RESEARCH PAPER | Botany | Volume : 4 | Issue : 11 | November 2014 | ISSN - 2249-555X

Characterization of Endophytic Bacteria Isolated from the Medicinal Plant CapparissinaicaVeill. and Analyze its Bioactive Flavonoid

KEYWORDS: *Endophytic bacteria, CapparissinaicaVeill.,Flavonoids, Quercetin.*

MagdyMohsen Mohammed Bahgat
Professor of Microbiology, Botany Department, Faculty of Sciences, Port-Said University, Port-Said, Egypt.

Mona Mahmoud El Bous
Lecturer of Plant Taxonomy, Botany Department, Faculty of Science, Port-Said University, Port-Said, Egypt

Salwa Ali Kawashty
Professor of Phytochemistry, Phytochemistry and Plant Systematics Department, National Research Centre, Dokki-12311, Cairo, Egypt

Nesma Ahmed Mohammed El
Demonstrator, Botany Department, Faculty of Science, Port-Said University, Port-Said, Egypt.

ABSTRACT *The present work focused on isolation, visualizing endophytic bacteria in its natural niche, inside plant tissue,and identification of cultivableendophytic bacteria that colonize aerial parts ofCapparissinaica, as anindigenous plant to Sinai-Egypt. C.sinaicais a medicinal plant was used in the treatment of several human diseasesin traditional medicines.The morphological, biochemical and 16Sr RNA gene sequence analysisrevealed thatBacillus species were the most dominantendophytic bacteria.Mixed microbial culture system of isolated endophyticbacterial species wasused for production of secondary metabolites.Paper Chromatography and HPLC analysis showed that bacterial extract producedquercetin andquercetin 3-O-glucopyranoside which were detected in plant extract. Hence, it was concluded that endophytic bacteria produced flavonoid compoundsin vitro similar to that originally produced by the plant itselfconfirming theidea of considering endophytes as the potential sources of novel natural products.*

Introduction

Endophytic bacteria can be defined as those bacteria that colonize the internal tissue of the plant showing no external sign of infection or negative effect on their host (Schulz and Boyle, 2006). Nearly 300 000 plant species that exist on the earth, each individual plant is host to one or more endophytes (Strobel et al., 2004).

Endophyticmicrobes have been known as possible useful sources of bioactive secondary metabolites (Strobel et al., 2004)as medicinally therapeutic important agents (Silvia-Firákováet al., 2007; Huang et al., 2008) like alkaloids, terpenoids, flavonoids etc. that have importance in medicine, agriculture and industries as well (Joseph and Mini Priya, 2011; Dhanya and Padmavathy, 2014).

The need for new natural and useful compounds to provide assistance and relief in all aspects of the human health is ever growing with the passage of time. During resistance in bacteria and the tremendous increase in the incidence of fungal infections in the world's population each day only underscore our inadequacy to cope with these medical problems, there is a general call for new antibioticsand chemotherapeutic agents that are highly effective, possess low toxicity, and have a minor environmental impact (Arundhati Pal and Paul, 2013). The search for plant bearing novel natural pharmaceutically active compounds continues to yield promising leads. However, in some cases, difficulties arise in securing a reliable source of potentially useful materials. Plants, compared to most drug-producing microbes employed by the pharmaceutical industry, are frequently difficult and expensive to propagate on large scale. Moreover, national and international regulations restrict the transport of many nonnative plant species (Gary et al., 1998).

Thus, if entophytes can produce the same rare and important bioactive compounds as their host plants, this would not only reduce the need to harvest slow growing and possibly rare plants but also preserve the world's ever-diminishing biodiversity. Furthermore, it is recognized that a microbial source of a valued product may be easier and more economical to produce, effectively reducing its market price.

Capparissinaica is a promising medicinal herb mentioned in ancient ayurvedic literature as having great economic potential. The plant is indigenous to Sinai and is used in traditional medicines to cure various illnesses. Phytochemicals studies of C.sinaica have shown the presence of many beneficial compounds such as rutin, quercetin, kaempferol, andtocopherols(Sharafet al., 1997; Hamedet al., 2007; Abd El-Hamedet al., 2011).Moreover, Biological studies reveal important antimicrobial, anti-oxidative, anti-inflammatory, immune-modulatory and antiviral properties (Tliliet al., 2011).Endophytes from C.sinaica in HammamFaraoun, Sinai, Egypt show a unique environmental setting of novel microbial endophytes. However, there is scarce information on the characterization of its endophytes.

Materials & Methods

Selection of plant material

The following characteristics were taken into consideration in order to isolate the endophytic bacteria from a plant (Strobel and Daisy, 2003):-

1. Plants from a unique ecological environmental niche and growing in special habitats, especially those with an unusual biology.
2. Plants that have an ethnobotanical history, and are used for traditional medicines.
3. Plants those are endemic, having an unusual longevity.
4. Plants growing in areas of great biodiversity.

Volume : 4 | Issue : 11 | November 2014 | ISSN - 2249-555X

Plant Collection

• For phytochemical screening, About 5 Kg fresh plant material (aerial parts) of C.sinaica was collected from HammamFaraoun area in Sinai; Egypt (Suez Gulf, N: 29: 12: 16, E: 32:57:16) on September 2012. The plant was found on the mountain; 4 meters height. Plant was identified according to Boulos, 1995, 1999.

• For the isolation of endophytic bacteria, healthy green aerial parts (internode) of plantwere collected, placed in sterile sealed plastic bags and immediately transported aseptically to the laboratory and were used within 24 hrs. for microbiological procedures.

Sample processing for endophytic bacteria isolation
Pretreatment of aerial parts

Aerial parts samples were cut into about 1cm long pieces and then washed in running tap water for 10 minutes to remove soil particles, microbes and adhered debris, then were washed in tap water and detergent (tween 20) and finally washed with distilled water.

Surface sterilization

Surface sterilization was done using methodology described by (Petrini, 1992; Werner et al., 1997) with some modifications to remove epiphytes. Samples were immersed 2 times in 70% ethanol for three minutes and immersed twice in 2-4% aqueous solution of sodium hypochlorite for 5 minutes and again immersed for 1 minute in70% ethanol. Finally, rinse samples 6-8 times with sterile water for 5 minutes and wash 2times in sterile distilled water for 5 min to remove surface sterilization agents with further drying in sterilized paper in a laminar flow hood.

Sterility check

To confirm that the plant surfaces were effectively decontaminated 1ml aliquots of the sterile distilled water that was used in the final rinse of surface sterilization procedures were plated onto nutrient agar medium N.A (McInroy and Kloepper 1994) and incubated at 28°C for 48 hrs. Bacterial growth was observed after 48 hrs.. Also, surface sterilized segments were rolled on nutrient agar plates, incubated at 28°C for 48 hrs. and checked for possible microbial growth (Hallmann et al., 1997).

