International Journal of Botany

ISSN: 1811-9700

science alert

ANSInet
an open access publisher
http://ansinet.com

International Journal of Botany 2 (3): 307-312, 2006
ISSN 1811-9700

Bioactivities of Endophytic Actinomycetes from Selected Medicinal Plants in the World Heritage Site of Saint Katherine, Egypt

Sahar El-Shatoury, Hesham Abdulla, Omnia El-Karaaly, Waleed El-Kazzaz and Ahmed Dewedar
Department of Botany, Faculty of Sciences, Suez Canal University, 41522 Ismailia, Egypt

Abstract: Endophytic actinomycetes were isolated from surface-sterilized living aerial parts of four 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. A total of 41 isolates were obtained, belonging to different genera with a prevalence of *Streptomyces*; tentatively identified according to microscopic examination and amino acid composition of the whole cell hydrolystates. The highest number of isolates was recovered from *Mentha longifolia* and represented 34% of the isolates. Organic extracts of all cultures were assayed for cytotoxicity against *Artemia salina* and 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 was against the clinical culture *Gardnerella vaginitis* and the reference strain *Shigella boydii* ATCC 9207, being represented in 29% of the isolates in both cases. For the first time, the isolation of 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

The emergence of drug-resistant microbial pathogens adding to the increased demand for potent antibiotics, antioxidants, antitumors and other natural bioactive compounds are among the problems posing challenges to the development of effective therapeutic agents. One approach, among many, in the search for new bioactive compounds is to continue the traditional method of screening large numbers of microbial isolates cultured from nature. The secret to continued success with this approach is the examination of hitherto under-explored habitats, combined with the use of novel cultural and genetic techniques that can reveal the full metabolic potential of the isolates under study (Demain and Davies, 1999).

The internal plant tissue, is a biologically complex and distinct microhabitat within the terrestrial ecosystem because of its varying content of alkaloids, terpenoids, steroids and aromatic compounds. Thus, healthy plant tissues represent an untapped reservoir of novel endophytic microorganisms producing bioactive metabolites (Strobel and Daisy, 2003). Endophytes exhibit a symptomless nature while occupying plant tissues and this has prompted focus on symbiotic or mutualistic relationships between endophytes and their host plants (Bacon and White, 2000). Some endophytes are thought to be making returns for the nutrition from the plant by producing special secondary metabolites to prevent the host from attack by fungi, pests and mammals. As a matter of fact, metabolites of endophytes were reported to inhibit a number of microorganisms (Gurney and Mantle, 1993) and to show identity with anticancer compounds produced by the host plants (Caruso *et al*., 2000).

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)

Corresponding Author: Sahar El-Shatoury, Department of Botany, Faculty of Sciences, Suez Canal University P.O. Box 41522, Ismailia Egypt Tel: 0020 64 3230418 Fax: 0020 64 3228200

of Sinai-Egypt, is one of the world's most biodiverse area; it is marked by high rate of endimism in flora, fauna and related microbiota merging from the existed altitude gradient. However, to the best of our knowledge, microbiological studies on the endophytes residing in its 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 evaluation of their metabolites in the screening for antimicrobial and cytotoxic activity.

MATERIALS AND METHODS

Site description and plant sampling: Plants were collected during the early summer of 2005 from six sites with characteristic granitic country rock, located in the ring dyke of the WHS. These were: Wadi Shreiag (33' 57" 29.8° E; 28' 33" 51.9° N; 1545.3 m Alt.), Wadi El Arbaen (33' 59" 18.2° E; 28' 40" 24.7° N; 1306.1 m Alt.), Al-Kharazen (33' 57" 53.2° E; 28' 43" 12.2° N; 1627.6 m Alt.), El-Talaa (33' 55" 55.8° E; 28' 34" 00.6° N; 1627.6 m Alt.), Wadi El Sheikh (33' 59" 08° E; 28' 40" 35.6° N; 1281.7 m Alt.) and Wadi El-Raha (33' 57" 14.6° E; 28' 33" 50.2° N; 1544.7 m Alt.). Four plant species were investigated: *Artemisia herba-alba* (Shieh) and *Echinops spinosissimus* (Khasheer), belonging to the family Compositae; *Mentha longifolia* (Habaq) and *Ballota undulate* (Ghassa), belonging to the family Labiatae. The four species have high medicinal value due to their rich 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 until 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 immersed in 70% ethanol for 5 min. for surface sterilization, followed by rinsing in sterile water and air drying under a laminar flow hood. Surface sterility check and processing of plant material for endophytes isolation was performed as shown in Fig. 1. All plants were processed in triplicates and all media were supplemented with cycloheximide antifungus (50 μg mL^{-1}) 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 on starch casein media and maintained as spore suspensions, in 20% glycerol, at -20°C (Hopwood *et al*., 1985). Isolates were characterized for micromorphology and diaminopimelic acid (DAP) isomer in whole cell hydrolysate. Microscopic observations 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 baffled flasks on shaking incubator at 100 rpm, 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 analysis. Actinomycetes were isolated after serial dilution in phosphate buffer on SC agar supplemented with cycloheximide (50 μg mL^{-1}) 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 broth and incubated in a shaker incubator for 5-7 days at 28°C, 100 rpm. Cultures were extracted using equal concentrations of ethyl acetate, for three successive times with vigorous shaking to thirty min. The 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^{-1}.

