

Microbial Community for Growing Pioneer Plants in a Lunar Greenhouse

Natalia Kozyrovska¹, Iryna Zaetz¹, Tetyana Lytvynenko¹, Tamara Voznyuk¹, Maria Kovalchuk¹, Ivan Rogutskyy², Olexander Mytrokhyn³, Dmitry Lukashov³, Svitlana Mashkovska⁴, Bernard Foing⁵

¹*Institute of Molecular Biology & Genetics of National Academy of Sciences, Acad. Zabolotnoho str., 150, 03143 Kyiv, Ukraine, kozyr@imb.org.ua, Tel.: +38-044-5265596;*

²*Institute of Physics of National Academy of Sciences, Nauky av., 46, 03125 Kyiv, Ukraine;*

³*T. G. Shevchenko Kyiv National University, Volodymyrska str., 64, 01033 Kyiv, Ukraine;*

⁴*Botanical Garden of National Academy of Sciences, Tymiryazivska str., 1, 01014 Kyiv, Ukraine;*

⁵*ESA Research and Scientific Support Department, ESTEC/SCI-SR, Postbus 299, 2200 AG Noordwijk, The Netherlands.*

Abstract. It may be assumed that the first plants in a lunar base will play a main role in forming a protosoil of acceptable fertility needed for purposively growing second generation plants like wheat, rice, tulips, etc. The residues of the first-generation plants could be composted and transformed by microorganisms into a soil-like substrate within a loop of regenerative life support system. The lunar regolith may be used as a substrate for plant growth at the very beginning of a mission to reduce its cost. The use of microbial communities for priming plants will allow to facilitate adaptation to stressful conditions and to support the plant development under growth limiting conditions. Well-defined plant-associated bacteria were used for growing three cultivars to colonize of French marigold (*Tagetes patula* L.) in anorthosite, a substrate of low bioavailability, analogous to a lunar rock. The consortium was composed of plant growth promoting rhizobacteria and the bacterium *Paenibacillus* sp. IMBG156 which stimulated seed germination, better plant development, and finally, the flowering of inoculated tagetes. In contrast, control plants grew poorly in a sterile anorthosite and practically did not survive until flowering. Analysis of bacterial community composition showed that all species colonized plant roots, however, the rate of colonization depended on the allelopathic characteristics of marigold varieties. Bacteria of consortium were able to liberate some elements (Fe, Si, Ni, Co, Cu, Zn, Cr) from substrate anorthosite. Plant colonization by mixed culture of bacterial strains resulted in increase of accumulation by the plant of potassium and cobalt and in lowering the level of toxic metal accumulation. It was assumed that rationally assembled consortium of bacterial strains promoted germination of marigold seeds and supported the plant development under growth limiting conditions by means of bioleaching plant essential nutritional elements and protecting of the plant against hyperaccumulation of some toxic metals.

THE PROTOTYPE PLANT-BACTERIA MICROCOSM FOR A LUNAR BASE

The ability to grow plants in space self-perpetuating gardens is topical for providing an advanced life support system for humans while inhabiting a permanently manned lunar base (PMLB). Plants could provide fresh food, oxygen, and clean water for explorers living in PMLB. A lunar garden has to supplement less appetizing packaged food brought from Earth. The ornamental plants will play role in reducing stress and in recovering emotional potency in PMLB personnel. Lunar agriculture has the potential to earn the needed export of fresh food to other space locations at a decided fiscal advantage over fresh products brought up from Earth. To reduce a cost of early missions to the Moon, it would be practical to use local materials such as a lunar regolith for growing plants in lunar greenhouses. The use of bacteria to govern a decomposition of silicate rocks, a liberation of essential growth elements for plants, and to deliver them to the plant is a key idea in precursory scenario of growing pioneer plants for a lunar base (Kozyrovska et al., 2004; 2005). The objectives of this study were to study bioleaching capacity of bacteria in batch experiments with anorthosite as a component of nutrient media, as well as in the model plant microcosms placed in plant growth chambers under controlled conditions.