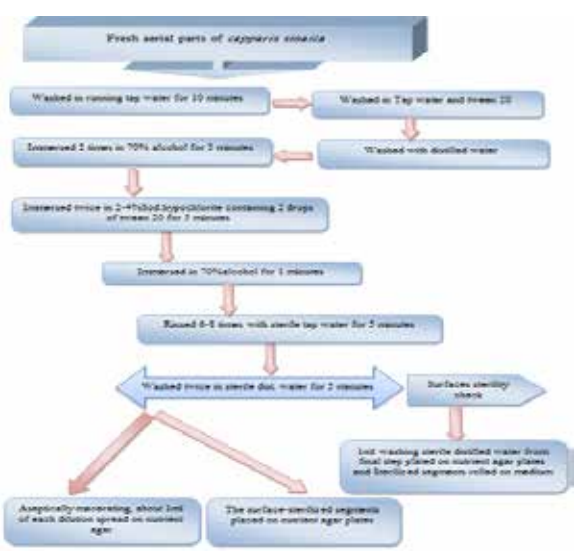


Figure 1Proposed strategy for selective isolation of endophytic bacteria from internal tissues of capparissinaica

Endophytic bacteria isolation

For this purpose two methods were followed:

1. Under aseptic conditions the surface-sterilized segments were cut into about 1×1×0.5 cm (length × width × thickness) pieces. The surface of shoots for each segmentapproximately 0.5 cm from the margin was removed using sterile surgical blades (Shah et al., 2010), then placed on N.A platesand incubated at 28°C for 2 days.

2. Surface-sterilized segments were aseptically macerated in a 10 mM$MgSO_4$ solution using sterile mortar and pestle and with ultra-turrax homogenizer (Wise Tis® HOMOGENIZER) (Hallmannet al., 2006). About 1 ml of the macerated tissue was serially diluted up to 10^{-3} using sterile10mM potassium phosphate buffer (pH 7). About 1ml from each dilution of intercellularfluid of its tissue was spread on N.A plates by sterile glass spreader. All plates were kept in an incubator at 28°C for 48hrs.

Purification, selection and preservation of endophytic bacterial isolates

After incubation, number of aerobic heterotrophic bacteria was recorded as colony-forming units (CFUs). And selection of colonies was under taken based on the variation in macro-morphology characteristics, results not shown. Colonies were purified through repeatedly re-streaking on N.A medium. Isolates were preserved on slants with fresh N.A medium covered with mineral oil at 4°c for further use.

Preliminary characterization of endophytic bacteria

Phenotypic characteristics such as microscopic characterization of gram reaction was carried according to method described by Süßmuthet al., 1999, endospore staining was done according to the Schaeffer-Fulton method and motility was determined with the Hanging Drop Slide for all isolates using a Trinocular phase contrast epi- fluorescent microscope (Olympus BIMAX 60). Catalase and Oxidase activity were performed using commercially available reagents according to Barrow and Feltham, 1993.

Microscopic examination and localization of endophytic bacteria within plant by vital staining technique

Examination of endophytic bacteria was performed following the protocol described by Pathak et al., 2009. 100 ml 50mMpotassium phosphate buffer (pH-7) and 0.0625 g malic acid were added into a conical flask and autoclaved it for 15 min. 0.15 g 2, 3, 5- triphenyltetrazolium chloride (TTC) was placed into sterile 50 mM malate potassium phosphate buffer (pH-7) in aseptic condition. Surface-sterilized of plant segments were dipped into 20 ml of the above sterile TTC solution and incubate overnight (not over 10 hrs.) at 30 °C.TTC-treated Cross sections wereplaced on glass microscopic slides and wet mount of the sections were prepared with phosphate buffer, thena glass cover slip was placed and the edges weresealedwith colorless nail polish.Sections were examined under compound microscope at 100_x magnification. The TTC-treated sections were counter stained with1% Aniline blue for 1 min and then the excess of stain was removed and wet mount was prepared and observed immediately under the microscope.Cross-sections werestained with working solution of Acridine orange for 3–5 min at room temperature. Sections were rinsedwith phosphate buffer for 2 min,finallywere observed under epi-fluorescence microscope.

Endophytic bacteria identification by 16S rRNA gene sequence analysis

Ten isolates were selected out of original twenty isolates.

Volume : 4 | Issue : 11 | November 2014 | ISSN - 2249-555X

The selection was carried out as the preliminary characterization showed similarity between some isolates. As a result of that 10 isolates were finally selected to progress further tests in this study.

Isolation of total DNA
The bacterial isolates were grown in nutrient broth medium at 28°c for 48 hrs. Genomic DNA was extracted followed the protocol described by Araujo et al., 2002 with minor modification.

16S rRNA gene amplification and sequencing
The 16S rRNA gene sequences were determined by PCR amplification (Kwon et al., 2003), followed by direct sequencing (Hiraishi, 1992).

Approximately 1,500 bp of the 16S rRNA gene fragment was PCR amplified from the purified genomic DNA of each isolate using the universal primer set 8–27F 5′-GAGTTTGATCCTGGCTCAG-3′ (Weisburget al., 1991) and 1492R 5′-GGTTACCTTGTTACGACTT-3′ (Reysenbachet al., 1992). PCR amplification was performed in an automated thermo cycler (GeneAmp® PCR System 9700 Dual 384-Well Sample Block Module) with the amplification profile: denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 92°C for 30 s, annealing at 50°C for 2 min, extension at 72°C for 1.5 min, and a final extension step at 72°C for 5 min. Gels photos were captured using gel documentation system then analyzed by Gel Docu advanced ver.2 software. Positive bands of PCR product were excised and purified after agarose gel electrophoresis by a gel extraction kit (QIAquick PCR Purification Kit, QIAGEN).

2nd PCR amplification was performed using BigDye Terminator v3.1 Cycle Sequencing Kit. Thermal profile for Cycle Sequencing PCR was initiated with denaturation at 96°C for 1 min, followed by 30 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 sec, and a final extension step at 60°C for 4 min and was performed with GeneAmp® PCR System 9700 .An additional step of purification for PCR product with CENTRI-SEP Columns (PRINCETON SEPARATIONS), finally Purified product was sequenced on an ABI 377 automated sequencer (3500 Genetic Analyzer, Applied BioSystems, Foster City, CA, USA).

