African Journal of Microbiology Research Vol. 6(45), pp. 7253-7258, 27 November, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR12.785
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Antimicrobial activities of secondary metabolites produced by endophytic bacteria from selected indigenous Kenyan plants

Purity Kaaria[1]*, Viviene Matiru[1] and Mary Ndungu[2]

[1]Department of Botany, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya.
[2]Department of Chemistry, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya.

Accepted 5 November, 2012

Endophytic microorganisms are to be found in virtually every plant on earth. These organisms reside in the living tissues of the host plant and do so in a variety of relationships, ranging from symbiotic to slightly pathogenic. Endophytes are a potential source of novel chemistry and biology products/compounds to assist in helping solve human health problems. In the present study, bacteria endophytes were isolated from the roots of *Cleodendrum myricoides, Lannea flavus, Dichrostachys cinerea, Gomphocarpus fruticosus, Balanites aegyptica, Jasminium floribundum* and *Hibiscus fuscus.* 21 endophytic bacteria were isolated and examined for their antimicrobial activities using the cross streak method against four human pathogenic bacteria (*Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus)* and one fungus (*Candida albicans)*. Five (5) endophytic bacterial isolates, which showed activity against all the test organisms were selected and were subjected to physiological, morphological and biochemical characterization and further cultured. The ethyl acetate extract of the culture broth was assayed for antimicrobial activity through disc diffusion method. The culture broth extracts were further separated by chromatographic methods and tested for biological activity. The natural products separated in different bands/fractions and the most active fraction(s) with an inhibition diameter of ≥7 mm from each sample was further characterized to detect the secondary metabolites and the active components present. It was discovered from this study that most of the bacterial extracts inhibited the growth of the test organisms to varying degrees, some of which were comparable to conventional antimicrobials, but showed a pronounced antibacterial effect against *Bacillus subtilis*. This study also shows that a large number and range of secondary metabolites were present in the products. Further work might show whether these metabolites can be used to develop antimicrobial agents to replace the existing ones, once resistance builds up or for emerging pathogenic microorganisms.

Key words: Chromatographic methods, natural products, test organisms.

INTRODUCTION

Historically, microbial secondary metabolites have been an important resource for drug development. It was the discovery of penicillin that led to later discoveries of potent antibiotics isolated from microbial broths. Despite the existence of varied antibacterial and antifungal agents available in the market, there is a continuous need to search for novel drug compounds as the numbers of drug resistant microorganisms are continuously increasing (Pinner et al., 1996). Over the last few years there has been increasing interest in the bioprospecting of endophytic bacteria and fungi. Although previous

*Corresponding author. E-mail: kinyakaaria@gmail.com. Tel: +254722979366. Fax: 254-(0)67-52164.

searches for bioactive compounds focused on soil fungi and bacteria, the rate of discovery of interesting new compounds from the soil has diminished (Verpoorte, 1998).

Research has turned to niches that have not yet been explored (Bills, 1995; Pelaez et al., 1998) for finding novel, pharmacologically active compounds for industrial screening programmes. One such niche is the healthy, green tissues of living plants, which are known to harbor a rich and diverse bacterial biota that is distinct from the soil mycobiota (Cannon and Simmons, 2002).

In this context, endophytic bacteria isolated from indigenous plants are promising, because it is possible that they have acquired some of the genes from their host plants (Tan and Zou, 2001). To investigate endophytic bacteria isolates from indigenous plants as sources of bioactive secondary metabolites the present study was undertaken to evaluate the antimicrobial properties of their natural extracts.

MATERIALS AND METHODS

Bacteria isolation and culture conditions

Endophytes were isolated from the roots of the indigenous plants: *Cleodendrum myricoides, Lannea flavus, Dichrostachys cinerea, Gomphocarpus fruticosus, Balanites aegyptica, Jasminium floribundum* and *Hibiscus fuscus*. Roots were chosen for this study, since they are the most likely to harbor endophytic bacteria as compared to other plant organs. Nutrient Agar (Oxoid) and Tryptic Soy Agar (Oxoid) media were used for isolation. They were prepared according to the manufacturers' instructions and (50 µg/ml) of Nystatin added to inhibit fungal growth (Williams and Davies, 1965), dispensed into sterile plates. Lateral roots from the indigenous plant species were pretreated as described by Denise et al. (2002).