Antimicrobial screening: The raw extracts were tested by disk diffusion at 0.1 mg per disk against a representative panel of human pathogenic microorganism 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 aeroginosa* (NCMB 8295); 6 clinical bacterial cultures *Vibrio* sp., *Proteus vulgaris*, *Klebsiella pneumonia*, *Gardnerella vaginitis*, *Staphylococcus aureus* and *Streptococcus* sp. and two clinical fungal cultures *Candida albicans* and *Candida* sp.

Brine shrimp cytotoxicity assay: The brine shrimp eggs were hatched in seawater (40 mg L^{-1}) as described by Svoboda and Hampson (1999). Hatched eggs were supplemented with 6 mg L^{-1} dried yeast and oxygenated for 48 h at room temperature. Crude organic extracts (1 mg in ethyl acetate) were transferred into sterile microtubes and evaporated under vacuum, to which 10-20 *A. salina* in 1 mL of seawater larvae were added. Control blind sample was accompanied by adding ethyl acetate and duplication was performed. Cytotoxicity was scored at intervals up to 84 h compared to the blind samples.

Statistical analysis: Significant differences between the different cultivation media and from 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^{-1} dry plant. 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 actinomycetes counts using those four media. As shown in Fig. 1 and 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 Fig. 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, 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 Fig. 4, high significant difference was noticed between soil actinomycete population in the three investigated localities ($p = 2.85E\text{-}06$). In contrast, endophytic actinomycetes 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

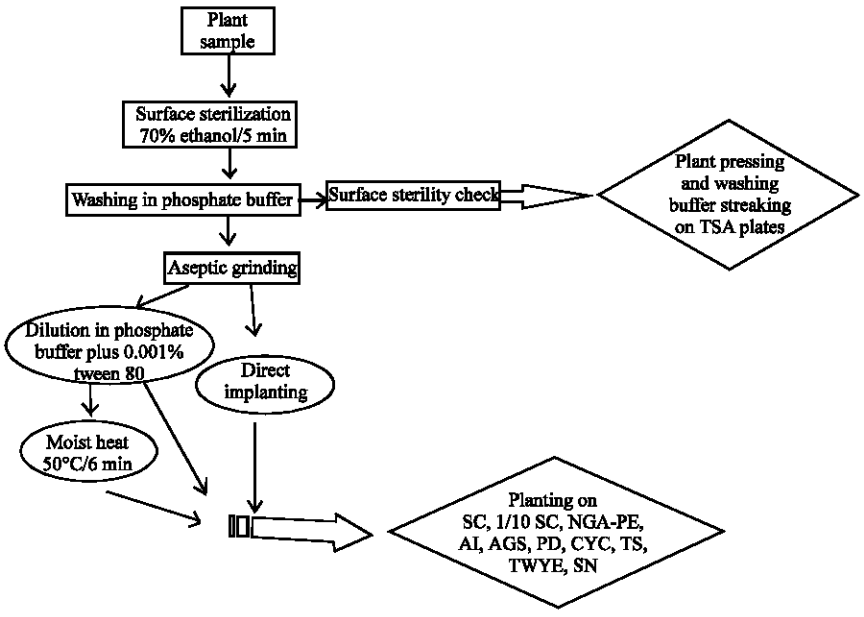

Fig. 1: Proposed strategy for selective isolation of endophytic actinomycetes from internal plant tissues

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

Actinomycetes (No. of isolates)[a]	Antimicrobial activity (avg±SD)[b]								Cytotoxicity death %
	Sa.	V.	Sh.	K.	Str.	Stp.	G.	C.	
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); Sa., *Salmonella typhimurium*; *V.*, *Vibrio* sp.; *Sh.*, *Shigella boydii* ATCC 9207; *K.*, *Klebsiella pneumonia; Str.*, *Streptococcus* sp.; *Stp.*, *Staphylococcus aureus; G., Gardnerella vaginitis; C., Candida albicans*