The prototype plant

The ornamental plant French marigold (*Tagetes patula* L.), undemanding to growth conditions, has been chosen as a model plant system to demonstrate of growing a plant with minimal expenses. The plant produces acceptable biomass which could be converted by microorganisms into a fertile protosoil assigned for growing first industrial plants. In future stages of lunar agroindustry, the marigold may be applied to recover of a tired plant-growing environment by producing secondary metabolites (allelochemicals) (Mashkovska, 2003). Beside the pragmatic side, the marigold could perform a role in esthetic decoration of the hostile environment of PMLB: the beautiful image and delicate fragrance of marigold cultivars, familiar to everybody and lovely, would remind of an earthly spirit to habitants of PMLB and, accordingly, correct the emotional comfort of lunar explorers. Marigold flowers are consumed widely as aromatic tea, and this experience could be proposed for application at PMLB in prophylactics of various diseases and protection from irradiation, to release pain (Vasilenko et al., 1990; Kasahara et al., 2002; Yasukawa et al., 1998). Both marigold flowers and leaves are excellent spices that could appetize tasteless packaged food and in that way supply it with vitamins and microelements (Mashkovska and Hryhoryuk, 2003). The set of these traits makes marigold a promising candidate as a pioneer plant for multipurpose application at lunar base. In these experiments a middle-sized cultivar Carmen and two dwarf cultivars, Petite Harmony and Petite Gold, served as the plant-hosts for a consortium of plant growth promoting rhizobacteria.

The model consortium of bacteria

The rationally assembled bacterial community - *Pseudomonas* sp. IMBG163, *Pseudomonas aureofaciens* IMBG164, *Xanthomonas maltophilia* IMBG147, *Paenibacillus* sp. IMBG156, *Klebsiella oxytoca* IMBG26, and *Pantoea agglomerans* IMV56 – aims to support plant growth in a substrate of low bioavailability using several mechanisms: priming resistance in plant to stresses, stimulating seed germination by providing phytohormones, improving nutrition by leached or biologically fixed elements, “cleaning” plant environment, etc. Cultures of bacterial strains were applied for seed inoculation, except *Paenibacillus* sp., which was introduced into a substrate. Model consortium of bacteria needs both organogenic elements N, P, C, O and additional elements essential for physiological activity like K, Na, Mg, Ca, Fe, etc., as well as microelements. In accordance with the idea of cultivating healthy crops in a lunar garden using low-cost technology, in these experiments bacteria were not provided with nutrients, except a water or 1 mM solution of potassium phosphate (PP) (controls).

The analogs of a lunar rock

The lunar highland regolith is predominantly composed of aluminosilicate basic rocks, mainly anorthosites, noritic anorthosites, and gabbroic anorthosites (Ashwal, 1983). The primary rock-forming minerals of lunar anorthosites are calcic plagioclase $\text{Ca}[\text{Al}_2\text{Si}_2\text{O}_8]$, pyroxene $(\text{Mg,Fe,Ca})(\text{Mg,Fe})[\text{Si}_2\text{O}_6]$, and olivine $(\text{Mg,Fe})_2[\text{SiO}_4]$. Therefore the plant could get the majority of elements essential for nutrition from the regolith, and the rest of it by lunar-sourced additions. The terrestrial anorthosites are usual rocks within the Precambrian Shields, for example, within the Ukrainian Shield. There are some differences between terrestrial and lunar anorthosites (Ashwal, 1983). While the first are “dry”, some earth rocks contain hydrated minerals. Another difference could be the absence of hydrocarbons. Despite these differences, terrestrial anorthosites may serve as simulants of lunar rocks in model experiments on plant cultivation under growth limited conditions. The Turchynka type anorthosite is composed of plagioclase $(\text{Ca,Na})[\text{Al}_2\text{Si}_2\text{O}_8]$, pyroxene of low calcium content, and olivine. The Penizevitchi anorthosite in addition to intermediate plagioclase, low-calcic pyroxene and olivine, contains minor quantities of ilmenite FeTiO_3 , orthoclase $\text{K}[\text{AlSi}_3\text{O}_8]$, biotite $\text{K}(\text{Mg,Fe})_3[\text{AlSi}_3\text{O}_{10}](\text{OH,F})_2$, and apatite $\text{Ca}_5[\text{PO}_4]_3(\text{F,OH,Cl})$ (Mytrokhyn et al., 2003). These types of anorthosite appeared to be a poor support of marigold growth, and the idea was to use natural bacterial residents of aluminosilicate rocks to leach the plant essential ions from a substrate and therefore to improve plant development (Kozyrovska et al., 2004). Two types of anorthosite of Turchynka and Pinizevitchi deposits (both Korosten Pluton, Ukraine) (Lychak, 1983; Mytrokhin et al., 2003), chemically and mineralogically similar to lunar anorthosites were used in model microcosms as substrates for plant growing.