The pretreated plant materials were macerated with sterile distilled water using a sterile pestle and mortar and homogenized. Fifty microlitres (50 µL) of homogenate was inoculated onto Nutrient Agar + Nystatin and Tryptic Soy Agar + Nystatin (3 plates per sample) and spread using a glass spreader. This was carried out aseptically on a clean bench. Incubation was done at 25°C for 2 to 4 days. The bacteria growing on Nutrient Agar and Tryptic Soy Agar showing clear zones of inhibition against other isolates were selected and sub cultured on Nutrient Agar to get pure cultures, which were further characterized.

Antimicrobial screening

To further test the ability of each individual isolate to inhibit the growth of *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans*, antimicrobial screening was carried out. Each bacteria isolate was cultured onto nutrient agar and incubated at 30°C for 18 to 24 h and the test organisms were cultured in nutrient broth and incubated at 37°C for 18 to 24 h. Fifty microlitres of each test organism was placed on different Nutrient agar plates; spread evenly using a glass spreader and each bacterium isolate was cross streaked horizontally per plate and incubated at 25°C for 18 to 24 h (Cappuccino and Sherman, 2002). The isolates that inhibited the growth of test organisms after 24 h were recorded as positive and were investigated further whereas isolates in plates where growth of the test organism was not to be inhibited at all were recorded as negative and were not investigated further.

Characterization of endophytic bacterial isolates

The bacterial isolates that showed broad spectrum activity on all the five test organisms were selected for further characterization. Morphological characteristics; the colony shape, color, elevation, texture and the bacteria Gram type were observed. Biochemical activities of microorganisms were studied for the purpose of identification as well as classification.

Effect of sodium chloride, temperature and pH on growth of endophytic bacteria

To study the effect of sodium chloride, pH and temperature on the growth of endophytic bacteria, nutrient broth medium was prepared by supplementing with different concentration of NaCl, H_2SO_4 acid and different temperatures respectively and the endophytic bacterial isolates were inoculated into it. All the plates were incubated at 25°C for 5 days and observed for every 24 h. This was carried out for the purposes of media optimization during fermentation.

Extraction of metabolites

Each bacteria isolate was inoculated in an Erlenmeyer flask containing 5 L of tryptic soy broth and incubated for 3 to 4 days. The fermentation flask was incubated at 110 rpm on a rotary shaker at room temperature for 7 days. After fermentation the culture broth was filtered and the filtrates extracted three times with ethyl acetate. The organic phase was passed through a pad of anhydrous sodium sulphate and evaporated to dryness. The yields of the extract were determined and recorded. Part (50%) of the crude extract was used for biological activity tests and the remainder was analyzed by Gas chromatography coupled mass spectrometry (GC-MS) to identify the compounds present in the extracts.

Separation of the metabolites

Preparative TLC was performed on pre-coated silica gel glass plates (silica gel 60 F_{254}Merck, 0.25 mm layer thickness) using hexane: ethyl acetate (3:2) as the eluent. The bands were viewed under UV light (wavelength λ-254 and λ-366 nm) and respective bands boundaries marked (Qin and Judith, 1999). Each band was then scrapped off onto a filter paper and the constituents eluted into a beaker using hexane: ethyl acetate (3:2) and evaporated to dryness and their bioactivity determined before analysis by GC-MS.

Antimicrobial assay

Natural extracts and the separated natural extract fractions were screened for their antibacterial and antifungal activity using the Kirby-Bauer disc diffusion method (Lorian, 1996) against potentially pathogenic bacteria; *B.subtilis, E. coli, P. aeruginosa, S. aureus* and fungi *C. albicans.* 10 µL /sample was pipette to impregnate 6 mm sterile paper discs with the sample (Katia et al., 2000). The discs were allowed to dry in a sterile fume chamber and placed on agar seeded with test organisms. Incubation was done at 37°C for 24 h. Diameter of zones of inhibition was then measured and recorded. 6 mm sterile paper discs with nothing impregnated were used as negative controls. Activity was also compared to conventional antibiotics; tetracycline and gentamycin (positive

controls).