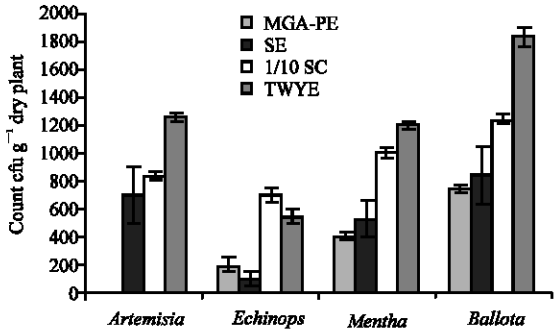

Fig. 2: Enumeration of endophytic actinomycetes on four selective media. Counts are averages of triplicates with error bars indicating standard error of the mean

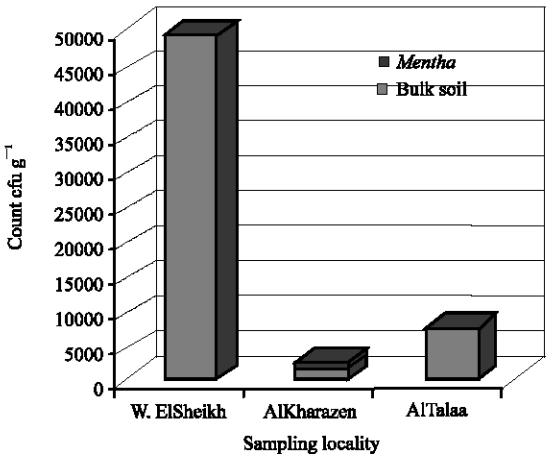

Fig. 4: Actinomycetes population from *Mentha longifolia* and the surrounding bulk soil. Counts are average of three replications

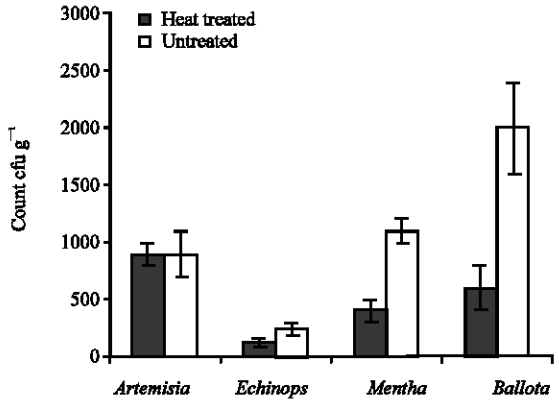

Fig. 3: Effect of heat treatment on recovery of endophytic actinomycetes from four plant species on 1/10 SC media. Counts are averages of triplicates with error bars indicating standard error of the mean

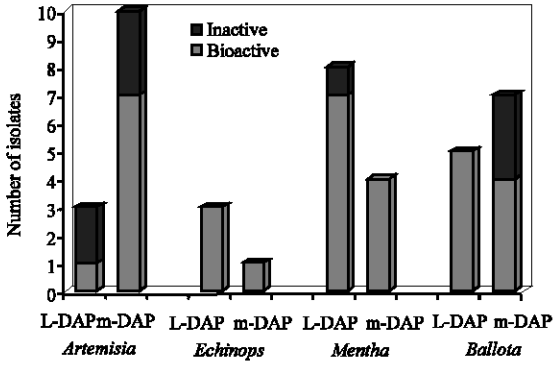

Fig. 5: Total number of endophytic actinomycetes isolates showing bioactivities. Isolates from the four investigated plant species are categorized into L-DAP and m-DAP containing groups

(Fig. 5). Of the 41 endophytic actinomycete isolates, 30 showed microbial inhibitory effect. The highest activity was against the clinical culture *Gardnerella vaginitis* and the reference strain *Shigella boydii* ATCC 9207, being represented in 29% of the isolates in both cases. None of the isolates showed bioactivity against *E. coli*, *Ps. aeroginosa* 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 h. Those were mainly *Artemisia and Echinops* isolates (Table 1).

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.* (2005) for isolation of stone actinomycetes using 1/10 SC. McCarthy and Williams (1992) have described an autochthonous behavior 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^{-1} dry plant in the four studied species, 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 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.

Overall, it is concluded that, using a simplified method actinomycetes could be selectively isolated from the internal tissues of wild medicinal plants in the WHS. 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.

ACKNOWLEDGMENTS

We are grateful to Dr. Kamal Ghodeif, Geology Dept., Faculty of Science, Seuz Canal University, for his invaluable guidance during the sampling trips. We also thank Dr. Raafat Abd El-Wahab, Botany Dept., Faculty of Science, Seuz Canal University, for his effort to identify the plants. This research was funded by grant C/3801-1 from the International Foundation for Science (IFS), Sweden.

REFERENCES

Bacon, C.W. and J.F. White, 2000. Microbial Endophytes. New York: Marcel Dekker.