DNA sequence analysis
16S rRNA gene sequences obtained from bacterial isolates were analyzed using BLASTn tool at the National Center for BiotechnologyInformation database (NCBI) GenBank database using the Basic LocalAlignment Search Tool (BLAST) analysis tools (Altschulet al., 1990) to identify the most similar 16S rRNA sequences available in the GenBank.

Chemical screening for plant material and endophytic bacterial extracts
Preparation of plant material
The fresh plant was harvested, cleaned and air dried under shade for 14 days and reduced to coarse powder using pestle and mortar and then grinded to fine powder using the Kenwood blender (Mabry et al., 1970, Markham, 1982). The powder was stored in an airtight bottle until needed for use.

Preparation of plant extract
The dried powdered aerial parts of C.sinaica were exhaustively extracted three times with 70% aqueous methanol at room temperature. The extract was filtered and dried under reduced pressure using a rotary evaporator (RE300 equipped with Stuart Vacuum Pump RE3022C) at 60 °C to yield residue. The resulting crude extract was dissolved in 1-3 ml methanol and was stored at – 20 °C.

Small-scale fermentation and extraction
Being able to obtain the exponential and stationary growth phases, the growth curve of the bacterial isolates was determined in optimum growth conditions using the absorbance method. Fermentation took place according to Bhoreet al., 2010 with some modification. it performed in 1 L Erlenmeyer flasks containing 500 ml of several media were tested (nutrient broth, nutrient broth supplemented with 3% glucose and enrichment medium (15 glucose, $3NaNO_3$, 0.5 KH_2PO_4, 2.5 KCl, 0.1 $MgSO_4$, 0.05 $FeSO_4$, 25 peptone, 15 beef extract in 500 ml distilled water). The cultures were incubated at 28 °C with 121 rpm agitation. After 3 days the culture was harvested by centrifugation for 15 min at 10000 rpm (16000x g, Centrifuge plc series).

The supernatants (extra cellular culture filtrates) were extracted with equal volumes of ethyl acetate in a separating funnel by shaking vigorously for 15 minutes. After separation, the organic phases were concentrated on aRotary Evaporator (RE300 equipped with Stuart vacuum pump RE3022C) at 40 °C and dried over Calcium Oxide (anhydrous). The resulting crude extract was dissolved in 1-3 ml methanol and stored at – 20 °C. The bacterial pellet was washed 2-3 times with sterile filtered saline solution. Extraction of washed cells was carried out with methanol overnight at 4 °C for three times. The organic phase was dried as filtrate ethyl acetate extract and stored at – 20 °C.

Phytochemical screening of the plant and bacterial extracts
1. Preliminary phytochemical screening
The extracts were subjected to preliminary phytochemical screening for the determination of major chemical groups by standard procedures. It was carried out on plant extract, all endophytic bacterial extra cellular culture filtrate extracts (F) and all endophytic bacterial pellet extracts (P) in order to test for the presence of tannins, flavonoids, glycoside and/or carbohydrates, alkaloids, phenols, saponins, sterols and triterpens according to methods described by Treaseet al., 1996 with some modifications.

2. Two Dimensional Paper Chromatography Analysis
Chromatographic screening of the previously prepared plant extract, all endophytic bacterial extracts were subjected to two Dimensional Paper Chromatography (2DPC) for qualitative identification on Whatmann paper No. 1(Whatman Ltd. Maidstone, Kent, England) using two solvent systems as (a) BAW for the first dimension and (b) 15% AcOHfor the second dimension (Mabry et al., 1970). The distribution of the flavonoids was compared with those previous reported in C. sinaica by CO-PC with authentic samples and confirmed using HPLC analysis.

3. HPLC analysis
Two grams of dried plant extract andextra cellular culture filtrate endophytic bacteria extract of enrichment medium were dissolved in 5 mL methanol of HPLC grade and analyzed by an agilent HPLC 1200 series equipped with diode array detector (agilent Technologies, Waldbronn, Germany). Chromatographic separations were performed using a waters column C18. The previously isolated compounds from C. sinaica (Sharaf et al., 1997;Abd El-Hamed, 2011) were used as reference standards to measure their

Volume : 4 | Issue : 11 | November 2014 | ISSN - 2249-555X

relatively in the extract. Separated peaks were identified by direct comparison of their retention times with those of standards. Standard solution wasthen added to the sample and peaks were identified by the observed an increase in their intensity.

Results

As natural habitat of C.sinaica at Ravins and rocks of hot deserts the representative specimen was collected from Sinai, HammamFaraoun, N: 29:44: 279, E: 32:41:865, 4 meters high; N: 29: 12: 219, E: 32: 57: 271.

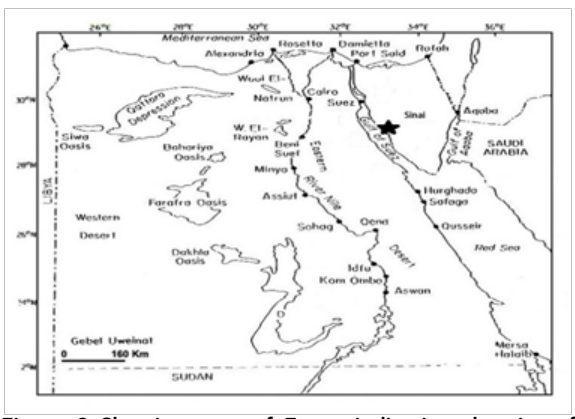


Figure 2 Showing map of Egypt indicating the site of collection of Capparis sinaica Veill

Figure 3overview of the sampling site showing the Capparis sinaica shrubs in its natural habitat at Hammam Faraoun,Sinai, Egypt

The surface sterilization protocol was successful in terms of isolating cultures representing endophytic bacteria. This was confirmed as sterility check was carried out on the final step. Samples of the final washing of plant segments and plant segments sterilized which were thoroughly rolled on the surface of NA plates under absolute aseptic conditions. Results showed absolutely no growth on the plates after incubation.Modifications were needed to improve the surface sterilizationprotocol which include:-

1. Increasing the immersion time of plant segments in 70% ethanol to 3 minutes instead of a minute was highly beneficial for better surface sterilization.
2. Plant segments were immersed twice in 2-4% hypochlorite instead of once.
3. Plant segments were rinsed with sterile water for 5 minutes instead of 3 minutes.

The growth of endophytic bacteria was observed as separate colonies in case of maceration of surface sterilized plant aerial parts. While growth of endophytic bacteria was in the form of emerging colonies close to the edge of the surface sterilized plant segmentsafter incubation on N.A plates (Figures 4, 5).