Identification of the compounds

The bands with inhibition activity of ≥7 mm were selected for GC-MS analysis. The samples were analyzed by combined GC-MS (7890 a series, GC Agilent technology), coupled to a 5975 C series mass spectrometer fitted with an 7683 B series auto sampler and a Triple Axis Detector. A HP5 MS 5% phenyl methyl silicone non-polar capillary column of 30 × 0.25 mm (internal diameter) and 0.25 µm (film thickness) was employed for separation of chromatograms. The GC was coupled to a HP monitor (L1710) onto which chromatographic data were acquired and evaluated by HP3365 CHEMSTATION software. Oven temperature was programmed at 35°C for 5 min followed by a rise at 10°C per min up to 280°C. The final temperature was maintained for 5 min. The injector and detector temperatures were set at 280°C. Helium gas was used as the carrier gas at a constant flow rate of 1.2 ml min^{-1}. For electron impact (EI), the ionization voltage was 70 eV and temperature of the ion source and the interface were 230 and 150°C, respectively. Matching mass spectra obtained within the John Wiley and NIST MS data libraries made tentative identification of the constituent compounds. In all cases, 1 µL of the sample was injected into the splitless mode with a 0.5 min delay before injection purging. From the GC-MS profiles of each fraction, different compounds were detected based on the retention time and the percentage of the total of each compound present.

Data analysis

All statistical analysis were performed with SAS software package, version 9.1 (SAS Institute, 2003) using two way analysis of variance (ANOVA) for the replicated treatments. Student –Newman Keuls (SNK) test was used for the separation of means.

RESULTS

Isolation of endophytic bacteria and characterization

A total of 21 antagonistic pure isolates were obtained from the indigenous plants and 5 isolates were further characterized based on their broad spectrum activity against the 5 human pathogens. Antagonistic bacteria endophytes were obtained from the plant *Gomphocarpus fruticosus* (33%), *Cleodendrum myricoides* (24%), *Lannea flavus* and *Jasminium floribundum* (14%) respectively, *Dichrostachys cinerea* (5%), *Hibiscus fuscus* (5%) *and Balanites aegyptica* (5%). Morphological characterization showed that the bacterial isolates had various forms of growth and pigmentation (morphological characteristics) on culture media and most isolates were gram positive (both rods and cocci). Biochemical characterization showed different biochemical reactions and is given in Table 1.

Antimicrobial activity of the natural products

Antimicrobial activity of the natural products obtained from the endophytic bacteria isolates against the test organisms was given in Table 2. The products from all the isolates showed inhibition against the strains tested.

Bioassay of separated fractions / bands of the natural products

The bioassay of the obtained different fractions/bands of the natural products was given in Table 3. The different fractions showed a range of inhibition activity against the test organisms. These are the fractions that had inhibition activity of > 7 mm.

Identification of chemical constituents

Identification of the compounds was done by analysis of the mass spectra and retention time. The fractions were chosen since they had considerable activity of more than 7 mm against the 5 test organism compared to the other fractions. From the analysis of the mass spectra of the fraction profiles, different compounds were identified and grouped in classes ranging from amines, amides, acids, quinines, indole derivatives, steroids, azoles, alcohols and hydrocarbons.

DISCUSSION

Various investigators reported endophytic microbes from various plant exists in different ecosystems. It is noteworthy that, of the nearly 300 000 plant species that exist on the earth, each individual plant is host to one or more endophytes. Only a few of these plants have ever been completely studied relative to their endophytic biology. Consequently, the opportunity to find new and interesting endophytic microorganisms among myriads of plants in different settings and ecosystems is great (Strobel and Daisy, 2003) .Plants have long provided mankind with a source of medicinal agents, with natural products once serving as source of all drugs. Microbial extracts have been and continue to be a productive source of new biologically active molecules for drug discovery (Arunachalam and Gayathri, 2010). According to a study by Denise et al. (2002), the variations are also attributable to plant age and time of sampling. In the present study, a total of 21 antagonistic endophytic bacterial isolates were obtained from the roots of 7 indigenous plants and they were selected since they are known to have an ethno botanical history (use by indigenous peoples) as a source of medicine particularly the roots.

Endophytes are the chemical synthesizers within plants. Many of them are capable of synthesizing bioactive compounds that can be used by plants for defense against pathogens and some of these compounds have been proved for useful drug discovery.

Table 1. Biochemical characterization of 5 bacteria endophytes.

Isolate code	Host plant	Triple Sugar Iron (TSI)				Sulphur Indole Motility (SIM)			SC	MR	VP	Urea	Gelatin	Catalase
		Butt	Slant	H_2S	Gas	Sulphur	Indole	Motility						
C25M	*Cleodendrum myricoides*	+	-	-	+	-	-	+	-	-	-	-	-	+
G20C	*Gomphocarpus fruticosus*	+	-	-	+	-	-	+	-	-	-	-	-	+
G43W	*Gomphocarpus fruticosus*	-	+	-	-	-	-	-	-	-	-	-	-	+
H11	*Hibiscus fuscus*	+	-	-	+	-	-	+	-	-	-	-	-	+
L13	*Lannea flavus*	+	-	-	+	-	-	+	-	-	+	+	-	+

+, A positive result for the reaction; **-,** A negative test for the reaction.