The marigold growing in anorthosite

French marigold seed germination in anorthosite substrate has been stimulated by bacterial consortium (FIGURE 1), and the rate of germination and survival approached 100%. Survival of germinated control seeds (without bacteria, moistened by distilled water or by the PP solution) in anorthosite was 20-30 % as compared to inoculated ones. Despite the addition of potassium and phosphorus, the germination rate of seeds did not increase. Inoculated marigold sprouts differed from control variants by a higher biomass, better branching of stems, and in more green color of leaves. After a period of 54 ± 3 days of co-cultivating the French marigold with the consortium of bacteria, the plant began to flower. Control plants did not survive as rule after 4-week period of development in a variant when they were moistened with distilled water or phosphate solution, however, accidentally survived plants sometimes even flowered. There was practically no difference between marigold cultivars tested in survival rate and in duration of the period before flowering. As compared to plants grown in the soil, inoculated tagetes began to flower 5-6 days later in anorthosite and produced 1.6-2.3 time less dry biomass compared to plants grown in a fertile soil. Inoculated plants gained 3.5-times higher dry weight as compared to control plants that accidentally survived in anorthosite at the age of 4 weeks (Table 1).

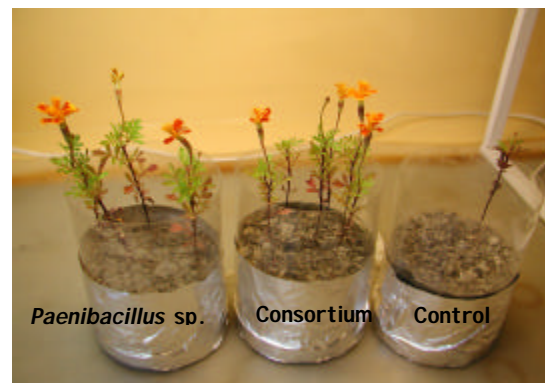
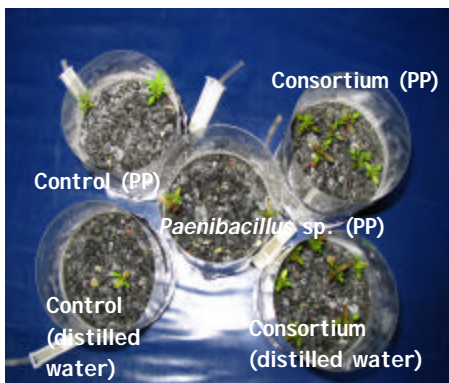


FIGURE 1. The rationally assembled bacterial community provided an accepted growth and flowering of a French marigold. 10 seeds of either variants in four replications have been planted into fragmented anorthosite of the Turchynka type. Two variants of microcosms have been

inoculated with suspension of *Paenibacillus* sp. or a mixture of bacterial strains; non-inoculated seeds were watered. Within a vegetative period marigolds were watered by distilled water (a separate variant – by 1 mM potassium phosphate, PP).

The survival of bacteria in the marigold rhizosphere

was tested in three cultivars with different allelopathic traits. There were no shifts in community composition in the roots of the Carmen cultivar within 6 weeks when plants were supplied by a PP solution or distilled water, in spite of putative deficit of nutrients in microcosms. At the beginning of the examination period, the *Paenibacillus* sp. IMBG156 came on roots from substrate anorthosite and generated a small-size population on the plant, and later, after 2 weeks, it was getting to rise of the population rapidly to log 8/g of fresh roots (Kozyrovska et al., 2005). The partners of *Paenibacillus* were rather competitive on the marigold roots and gained the log 6-7 populations. In contrast, the roots of the dwarf marigold cultivars were less colonized by the bacterial assemblage. Total number of bacteria per gram of Petite Harmony root did not exceed log 7, and the most active colonizers were *Paenibacillus* sp. IMBG156 and *P. aureofaciens* IMBG164. Poor survival of some bacterial species on the dwarf marigold roots correlated with a higher rate of phenolcarboxylic acids produced by plants which appeared to inhibit bacterial growth (Mashkovska, 2003). It was concluded that Carmen cultivar was more suitable than dwarfs for further experiments.