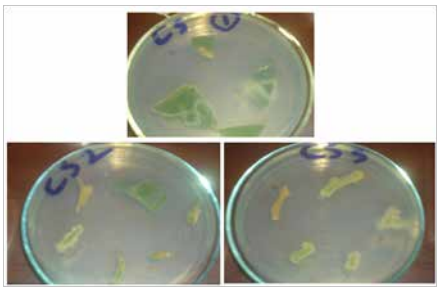


Figure 4 Emerging bacterial endophytes from aerial parts ofC.sinaica

Figure 5 Generated bacterial endophytes colonies from original solution

of macerated aerial parts of C.sinaica (spread plate technique)

Twenty strains of endophytic bacteria were isolated and only ten selected isolates fromthemwere chosen for further tests.The preliminary identification of the bacterial isolates was done based on morphological and various biochemical characteristics.90% of the isolated strains of endophytic bacteria were gram positive type, all are catalase positive, 70% were spore former and 60% of isolates are motile.

The stained transverse sections of plant with (TTC) revealed the presence of pink to purple stained bacteria in cortex and pith area (Figure 7, a, b) upon microscopic examination. They appeared to be localized in intercellular spaces, intracellular of cortex cells as well as around xylem vessels (Figure 7, c).

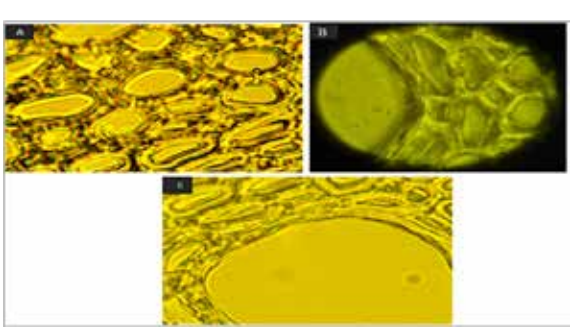


Figure 7 Light micrograph 100x of T.S. cortical cells of TTC treated C. sinaica roots showing the distribution of bacterial cells within the intercellular spaces and around xylem vessels

Volume : 4 | Issue : 11 | November 2014 | ISSN - 2249-555X

Endophytic bacteria identification by 16SrRNA gene sequence analysis was carried out for ten isolates. 16S rRNA fragments were PCR-amplified in which fragments with approximately 1,500 bp were observed (Figure 8).

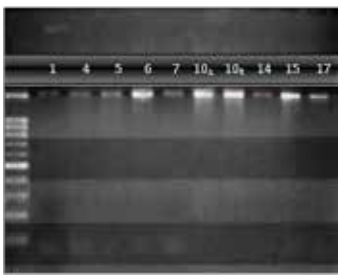


Figure 8 PCR products of the amplification of the 16S rRNA encoding regions/fragments on Agarose gel 1% (w/v) for 10 isolates (1,4,5,6,7,10A,10B,14,15,17), lane 0; 1500 pb DNA ladder (DNA marker)

The identification of endophytic bacteria based on the comparison between the16SrRNA gene sequences obtained from bacterial isolates and those deposited in (NCBI) GenBank database using BLASTn tool to obtain the sequences that displayed maximum similarity (Table 1). Results of this analysis showed that one given sequence was actually similar to those sequences of more than one species.Most sequences reported by BLASTn tool revealed the prevalence of genus Bacillus commonly associated with plants as endophyte as reported in similar works (Krid, 2010; Borriss and Maheshwari, 2012; Jagadesan andBhore, 2014). 70% of the analyzed isolates belonged to different strains of genus Bacillus(B. subtilis, B. amyloliquefaciens subsp. Plantarum and Bacillus benzoevorans). While it also revealed that one analyzed isolate (14) was in closest match to Uncultured Staphylococcus sp. clone WS08B_D02which recorded as endophytic bacteria according to Khianngamet al., 2013.

Table 1Endophytic bacteria isolated from Capparissinaica identification based on 16S rRNA gene sequence compared to sequences deposited in (NCBI) GenBank database using BLASTn tool

Culture ID	Closest NCBI match/identity 16S ribosomal RNA gene	Max Identity %	Suggested identification pb length	16SrRNA sequence (pb length)	GenBank Accession number	Max. score	Gaps %	Suggested phylogeny
1	Paucisalibacillusglobulus	91%	882	1084	JN615418.1	75	3	Firmicutes
4	unidentified diplococcic gram negative motile catalase and oxidase positive							
5	Bacillus sp. CBMAI 1211	100%	844	1052	HQ433240.1	62.1	0%	Firmicutes
6	Bacillus tequilensis	92%	1435	1099	JN700167.1	460	6%	Firmicutes
	Bacillus methylotrophicus	92%	1453		JN700132.1	460	6%	Firmicutes
	Bacillus amyloliquefaciens subsp. plantarum	92%	1457		HQ831402.1	459	6%	Firmicutes
7	Uncultured endophytic bacterium clone WBac32	87%	531	957	KF709601.1	320	11%	environmental samples
10A	Bacillus circulans	90%	502	1037	JN626207.1	497	8%	Firmicutes
10B	Bacillus benzoevorans 'PapViBa6b'	93%	880	858	DQ346735.1	438	8%	Firmicutes
14	Uncultured Staphylococcus sp. clone WS08B_D02	90%	747	981	gb|DQ171657.1|	63.9	2%	Firmicutes
15	Bacillus nealsonii	83%	1455	1058	JQ579625.1	276	9%	Firmicutes
17	Bacillus subtilis	89%	505	1092	EU158264.1	501	8%	Firmicutes
	Bacillus cereus	89%	1142		JX847620.1	468	9%	Firmicutes

Volume : 4 | Issue : 11 | November 2014 | ISSN - 2249-555X

The results obtained from growth curves of the studied endophytic bacterial isolates showed that the optimum period for harvesting the secondary metabolites was considered to be about three days. Preliminary phytochemical screening of both plant extract and endophytic bacterial extra cellular cultureextractsofnutrient broth supplemented with 3% glucose (F_2) and ofenrichment medium (F_3) indicates the presence of flavonoids,terpenoids and glycosidesin different concentrations. Bacterial pellet extracts screening showed absence of all bioactive compounds except carbohydratesas shown in (Table 2).

Paper Chromatographyand HPLC analysis were conducted for tracking flavonoids as these compounds represent the major constituent of plant extractas well as (F_3) bacterial extract.All Extracts of plant and endophytic bacteria were subjected to 2DPC and CO-PC with the authentic samplesunder the same conditions. Five flavonoids namely quercetin, quercetin 3-O-glucopyranoside,quercetin 3-rutinoside,isorhamnetin 3-rutinoside and kaempferol 3-rutinoside were detected in plant extract, while quercetin andquercetin 3-O-glucopyranoside were detected in both bacterial extracts (F_2; F_3)but in different concentrations (Table 3).