Table 2. Inhibition diameters (mm) ± Standard error (se) for the isolates' natural (crude) extracts against the test organisms.

Isolate	Test organisms (Diameter in mm, n=3)				
	Staphylococcus aureus	***Pseudomonas aeruginosa***	***Bacillus subtilis***	***Escherichia coli***	***Candida albicans***
C25M	10.7 ± 0.3^{cdB}	9.3 ± 0.3^{dBC}	15.0 ± 0.0^{dA}	8.3 ± 0.3^{deC}	9.7 ± 0.9^{cdBC}
G20C	10.0 ± 0.0^{dA}	7.0 ± 0.0^{efB}	9.7 ± 0.3^{efA}	9.0 ± 0.6^{deA}	9.7 ± 0.3^{cdA}
G43W	7.0 ± 0.0^{eA}	7.7 ± 0.3^{eA}	8.7 ± 1.2^{efA}	9.0 ± 1.0^{deA}	7.0 ± 0.0^{deA}
H11	11.7 ± 0.9^{cAB}	7.0 ± 0.0^{efC}	10.3 ± 0.3^{efB}	9.7 ± 0.9^{dB}	13.7 ± 1.2^{bA}
L13	11.3 ± 0.7^{cdB}	11.3 ± 0.7^{cB}	17.7 ± 2.7^{cA}	10.3 ± 0.3^{dB}	12.3 ± 0.3^{bcB}
-Ve control	6.0 ± 0.0^{eA}	6.0 ± 0.0^{fA}	6.0 ± 0.0^{fA}	6.0 ± 0.0^{fA}	6.0 ± 0.0^{eA}
TET	23.3 ± 0.7^{aB}	12.7 ± 0.9^{bC}	29.3 ± 0.7^{aB}	24.0 ± 0.6^{aB}	11.3 ± 0.7^{bcC}
GENT	21.0 ± 0.6^{bB}	18.7 ± 0.3^{aC}	20.7 ± 0.3^{bB}	22.3 ± 0.3^{bAB}	21.7 ± 0.3^{aAB}

Within a column, means compare inhibition diameters among the isolates natural extracts with the same test organism and means with the same lower case letter are not significantly different (P=0.05, SNK test). Within a row, means compare inhibition diameters among the individual isolate crude extract with different test organisms and means values with same upper case letter are not significantly different (P=0.05, SNK test). TET, Tetracycline; GENT, Gentamycin.

Table 3. Inhibition diameters (mm) ± Standard error (se)) of bands/fractions against the test organisms.

Isolate	Band/ fraction no.	Test organisms (diameter in mm, n=3)				
		Staphylococcus aureus	***Pseudomonas aeruginosa***	***Bacillus subtilis***	***Escherichia coli***	***Candida albicans***
C25M	4	9.0 ± 0.6^{defAB}	7.0 ± 0.0^{efC}	9.3 ± 0.9^{defAB}	9.7 ± 0.3^{cdeA}	7.7 ± 0.7^{efghBC}
G20C	12	8.3 ± 0.9^{defA}	$8.3 \pm 0.31.2^{cdefA}$	10.0 ± 1.2^{defA}	7.7 ± 0.3^{deA}	7.7 ± 0.7^{efghA}
	13	7.3 ± 0.3^{efA}	8.3 ± 0.3^{cdefA}	10.0 ± 1.2^{defA}	9.0 ± 1.2^{lcdeA}	0.0 ± 0.0^{iB}

Table 3. Continued.

