to dominate the isolation plates. These results were in agreement with Basil *et al.* (2004) for isolation of soil actinomycetes using TWYE and with May *et al.* (2003) for isolation of stone actinomycetes using 1/10 SC. McCarthy and Williams (1992) have described an autochthonous behaviour, i.e. sustained growth at low nutrient concentrations amongst actinomycetes, which may explain the successful selectivity of these low nutrient-content media for actinomycetes.

Although average endophytic actinomycetes population was 10^2 to 10^3 cfu/g dry plants in the four plant species studied, the bioactivities of these endophytes varied widely. For example, 92% of *M. longifolia* endophytes showed broad antimicrobial activities and inhibited eight representatives of Gram positive, Gram negative bacteria and yeast strains. In contrast, only 31% of *A. herba-alba* endophytes exhibited antimicrobial activities against six of the tested microbial strains. Similarly, the cytotoxicity of *M. longifolia* isolates was 33% compared to 62% for *A. herba-alba* isolates. The high ratio of bioactive isolates obtained from this study (81%) compares well with other investigations, for example antimicrobial activity was 14% for actinomycetes endophytic in *Taxus* plant (Caruso *et al.*, 2000) and 20% for endophytic actinomycetes isolated from a variety of Japanese wild plants (Igarashi *et al.*, 2002). However, it should be emphasized that attempts to compare and contrast antimicrobial potential are susceptible to differences in protocols used.

Although the available literature doesn't provide knowledge on the endophytes of the currently investigated plants, the four species are rich in metabolites of high medicinal value (Hanafy *et al.*, 2000) and thus represent a promising source for bioactive microbial products. The recent discovery of different genera of actinomycetes and fungi that can produce quinoline alkaloids and diterpenoids identical to those originally characteristic of their host plants (Caruso *et al.*, 2000; Puri *et al.*, 2005) have arisen an interesting aspect that microbial endophytes may exhibit genetic recombination with their host plant to produce metabolites that facilitate the domination of its biological niche within the plant or even provide protection to the plant from harmful invading pathogens.

While there was a significance difference in the culturable actinomycetes numbers in bulk soil of the studied localities, there were no qualitative differences in numbers and bioactivities of endophytic actinomycetes from *M. longifolia* collected from these localities. Bills *et al.* (2002) described a metabolic distinction between endophytes of the same plant origin in tropical regions and suggested the importance of the host plant in influencing the general metabolism of its endophytic microbes.

Table (1): Source of actinomycetes and their bioactivities measured as diameter of the inhibition zone for antimicrobial activities and as percentage of dead larvae for cytotoxicty. Results were scored after 24 hours of incubation.

Actinomycetes group (no. of isolates) [a]	Antimicrobial activity (avg ± SD) [b]								Cytotoxicity (Death %)
	S. typhimurium	*Vibrio* sp.	*S. boydii*	*K. pneumonia*	*Streptococcus* sp.	*S. aureus*	*G. vaginitis*	*C. albicans*	
Artemisia herba-alba									
L-DAP group (2)	9 ± 0.7	9 ± 0.7	12 ± 0.5		16 ± 0.6		6 ± 0.7		17 ±.01
m-DAP group (5)					9 ± 0.6		7 ± 1.4	27± 0.7	100 ± 25
Echinops spinosus									
L-DAP group (3)			18 ± 0.3				7 ± 0.5	6 ± 0.6	100 ± 0.01
m-DAP group (1)			7 ± 0.3						100 ± 0.01
Mentha longifolia									
L-DAP group (7)	11 ± 0.7		10 ± 4.6	6 ± 0.7	15 ± 0.6	13 ± 0.6	7 ± 0.6	7 ± .05	33 ± 0.7
m-DAP group (4)	13 ± 7	12 ± 0.6	18 ± 3		12 ±0.7	8 ± 0.5	7 ± .01		67 ± 33
Ballota undulate									
L-DAP group (5)	7 ± 0.5		12 ± 0.5		7 ± 0.6		8 ± 0.7	9 ± 4	66 ± 40
m-DAP group (3)					15 ± 0.7		7 ± 0.6		0

[a]: Number of actinomycetes isolates in each group. [b]: Average (avg) of inhibition zone diameter ± Standard deviation (SD).

Overall, it is concluded that, actinomycetes could be selectively isolated from the internal tissues of wild medicinal plants in the WHS using a simplified method. The bioactivities of these isolates varied with plant origin and the higher percentage of wide-spectrum antimicrobial strains were from *M. longifolia*. The reason for this requires further investigation. We hypothesize that actinomycetes which colonize internal tissues of these plants are highly adapted to this unique little-studied habitat and should be investigated for detailed identification and potential use as source of bioactive agents.

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الأكتينوميسيتات داخلية المعيشة فى بعض النباتات الطبية بمنطقة سانت كاترين، مصر

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الملخص العربـــى

تختص هذه الدراسة بالعزل الميكروبى وتوصيف النشاط الحيوى للأكتينوميسيتات المتواجدة فى الأنسجة الداخلية لأربعة نباتات طبية وهى: *Artemisia herba-alba* و *Echinops spinosus* و *Mentha longifolia* و*Ballota undulate* حيث أمكن تحديد طريقة مبسطة للعزل الإختيارى للأكتينوميسيتات ساكنة تلك النباتات، وأوضحت النتائج أفضلية العزل على الأوساط الغذائية: آجار النشا والكازين، وآجار النشا والكازين المخفف إلى العشر، وآجار مستخلص الخميرة، وآجار المستخلص النباتى.

وقد تم الحصول على عدد 41 عزلة أكتينوميسيتات متباينة فى الشكل الظاهرى، أظهر التعريف المبدئى لها إنتشار جنس *Streptomyces* كما أوضحت النتائج أن 34% من إجمالى العزلات كان من أنسجة نبات *Mentha longifolia*. وقد تم تقييم النشاط الحيوى للمستخلصات العضوية لمزارع الأكتينوميسيتات وذلك من حيث السمية الخلوية ضد *Artemia salina*، والنشاط الميكروبى ضد 10 سلالات بكتيرية مرجعية وإكلينيكية إضافة إلى سلالتى فطر إكلينيكية. وجاءت النتائج موضحة لوجود نشاط حيوى واسع ومتباين لعزلات الأكيبنوميسيتات من المصادر النباتية المختلفة. وكان أعلى نشاط ضد ميكروبى للعزلات مع سلالة *Shigella boydii* ATCC 9207 المرجعية والسلالة *Gardnerella vaginitis* الإكلينيكية، حيث سجلت 29% من الأكتينوميسبتات نشاطاً ضد كل من هاتين السلالتين.

تلك الدراسة هى الأولى عن إمكانية العزل الإختيارى للأكتينوميسيتات ساكنة النباتات فى منطقة سانت كاترين وفعالية مثل هذه العزلات كمصدر جديد للمركبات الحيوية النشطة.