Table 2 phytochemical screening for C. sinaica and its endophytic bacterial extracts

Tested extract	Phytochemical constituents					
	Tannins	Flavonoids	Glycosides or carbohydrates	Alkaloids	Saponins	Steroids or Terpenoids
Plant extract	--	+ + +	++	+	++	++
F_1	--	--	+	--	--	--
F_2	--	(+)	+	--	--	(+)
F_3	--	+ +	+	--	+	(+)
P_1	--	--	--	--	--	--
P_2	--	--	+	--	--	--
P_3	--	--	+	--	--	--

MeOH C.s for 70% methanol extract of C.sinaica aerial parts, F for extra cellular culture filtrate, F_1; Culture filtrate of nutrient broth medium, F_2; Culture filtrate of nutrient broth supplemented with 3% glucose, F_3; Culture filtrate of enrichment medium, P for 80% methanol extract of bacterial pellet, P_1; Bacterial pellet of nutrient broth, P_2; Bacterial pellet of nutrient broth supplemented with 3% glucose, P_3;Bacterial pellet of enrichment medium. +++; Very strong, ++; Strong, +; Present, (+); Trace, (-); Absent.

Paper chromatography results showed that flavonoids were found in relatively high concentration in plant extract while they were present in moderate concentration in endophyticbacterial extract (F_3). Therefore, HPLC analysis was performed on both plant and endophytic bacterial(F_3) extracts.

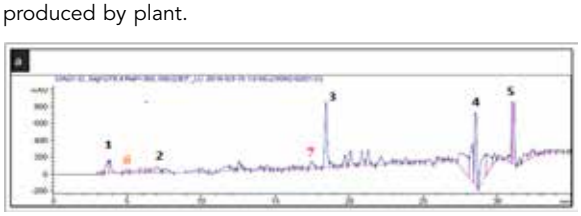
HPLC analysis was performed to identify the flavonoids of plant and endophytic bacterial (F_3) extracts as shown in Figure 9a and 9b, respectively. The flavonoids were identified by comparison their retention times with those of the authentic samples (Table 4).

Table3 Detected compounds in this study compared with previously isolated flavonoids from C. sinaica

Flavonoids reported previously in C. sinaica	References	Compounds detected in this study			Color reactions		
		MeOH C.s	F2	F3	UV	UV & NH_3	UV&$AlCl_3$
Quercetin	Abd El-Hamedet al., 2011	+	+	+	Y	Y	Y-G
Quercetin 3-glucoside	Sharafet al., 1997	+	trace	+	D-P	Y	Y
Quercetin 3-rutinoside (Rutin)	Hamedet al., 2007	+	-	-	D-P	Y	Y
kaempferol 3-rutinoside	Hamedet al., 2007	+	-	-	D-P	Y	Y
Isorhamnetin 3-rutinoside	Abd El-Hamedet al., 2011	+	-	-	D-P	Y	Y

MeOH C.s for 70% methanol extract of C.sinaica aerial parts, F for extra cellular culture filtrate, F_2; Culture filtrate of nutrient broth supplemented with 3% glucose, F_3; Culture filtrate of enrichment mediumcolors abbreviations Y; Yellow, D; Deep, G; Green, P; Purple

The result showed plant have five peaks in its chromatogram at retention time Rt (minutes) 3.71, 6.98, 18.43, 28.53 and 31.14 for quercetin, quercetin 3-O-glucopyranoside, quercetin 3-rutinoside, isorhamnetin 3-rutinoside and kaempferol 3-rutinoside, respectively as in Table (4) andFigure 9a which exhibited diversity of flavonoids compounds in its crude extract. Similarly the chromatogram of endophytic bacteria (F_3) showed two peaks at Rt (min) 3.74 and 6.82 relative to quercetin and quercetin 3-O-glucopyranoside, respectively (Table 4, Figure 9 b).HPLC chromatograms also showed two peaks (no. 6, 7) of the two different extracts (plant and endophytic bacteria) are separated at the same retention time suggesting similar phenolic compound which indicated that endophytic bacteria produce several bioactive compounds which are originally produced by plant.



Volume : 4 | Issue : 11 | November 2014 | ISSN - 2249-555X

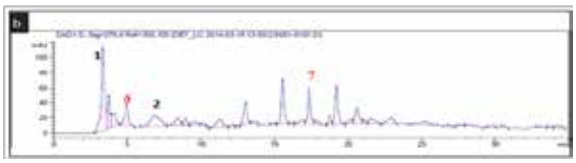


Figure 9 Analytical HPLC chromatograms of the crude extract of C.sinaica aerial parts, (a) and endophytic bacteria consortium (co- culture), (b). Recorded at 278 nm and performed using a waters column C18 and acetonitrile, 0.1 % acidified water with formic acid as mobile phase.

Table 4 HPLC analysis of phenolic compounds (flavonoids) from C.sinaica aerial parts methanol extract and endophytic bacteria extracellular cultures filtrate of enrichment medium

Peak no.	Assigned flavonoid compound	Authentic sample Rt			F3	
			Rt	Area	Rt	Area
1	Quercetin	3.41	3.71	1783.69	3.74	501.15799
2	Quercetin 3-O-β-glucopyranoside	6.90	6.98	1821.39	6.82	617.09467
3	Quercetin 3-O-rutinoside (Rutin)	18.57	18.43	7947.65	-	–
4	Isorhamnetin 3-rutinoside	28.92	28.53	8911.53	-	–
5	Kaempferol 3-O-rutinoside	29.89	31.14	6456.98047	-	–
6	Not determined	-	4.954	405.52859	4.954	525.48596
7	Not determined	-	17.4	1372.80017	17.4	690.53638

Rt for retention time of each separated peak, areafor area under each peak

.Discussion

The knowledge of the diversity of endophytic bacteria in plants and medicinal plants in particular is important to explore their capabilities in various fields of biotechnology (Bhoreet al., 2010).

It is well known that aerial parts of C. sinaica were used in the treatment of swellings, bruises, rheumatic joints inflammation (MEDUSA n.d., 2012), pains after child birth, skin inflammation, knee problems, tendinitis, muscular contractions, headache, earache and as an antiseptic (Rivera et al., 2003).And hence cultivable endophytic bacterial isolation was confined to the aerial parts of the plant.

However, the endophytic bacteria have been reported from various other traditional medicinal plants; for instance, Gynuraprocumbens, (Bhoreet al., 2010) and Artemisia annua. (Li et al., 2012) But, to ourknowledge, this study is the first to isolate and describe endophytic bacteria in C.sinaica.

Gao et al., 2005 had reported that in medicinal plants a seasonal fluctuation of the endophytes occurs. Rainy season was chosen for collection of plant samples (Raghunath, 2011) in this study.