G43W	8	8.7±0.7^{defB}	9.7±0.3^{bcdeB}	14.3±0.9^{cdA}	8.7±0.3^{cdeB}	10.3±0.3^{cdefB}
H11	6	7.7±0.7^{efAB}	7.7±0.3^{cdefAB}	9.3±0.3^{defA}	8.0±1.0^{cdeAB}	7.0±0.0^{ghB}
	7	8.3±0.9^{defA}	7.7±0.7^{cdefA}	10.3±1.8^{defA}	8.3±0.9^{cdeA}	7.3±0.3^{fghA}
L13	8	14.3±0.7^{bcA}	10.0±1.2^{bcdeB}	12.0±1.2^{cdeAB}	10.0±1.7^{cdeB}	15.3±0.7^{bA}
	11	15.3±0.9^{bA}	18.0±1.2^{aA}	15.7±1.2^{bcA}	15.7±0.7^{bA}	11.7±0.0^{cdB}
TET		23.3±0.7^{aB}	12.7±0.9^{bC}	29.3±0.7^{aB}	24.0±0.6^{aB}	11.3±0.7^{cdC}
GENT		21.0±0.6^{aB}	18.7±0.3^{aC}	20.7±0.3^{bB}	22.3±0.3^{aAB}	21.7±0.3^{aAB}
-Ve control		6.0±0.0^{fA}	6.0±0.0^{fA}	6.0±0.0^{fA}	6.0±0.0^{fA}	6.0±0.0^{fA}

Within a column, means compare inhibition diameters among the isolates different fractions and means with the same lower case letter are not significantly different (P=0.05, SNK test). Within a row, means compare inhibition diameters among the individual isolate fraction with different test organisms and means with the same uppercase letter are not significantly different (P=0.05, SNK test). TET, Tetracycline; GENT, Gentamycin.

Up till date, most of the natural products from endophytes are antibiotics, anticancer agents, biological control agents' antivirals, antidiabetic agents and other bioactive compounds by their different functional roles (Guo et al., 2008). From the cross streak antimicrobial screening, the isolates had considerable inhibition activity against pathogenic bacteria and fungi. 5 out of 21 isolates showed broad antagonistic activity against the all the test organisms. This could be due to the expected different modes of action, the level of isolate inoculation and activity of the individual biochemical constituent of the respective isolates (Mao et al., 2006).

In the present study, the natural products were extracted from the selected isolates and yields differed from one bacterial isolate to another. The yield difference could be attributed to the chemical composition (metabolites) and the individual genetic composition of the isolates. Different isolates produce a host of different number of compounds/metabolites; these metabolites also have different molecular weights causing the difference in yields (Newman et al., 2003). The natural products were subjected to an antimicrobial activity, which differed from one bacteria extract to another against the different test organism. Most of the bacterial extracts exhibited activity against the five test organisms. The difference in the *in vitro* activity among the various crude extracts could be due to the production of either a broad spectrum antimicrobial compound, or several compounds with different activities (Foldes et al., 2000). In a study conducted by Omura (1992), he concluded that the differences in levels of antagonism are dependent on concentration of the active substance(s).

Comparing the activity of the positive controls and the fractions against *S. aureus, B.subtilis* and *E.coli*, the positive controls had more inhibition activity. This could be due to the fact that the standard drugs may be more concentrated, hence show more activity. The zones of inhibition of the isolates fractions may be similar to the standard drugs, but if they have different modes of activity, this would still make them promising (Fatope, 1995). However some of the test isolates had fairly high activity as well. It is possible that there were novel compounds from the test isolates and this could be promising agents to replace drugs which resistance has developed. However further chemical analysis is required to determine this that is, if there are novel compounds. Consequently, the search for the secondary metabolites as lead compounds or templates in drug development is encouraged. From the results, the different natural products fractions, are a potential in the provision of the basis for the synthesis of novel therapeutics, that would aid in the fighting of life threatening diseases once resistance builds up and since gram negative bacteria are among the recalcitrant pathogens

found in hospitals that have acquired resistance to several antibiotics in the past, the search for the secondary metabolites that could enable the synthesis of drugs should be encouraged.

From the GC-MS analysis, the constituent compounds present in the fractions were a mixture of amines, amides, indole derivatives, quinones and steroids. Some of these compounds have been documented as antimicrobial compounds in a study carried out by Hua et al. (2006) and Tan and Zou (2001). Other unique groups of compounds that were detected include alcohols, azoles and vanillin.

From our results we could demonstrate the potential value of investigating metabolite production by endophytic bacteria from indigenous plants, since they are a source promising antimicrobial compounds against various human pathogenic microbes. It would be of interest to find out which functional group is responsible for the bioactivity and also whether any of them is a novel compound with antimicrobial activity which would make it a promising candidate for the production of new antimicrobials. Further work on these metabolites will reveal this and will also aid in gaining insight of synergism among the different functional groups. Molecular characterization should be carried out to fully identify the 5 endophytic bacterial isolates responsible for the production of the active secondary metabolites.