Bioleaching anorthosite by Paenibacillus sp. IMBG156 and by consortium of bacteria

Siliceous bacteria as well as other microorganisms could be used for bioleaching the lunar regolith in a program of growing pioneer plants. Some species of *Bacillus/Paenibacillus* and *Pseudomonas* genera are known as destructors of aluminosilicates (Alexandrov and Zak, 1950; Natarjan et al., 1997). In this respect *Paenibacillus* sp. IMBG156, isolated from a silica rock, has been chosen as the model bacterium in the simulation of bioleaching anorthosite. In batch experiments with monoculture, *Paenibacillus* sp. IMBG156 cells attached to anorthosite fragments and changed electrokinetic potential of the rock surface apparently due to exopolysaccharide (EPS) capsule (Kozyrovska et al., 2004). The population size of IMBG156 culturable cells was different in different microcosms; the attached cells gained larger populations and looked like young vegetative cells, covered with EPS capsules and thus protected from toxic actions of some released cations of metals, in contrast, lonely cells, freely floating in the medium, were middle-sized, mainly spore-conferring. *Paenibacilli* caused the corrosion resulted from formation of iron(III) oxide of the rock within 28 days of incubation in the presence of the Pinizevitchi anorthosite fragments. No signs of changes on the anorthosite surface were observed in the variant without bacteria. Cultural media were examined by flame atomic adsorption spectrophotometry using C115-M1 (Selmi, Ukraine). SiO_3^{2-} was detected with the colorimetric method. Results represented in Table 2 show that strain IMBG156 was able to liberate Fe^{3+} and SiO_3^{2-} from anorthosite under normal pH within the 6-week period of incubation of monoculture in a minimal medium A3 (Alexandrov and Zak, 1953). The level of released elements increased 6-15-fold compared to control. Model bacterial consortium released approximately the same concentration of Fe like IMBG 156, and also other elements (Zn, Cu) in culture medium (see Table 2). However, in a pellet of mixed culture a 4-times higher concentration of Fe was detected. Bacterial cells also accumulated Mn and Ni which have not been revealed in the culture medium. Results of this series experiments demonstrated that both monoculture *Paenibacillus* sp. IMBG156 and mixed bacterial culture were able to leach anorthosite.

TABLE 1. Accumulation of elements from anorthosite by inoculated tagetes*, $\mu\text{g/g}$

Microcosm	Dry weight, g/plant	Zn ²⁺	Mn ²⁺	Fe ³⁺	Ni ²⁺	Cr ³⁺	Co ²⁺	?? ²⁺	Mg ²⁺	Na ⁺	K ⁺
Anorthosite, control	0.047±0.03	80.4±14.3	306±86	340±17	27.2±3.1	35.4±11.8	<0.5	179050±51548	1752±469	1162±71	19551±1948
Anorthosite, <i>Paenibacillus</i> sp.	0.106±0.08	30.1±10.4	384±81	143±42	13.6±0.1	3.1±1.4	2.6±0.9	81505±3279	3005±652	678±148	20629±2285
Anorthosite, consortium	0.153±0.03	29.4±6.5	423±62	81±13	14.2±3.9	18.0±5.5	1.5±0.4	63373±13435	1786±421	383±23	23705±2780
Soil, Control	0.238±0.01	69.4±4.0	601±4	95±0	3.1±0.5	2.7±3.0	0.6±0.1	61710±5882	8940±1560	451±76	12747±1443
Soil, <i>Paenibacillus</i> sp.	0.248±0.03	58.2±11.4	520±140	70±22	5.1±0.9	1.9±1.1	0.9±0.5	57392±7855	8493±2574	435±63	15010±2870
Soil, consortium	0.221±0.05	71.0±2.7	835±104	75±3	6.6±2.7	2.0±2.2	1.3±1.1	55966±7419	9604±382	373±58	14850±2335

*All variants where watered by distilled water once in two days.