Proper surface sterilization of the plant is necessary to ensure the isolation of true endophytic bacteria. Great caution and strict methodology was undertaken to achieve such goal. It was proved that the surface sterilization protocol followed in this study was effective in removing epiphytic microorganism, as increasing exposure time and repeating sterilization procedures for several times led to no contamination was observed in sterility check procedures. Accordingly, it was proved that bacteria isolated during the study can be considered to be true endophytic bacteria.

Most of the endophytic bacteria isolated in this study were Gram positive constituted 90% in agreement with Narayan Chandra et al., 2013 as they reported that 80% of isolated endophytic bacteria from chili pepper (Capsicum annuumL.) were Gram positive.

Following the successful isolation of endophytic bacteria as confirmed through the sterility check which proved the absolute absence of any growth of epiphytic bacteria, the following step was designed to visualize endophytic bacteria in its natural niche inside plant tissue.Microscopic examination of transverse sections of plant roots after staining with TTC revealed the presence of pink color stained bacteria typical appearance of endophytic bacteria in cortex and pith area. Endophytic bacteria also appeared to be localized in intercellular spaces as well as around xylem vessels. Indeed, the microscopic observations as well as the effective surface sterilization procedure, confirmed the endophytic nature of isolated bacterial strains.

16S rRNA gene sequence-based bacterial identificationhas been used for the rapid and accurate identification of endophytic bacterial isolates(Clarridge, 2004). Among the bacterial strains, Bacillus species were dominant in C. sinaica aerial parts sample and it is well known that it commonly associated with plants as endophyte as reported in similar works (Borrissand Maheshwari, 2012; Ma et al., 2013; Jagadesan and Bhore, 2014).

It was suggested that endophytic bacteria could produce the same bioactive metabolites that also produced by the plant itself (Mehanni and Safwat, 2010). In this study, bioactive metabolites produced by plant was identified using three successive tests namely, preliminary phytochemical screening, paper chromatography and HPLC analysis. Although, information of bioactive metabolites produced by plant were available in the literature from previous studies (Sharafet al., 1997; Hamedet al., 2007; Abd El-Hamedet al., 2011), In this study, investigation was done in case that particular environmental feature of Sinai such as geographical variation (high altitude area plant collected from) El-Shatouryet al., 2006, 2013, time of collection and Stage of maturity and age may induce the plant to produce specific metabolites that may be unique for C.sinaica growin this geographical part of Egypt (Hammamfaraoun).Considering these particular environmental features of Sinai investigation were conducted to detect the types of bioactive metabolites produced by theplant.It is important to note that this detection was carried out simultaneously with the isolation of endophytic bacteria.

Mixed fermentation (Co-cultivation of microbes or microbial consortium) represents a potentially valuable strategy for discovery of novel metabolites (Zhu and Lin, 2006) and

Volume : 4 | Issue : 11 | November 2014 | ISSN - 2249-555X

increase the chemical diversity and yield of microbial secondary metabolites (Shalinet al., 2013; Degenkolbet al., 2002). In this study, mixed microbial culture system include isolated endophytic bacterial strains were evaluated for production of novel bioactive compounds.

It was observed that flavonoids represented a major constituent of plant extract inPreliminary phytochemical screening of methanol extract of plant aerial parts. On the other hand endophytic bacterial extra cellular culture filtrate extracts of nutrient broth supplemented with 3% glucose (F_2) and of enrichment medium (F_3) were found to contain flavonoids, terpenoids and glycosides in different concentrations.

HPLC chromatograms of endophytic bacterial extract (F_3) with significant number of well separated peaks indicate that these endophytic bacterial isolates have the ability to produce diversity bioactive compounds simultaneously at relatively moderate concentrations. For example, it was noticed that two peaks were separated at the same retention time for both plant and endophytic bacteria extracts in HPLC chromatogrameindicating that these are similar compounds found in both of plant and endophytic bacteria extracts.

Quercetin and quercetin 3-O-glucopyranoside were detected in both endophytic bacterial extracts (F_2, F_3). Hence, it was concluded that endophytic bacteria produced flavonoids compounds similar to that produced by the plant itself. These revealed that the consortium of endophytic bacteria isolated in this study could synthesize quercetin and quercetin glycoside in vitro.It is worth mention that the synthesis of quercetin and quercetin 3-O-glucopyranoside by endophytic bacteria in vitro has never been recorded in previous studies.

The production of similar bioactive compounds by both endophytic bacteria and plant is supporting the idea that the presence and selection of these bacteria by the plant is sort of a symbiotic relationship and that bacteria may induce the plant to synthesize specific compounds and they could improve the in plant content of bioactive compounds if they utilized as bio-inoculants (Koulet al., 2013). Proving that endophytes can produce the same important bioactive compounds as their host plants, this would not only reduce the need to harvest possibly rare plants but also preserve the world's ever diminishing biodiversity.

The study revealed several particular remarks that may be beneficial for the future studies on the same field of study.

Although, several bioactive compounds were found in bacterial filtrate extracts, Bacterial pellet after washing methanol extracts showed absence of all bioactive compounds tested except carbohydrates. This is to confirm that bioactive compounds are synthesizedextracellular in vitro/within plant tissue.

Three different types of media were used to stimulate synthesis of novel bioactive products by isolated endophytic bacterial consortium and increase yield of bioactive compounds. Thus, it could be concluded that increasing supplemented glucose in nutrient agar medium could stimulate production of compounds which not produced in case of nutrient broth medium. Additionally, supplementary glucose and enrichment additives (peptone, beef extract) resulted in an increase in the yield of bioactive compounds.

Owing to the huge microbial biodiversity of endophytes, these are still the less investigated group of microorganisms that need to be explored for their huge potential of being used as the sources of pharmacologically active therapeutic compounds.

Conclusion

The study aimed to isolate endophytic bacteria from the medicinal plant Capparissinaica collected from Sinai, Egypt. Strict protocol was followed to ensure isolation of true endophytic bacteria and to avoid possible contamination from epiphytic bacteria on surface of the plant. Also, bacteria were visualized inside its natural niche plant tissues.16S rRNA gene sequence analysis reveals that Bacillus species were dominant in C. sinaica aerial parts. Both endophytic bacterial extracts as well as plant extract were proved to contain same bioactive compounds, namely quercetin and quercetin 3-O-glucopyranoside, which ensure the symbiotic relationship between endophytic bacteria and their host plant. In conclusion the study is giving the start for future research in producing such valuable compounds from bacteria cultures in a mega scale. Moreover, on a scientific ground the work produces strict excellent protocol that may facilitate future researcher trials to isolate endophytic bacteria.