Basil, A.J., J.L. Strap, H.M. Knotek-Smith and D.L. Crawford, 2004. Studies on the microbial populations of the rhizosphere of big sagebrush (*Artemisia tridentata*). J. Ind. Microbiol. Biotechnol., 31: 278-288

Bills, G., A. Dombrowski, F. Pelaez, J. Polishook and Z. An, 2002. Recent and Future Discoveries of Pharmacologically Active Metabolites from Tropical Fungi. In: Tropical mycology: Watling, R., J.C. Frankland, A.M. Ainsworth, S. Issac and C.H. Robinson (Eds.), Micromycetes, vol. 2. CABI Publishing, New York, pp: 165-194.

Caruso, M., A.L. Colombo, L. Fedeli, A. Pavesi, S. Quaroni, M. Saracchi and G. Ventrella, 2000. Isolation of endophytic fungi and actinomycetes taxane producers. Ann. Microbiol., 50: 3-13.

Castillo, U.F., G.A. Strobel, E.J. Ford, W.M. Hess and H. Porter *et al*., 2002. Munumbicins, wide-spectrum antibiotics produced by Streptomyces NRRL 30562, endophytic on Kennedia nigriscans. Microbiology, 148: 2675-2685.

Coombs, J.T., P.P. Michelsen and M.M. Franco, 2004. Evaluation of endophytic actinobacteria as antagonists of *Gaeumannomyces graminis* var. tritici in wheat. Biological Control, 29: 359-366.

Demain, A. and J. Davies, 1999. Manual of industrial microbiology and biotechnology. ASM. Washington. DC.

El-Tarabily, K.A., 2003. An endophytic chitinase-producing isolate of *Actinoplanes missouriensis*, with potential for biological control of root rot of lupine caused by *Plectosporium tabacinum*. Aust. J. Bot., 51: 257-266.

Gurney, K.A. and P.G. Mantle, 1993. Biosynthesis of 1-N-methylalbonoursin by an endophytic *Streptomyces* sp. isolated from perennial ryegrass. J. Nat. Prod. (Lloydia), 56: 1194-1198.

Hanafy, Y., M. Abd El-Wahaab, I. Amin, A. El-Haggawy and K. Rida, 2000. Wild Medicinal Plants of Sinai. 1st Edn. Arabian Gulf Est.

Hopwood, D.A., M.J. Bibb, K.F. Chater, T. Kieser, C.J. Bruton *et al*., 1985. Genetic Manipulation of *Streptomyces*: A Laboratory Manual. John Innes Foundation. Norwich, UK.

Igarashi, Y., T. Iida, T. Sasaki, N. Saito, R. Yoshida and T. Furumai, 2002. Isolation of actinomycetes from live plants and evaluation of antiohytopathogenic activity of their metabolites. Actinomycetologica, 16: 9-13.

Igarashi, Y., 2004. Screening of novel bioactive compounds from plant-associated actinomycetes. Actinomycetologica, 18: 63-66.

Kunoh, H., 2002. Endophytic actinomycetes: attractive biocontrol agents. J. Gener. Plant Pathol., 68: 249-252.

May, E., S. Papida and H. Abdulla, 2005. Consequences of microbe-biofilm-salt interactions for stone integrity in monuments. In: Art, Biology and Conservation. Koestler, R.J. (Ed.), Metropolitan Museum of Art, N.Y., pp: 452-471.

McCarthy, A.J. and S.T. Williams, 1992. Actinomycetes as agents of biodegradation in the environment. A Rev. Gene, 115: 189-192.

Puri, S.C., V. Verma, T. Amna, G.N. Qazi and M. Spiteller, 2005. An endophytic fungus from *Nothapodytes foetida* that produses camptothecin. J. Nat. Prod., 68: 1717-1719.

Rimbau, V., C. Cerdan, R. Vila and J. Iglesias, 1999. Anti-inflammatory activity of some extracts from plants used in traditional medicine of North-African countries. Phytother. Res., 13: 128-132.

Schaal, K., 1985. Identification of Clinically Significant Actinomycetes and Related Bacteria Using Chemical Techniques. In: Chemical Methods in Bacterial Systematics. Goodfellow, M. and D.E. Minnikin (Eds.), Academic Press. London, 359-381.

Shirling, E. and D. Gottlieb, 1966. Methods for Characterization of *Streptomyces* sp. Intl. J. Bacteriol., 16: 313-340.

Stamford, T.L., N.P. Stamford, L.C. Coelho and J.M. Araujo, 2002. Production and characterization of a thermostable glucoamylase from *Streptosporangium* sp. endophyte of maize leaves. Bioresour. Technol., 83: 105-109.

Strobel, G. and B. Daisy, 2003. Bioprospecting for microbial endophytes and their natural products. Microbiol. Mol. Biol. Rev., 67: 491-502.

Svoboda, K.P. and J.B. Hampson, 1999. Bioactivity of essential oils of selected temperate aromatic plants: Antibacterial, anti-inflammatory and other related pharmacological activities. Aromatopia, 35: 50-54.