ACKNOWLEDGEMENTS

We wish to sincerely record our deepest gratitude to JKUAT (RPE) for the financial support. We are thankful to ICIPE-BCED staff for their support in chemical characterization using GC-MS and all the JKUAT Botany and Chemistry staff for their unending support during the entire study.

REFERENCES

Arunachalam C, Gayathri P (2010). Studies on bioprospecting of endophytic bacteria from the medicinal plant of *Andrographis paniculata* for their antimicrobial activity and antibiotic susceptibility pattern. Int. J. Curr. Pharm. Res. 2(4):63-68

Bills GF (1995). Analysis of micro fungal diversity from a user's perspective. Can. J. Bot. 73:33-41.

Cannon PF, Simmons CM (2002). Diversity and host preference of leaf endophytic fungi in the Iwokrama Forest Reserve, Guyana. Mycologia 94:210-220.

Cappuccino JG, Sherman N (2002). Microbiology. A laboratory manual. 6th edition. Pearson education inc. San Francisco, California pp. 149-224.

Denise KZ, Pat LN, Harris BN, Feng Z, Daniel K, Higley P, Carol AI, Alahari A, Raul GB, Anne KV (2002). Isolation and characterization of endophytic colonizing bacteria from agronomic crops and prairie plants. Appl. Environ. Microbiol. 68:2198-2208.

Fatope MO (1995). Phytocompounds: their bioassay and diversity. Discov. Innov. 1:229-236.

Foldes T, Banhegyi I, Herpai Z, Varga L, Szigeti J (2000). Isolation of *Bacillus* strains from the rhizosphere of cereals and *in vitro* screening for antagonism against phytopathogenic, food-borne pathogenic and spoilage micro-organisms. J. Appl. Microbiol. 89:840-846.

Guo BY, Wang X, Sun, Tang K (2008). Bioactive natural products from endophytes: A review. Appl. Microbiol. Biotechnol. 44(2):136-142.

Hua WZ, Yong CS, Ren XT (2006). Biology and chemistry of endophytes. Nat. Prod. Rep. 18:448

Katia FM, Manfred, Christa W (2000). Antimicrobial activities of secondary metabolites produced by endophytic fungi *Spondias mombin*. J. Basic Microbiol. 40:261-267

Lorian V (1996). Antibiotics in Laboratory Medicine, 3rd edition. Williams and Wilkins.

Mao S, Lee SJ, Hwangbo H, Kim YW, Park KH, Cha GS, Park RD, Kim KY(2006). Isolation and characterization of antifungal substances from *Burkholderia* spp. culture broth. Curr. Microbiol. 53:358-364.

Newman DJ, Cragg GM, Snader KM (2003). Natural products as sources of new drugs over the period 1981-2002. A detailed analysis and description of current natural product derived therapeutic agents. J. Nat. Prod. 66:1022-1037.

Omura S (1992). Trends in the search for bioactive microbial metabolites. J. Ind. Microbiol. 10:135-136.

Pelaez F, Collado J, Arenal F, Basilio A, Cabello A, Diez matas MT, Garcia JB, Gonzalez del val A, Gonzalez V, Gorrochategui J, Hernandez P, Martin I, Platas G, Vicente F (1998). Endophytic fungi from plants living on gypsum soils as a source of secondary metabolites with antimicrobial activity. Mycol. Res. 102:755-761.

Pinner R, Teutsch S, Simonsen L, Klug L, Graber J, Clarke M, Berkelman R (1996). Trends in infectious diseases mortality in the United States. J. Am. Med. Assoc. 275:189-193.

Qin L, Judith LK (1999). Preparative thin layer chromatographic separation and subsequent gas chromatographic –mass spectrometric analysis of monoacylglycerols derived from butter oil by fungal degradation. J. Chromatogr. 855:617-624.

Strobel GA, Daisy B (2003). Bioprospecting for microbial endophytes and their natural products. J. microbiol. Mol. Biol. 67:491-502.

Tan RX, Zou WX (2001). Endophytes: a rich source of functional metabolites. Nat. Prod. Rep. 18:448-459.

Verpoorte R (1998). Exploration of nature's chemo diversity: the role of secondary metabolites as leads in drug development. Drug Discov. Today 3:232-238.

Williams ST, Davies FL (1965). Use of antibiotics for selective isolation and enumeration of actinomycetes in soil. J. Gen. Microbiol. 38:251-261.