Volume : 4 | Issue : 11 | November 2014 | ISSN - 2249-555X

REFERENCE

Abd El-Hamed, S. M .D. (2011): Phytochemical and biological evaluation of CapparissinaicaVeill.With special emphasis on liver diseases.M.Sc in Pharmacognasy, Pharmacognasy Department, Faculty of Pharmacy, Cairo University, Egypt. | Altschul, S.F.; Gish, W. Miller, W. Myers, E.W. Lipman, D.J.(1990): Basic local alignment search tool. J MolBiol, 215:403–410. | Araujo, W. L.; Marcon, J. Maccheroni, W. Jr. Van, Elsas. J.D. Van, Vuurde .J .W. L. Azevedo, J.L. (2002): Diversity of endophytic bacterial populations and their interaction with Xylellafastidiosain citrus plants.Appl Environ Microbiol,68:4906–4914. | Arundhati, Pal.; Paul, A .K. (2013): Bacterial Endophytes of the Medicinal Herb Hygrophilaspinosa T. Anders and Their Antimicrobial Activity. British Journal of Pharmaceutical Research, 3(4). | Barrow, G. I.; Feltham, R. K. A. (1993):Cowan and Steel's Manual for the Identification of Medical Bacteria, 3rd edn. Cambridge: Cambridge University Press. | Bhore, J.; Nithya,Ravichantar.Chye, Ying Loh. (2010):Screening of endophytic bacteria isolated from leaves of SambungNyawa [Gynuraprocumbens (Lour.)]Merr.forcytokinin-like compounds.Bioinformation, 5(5): 191–197. | Borriss, R.;Maheshwari,D. K.(2012): Use of plant associated Bacillus strains as bio-fertilizers and bio-control agents in agriculture. Chapter3. In: Bacteria in Agrobiology:Plant Growth Responses. Springer-Verlag, Berlin, Germany. | Boulos, L. (1995):Flora of Egypt checklist, 38-48. Al-HadaraPuplishing, Cairo, Egypt. | Boulos, L. (1999):Flora of Egypt. Vol. I. Azolliaceae- Oxallidaceae, 131-230. Al Hadara Publishing, Cairo, Egypt. | Clarridge, J.E. 3rd. (2004): Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. ClinMicrobiol Rev,17:840 62. | Degenkolb, T.; Heinze, S. Schlegel, B. Strobel, G. Gräfe, U. (2002): Formation of new lipoaminopeptides, acremostatins A, B, and C, by co-cultivation of Acremonium sp. BiosciBiotechnolBiochem, 66:883–886. | Dhanya, N. Nair.; Padmavathy, S.(2014): Impact of Endophytic Microorganisms on Plants, Environment and Humans.Scientific World Journal,Volume 2014, Article ID 250693, 11 pages. | El-Shatoury, SaharA.; Abdulla, Hesham; Al-Karaaly, Omnia; El-Kazzaz, Waleed; Dewedar, A. (2006): Bioactivities of endophyticactinomycetes from selected medicinal plantsin the world Heritage site of Saint Katherine,Egypt. International journal of botany, 2(3):307-312. | El-Shatoury, Sahar A.; El-Kraaly, Omnia A.; Trujillo, Martha E.; El-Kazzaz, Waleed; Gamal El-Din, El-Sayeda;Dewedar, Ahmed (2013): Generic and functional diversity in endophyticactinomycetes from wild Compositae plant species at South Sinai Egypt. Research in Microbiology, 164:761-769. | Gao, X .X.; Zhou, H. Xu, D.Y. Yu, C.H. Chen, Y.Q. Qu, L.H. (2005): High diversity of endophytic fungi from the pharmaceutical plant, HeterosmilaxjaponicaKunth revealed by cultivation-independent approach, FEMS MicrobiolLett, 249(2):255-66. | Gary, A. Strobel, David. Long, M. (1998): Specific associations of fungal endophytes with plant hosts represent a large untapped area for discovery. Endophytic Microbes Embody Pharmaceutical Potential, 64(5). | Hallmann,J.; Berg,G. Schulz, B.(2006): Isolation procedures for Endophytic microorganisms. In: Schulz BJE, Boyle CJC, Sieber TN (Eds) Microbial root endophytes,Soil biology .Springer, Heidelberg, 300–319. | Hallmann, J.; Quadt-Hallmann, A. Mahaffee, W.F. Kloepper, J.W. (1997): Bacterial endophytes inagricultural crops. Canadian Journal of Microbiology,43: 895-914. | Hamed, A.R.; Abdel-Shafeek, K.A. Abdel-Azim, N.S. et al. (2007):Chemical Investigation of some Capparisspecies growing in Egypt and their antioxidant activity.Evid Based Complement Alternat Med, 4(1): 25–8. | Hiraishi, A.(1992): Direct automated sequencing of 16S rDNA amplified by polymerase chain reaction from bacterial cultures without DNA purification. LettApplMicrobiol, 15:210–213. | Huang,W.Y.; Cai,Y.Z. Hyde,K.D. Corke,H. Sun, M. (2008): Biodiversity of endophytic fungi associated with 29 traditional Chinese medicinal plants. Fungal Divers,33:61–75. | Jagadesan, Preveena.;Subhash, J. Bhore. (2014): Identification of bacterial endophytes associated with traditional medicinal plant TridaxprocumbensLinn, Ancient Science of Life, 32 (3), IP: 41.238.55.7. | Joseph, B.; Mini, Priya R. (2011): "Bioactive compounds from endophytes and their potential in pharmaceutical effect: a review," The American Journal of Biochemistry and Molecular Biology, 1(3): 291–309. | Khianngam, S.; Pranburi, P. Khiri, Khan. Thailand, Schneider, C. Leifert, C. Feldmann, F. (2013): Isolation and screening of endophytic bacteria for hydrolytic enzymes from plant in mangrove forest Endophytes for plant protection, the state of the art, pp.279-284. Deutsche PhytomedizinischeGesellschaft, Braunschweig, ISBN: 978-3-941261-11-2. | Koul, M.; Lakra, N.S. Chandra, R.Chandra, S. (2013):Catharanthusroseusand prospects of its Endophytes: A new avenue for production of bioactive metabolites. Int J Pharm Sci Res, 4(7): 2705-2716. doi: 10.13040/IJPSR. 0975-8232. 4(7): 2705-16. | Krid,S.; Rhouma, A. Mogou, I. Quesada, J.M. Nesme, X. Gargouri, A. (2010):PseudomonasSavastanoiEndophytic Bacteria In Olive Tree Knots And Antagonistic Potential Of Strains Of Pseudomonas fluorescens And Bacillus Subtilis .Journal Of Plant Pathology,92 (2):335-341. | Kwon, S.W.; Kim, J.S.Park, I.C. Yoon, S.H. Park, D.H. Lim, C.K. Go, S.J. (2003):Pseudomonas koreensis sp.nov., Pseudomonas umsongensis sp.nov., and Pseudomonas jinjuensis sp. nov., novel species from farm soils in Korea. Int J SystematEvolMicrobiol, 53:21–27. | Li, J.; Zhao, G.Z. Huang, H.Y. et al.(2012): Isolation and characterization of culturableendophyticactinobacteria associated with Artemisia annuaL.,Antonie van Leeuwenhoek, 101(3): 515–527. | Ma,L.; Cao, Y.H. Cheng, M.H. Huang,Y. Mo, M.H. Wang,Y. Yang, J.Z. Yang, F.X. (2013): Phylogenetic diversity of bacterial endophytes of Panaxnotoginseng with antagonistic characteristics towards pathogens of root-rot disease complex . JOURNAL Antonie Van Leeuwenhoek, 103 (2): 299-312. | Mabry, T.J.; Markham, K. R.Thomas, M.B. (1970): The systematic identificationof flavonoids, pp. 35-109.Springer.Verlag and New York. | Markham, K.R. (1982): Techniques of flavonoids identification, PP, 1-113. Academic Press, New York. | McInroy, J.A.; Kloepper, J.W.(1994): Studies on indigenous endophytic bacteria of sweet corn and cotton. In: O'Gara F, Dowling DN, Boesten B (Eds). Molecular ecology of rhizosphere microorganisms. Wiley-VCH,Weinheim, pp 19–28. | MEDUSA n.d. (2012):The Medusa Database.n.d.(http://medusa.maich.gr) and references contained therein (accessed 12 November 2012). | Mehanni, M.M.; Safwat, M.S.(2010):Endophytes of medicinal plants. ActaHort (ISHS), 854:31-39. | Mei, Z. S.; Qing, S. C. Xia, W. Y. Jing, L. Yu, Z. X. Cheng, Z. X. (2008): Isolation and characterization of antifungal endophytic bacteria from soybean. Micobiology, 35: 1593-1599. | Narayan, Chandra. Paul.;Seung, Hyun.Ji. Jian, Xin. Deng.Seung, Hun. Yu. (2013): Assemblages of endophytic bacteria in chili pepper(Capsicum annuum L.) and their antifungal activity against phyto-pathogens in vitro. Plant Omics Journal,(6):441-448 .ISSN:1836-3644. | Pathak, K.; Keharia, H. Kharkwal, A.C. (2009):Isolation, cultivation and in planta visualization of bacterial endophytes from hanging roots of Banyan tree (Ficusbengalensis). Symbiotic fungi, Principle & Practice, Soil Biology Series book,Verma, A, Kharkwal, AC, (EDs). Springer link-verlag, 18: 211-227. | Petrini,O.; Fisher, P.J. Petrini, L.E. (1992a): Fungal endophytes of bracken (Pteridiumaquilinum),with some reflections on their use in biological control. Sydowia,44:282–293. | Raghunath, T. Mahajanisolatio.(2011): characterization of endophytic bacteria from roots of vent. International Journal of Pharma and Bio Sciences, 2(1): 0975-6299. | Reysenbach, A.L.; Giver, L.J. Wickham, G.S. Pace, N.R.(1992): Differential amplification of rRNA genes by polymerase chain reaction. Appl Environ Microbiol, 58:3417–3418. | Rivera, V.D.; Inocencio, C. Obon, C. Alcaraz, F.(2003): Review of food and medicinal uses of CapparisL. subgenus Capparis(Capparidaceae). Econ Bot,57:515–34. | Schulz, B.; Boyle, C.(2006):What are endophytes? Microbial Root Endophytes, Springer-Verlag, Berlin, pp. 1–13. | Shah, Md. Asraful, Islam; Renukaradhya, K. Math Jong, Min. Kim.Myoung, Geun. Yun.Ji,Joong . Cho.Eun, Jin. Kim. Young, Han. Lee.Han,Dae. Yun.(2010): Effect of Plant Age on Endophytic Bacterial Diversity of Balloon Flower (Platycodongrandiflorum) Root and Their Antimicrobial Activities. Springer Science+Business Media, LLC CurrMicrobiol. 61:346–356. | Shalin, Thomas; Raveendran, Sindhu Parameswaran, Binod Carlos, Ricardo Soccol Ashok, Pandey (2013): Mixed Cultures Fermentation for the Production of Poly- ⊠- hydroxybutyrate. Brazilian archives of biology and technology,54(4): 783-794. | Sharaf, M.; El-Ansari, M.A. Saleh, N.A.M.(1997): Flavonoids of four cleome and three Capparisspecies. BiochemSystEcol,25:161–6. | Silvia, Firáková; Mária, Šturdíková Marta, MúⅧkováBiologia, Bratislava (2007): Bioactive secondary metabolites produced by microorganisms associated with plants, Section Botany, Institute of Botany, Slovak Academy of Sciences 62(3): 251—257. | Strobel G.; Daisy B. (2003):Bioprospecting for microbial endophytes and their natural products. Microbiology and Molecular Biology Reviews 67: 491–502. | Strobel, G.; Daisy, B. Castillo, U. Harper, J. (2004): Natural products from endophytic microorganisms. J Nat Prod, 67: 257–268. | Süßmuth, R.; Eberspächer, J. Haag, R. Springer, W. (1999):MikrobiologischbiochemischesPraktikum. Georg ThiemeVerlag, Stuttgart. | Tlili,Nizar.;Walid, Elfalleh. Ezzeddine, Saadaoui. Abdelhamid, Khaldi. Saida, Triki. Nizar, Nasri.(2011):The caper (Capparis L.): Ethnopharmacology, phytochemical and pharmacological properties. Fitoterapia, 82:93–101. | Trease, G.E. (1966): Textbook of Pharmacognosy, 8thEdn. P. 596, Tyndall and Cassel, London. | Weisburg, W.G.;Barns, S.M. Pelletier, D.A. Lane, D.J. (1991): 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol,173:697–703. | Werner, C.; Petrini,O.Hesse,M.(1997):Degradation of the polyamine alkaloid aphelandrine by endophytic fungi isolated fromAphelandratetragona.FEMS MicrobiolLett,155:147–153. | Zhu, F.; Lin, Y. (2006):Marinamide, a novel alkaloid and its methyl ester produced by the application of mixed fermentation technique to two mangrove endophytic fungi from the South China Sea. Chin Sci Bull, 51:1426–1430. |