Bioleaching anorthosite by plant microcosm

The marigold is known to accumulate metal cations (Bessonova, Ivanchenko, 2004). In this study results revealed that *T. patula* cv. Carmen accumulated macroelements K, Na, Ca and microelements Fe, Zn, Ni, Cr in anorthosite substrate in a higher concentration than when grown in a podzol soil (organic matter, 1,2%; ?? 6,2; N-4,3; P-7,6; K- 8,4 mg in 100 g of a soil) (see Table 1). In association with bacteria, tagetes accumulated more K⁺ and Co²⁺ than in control soil. Bacteria corrected hyperaccumulation by the plants of Na, Ca, Fe, Ni, Cr and in such a way prevented the intoxication by these elements. The plant root microinhabitants accumulated Co²⁺, in contrast to the control marigold. This phenomenon may be connected with a resistance to cations of toxic metals known for some species of bacteria (Trajanovska et al., 1997), for example, some representatives of

Pseudomonas and *Klebsiella* genera are tolerant to toxic concentrations of heavy metals (Stoppel, Schlegel, 1995). Bacteria have developed a variety of resistance mechanisms to counteract heavy metal stress. These mechanisms include the formation and sequestration of heavy metals in complexes, reduction of a metal to a less toxic species, and the direct efflux of a metal out of the cell (Nies, 1999). Mobile genetic elements (MGE) are responsible for such activity in some cases (Tibazarwa et al., 2000). Either deleting MGE or substituting some species of bacteria, possessing resistance to chromium and cobalt, can resolve the problem with accumulation of undesirable elements.

Analysis of the results of this study show that inoculated marigold plants have got in full practically all elements, except Mg and Mn which were in deficit. However, due to bacteria, it is possible to save up to 70% of needed Mn^{2+} . Summarizing advantage of marigold inoculations by mono- or mixed culture we can conclude that application of consortium of bacteria is more profitable than *Paenibacillus* sp. alone.

TABLE 2. Concentration of elements released by bacteria from anorthosite, mg/l

Microcosm	Zn ²⁺	Mn ²⁺	Fe ³⁺	Cu ²⁺	Ni ²⁺	SiO ₃ ²⁻
Control (a nutrient medium without bacteria and anorthosite)	0	0	0	0	0	0
Control (a nutrient medium without bacteria)	0	0	0.325	0	0	0.8
Consortium:						
A cultural medium	0.133	0	2.340	0.036	0	ND
A pellet (bacterial cells)	0.310	0.223	10.129	0.049	0.072	ND
<i>Paenibacillus</i> sp. IMBG156 (a cultural medium)	ND	ND	2.250	ND	ND	12.0

Titre of bacteria log 6-7 CFU/ml. Anorthosite rocks of the Turchynka type contain (ppm) Fe (46722-75426), Si (228326-240499), Mn (924-693), Zn (44.0-24.0), Cu (18.2-16.7), Ni (68.8-42.7) (Mytrokhin et al., in preparation). ND, not determined. The cultural medium A3 (Alexandrov and Zak, 1953).

CONCLUSIONS

In model experiments, the rationally assembled consortium of bacterial strains promoted the growth of *T. patula* L. and supported the plant development under growth limiting conditions by stimulating of seed germination (1); bioleaching and delivering essential nutritional elements to the plant (2); preventing intoxication of the plant by excess of metal cations released from anorthosite. Application of mixed populations of bacteria for seed inoculation resulted in 100% seed germination and survival of sprouts in anorthosite. In contrast, watered seeds survived in 20-30%, even when potassium and phosphorus were added to microcosm. Due to the bacterial consortium, the model plant was supplied with an additional amount of basic macro- and microelements. French marigold grown without bacteria appears to be intoxicated by threshold accumulation of some metals, and the presence of bacteria on the plant roots protected it against excessive accumulation of some elements. So the bacteria were able to correct both hyperaccumulation and deficit of elements needed for plant nutrition. Growing first generation plants of such as the French marigold in the presence of a community of microorganisms, including eubacteria, cyanobacteria, mycorrhiza fungi, etc and converting the plant residues by microorganisms into a soil-like substrate may give the beginning of agro-industry at PMBL, however, bioaugmentation strategy of growing plants for lunar bases needs comprehensive study and a wider body of evidence.

ACKNOWLEDGMENTS

The authors are thankful to Olya Nimenko for technical assistance. The project is being fulfilled on a voluntary basis.

REFERENCES

- Alexandrov, V.G., and Zak, G.A., Bacteria, destroying aluminosilicates (silicious bacteria), *Microbiologia*, 19, 97-104 (1950) (In Russian).
- Ashwal, L.D., *Anorthosites*. Ed. Springer-Verlag, 1993.
- Bessonova, V.P., and Ivanchenko, O.E., Iron and chrome excess effect on the activity of nitrate reductase in vegetative organs of *Tagetes patula* L. and *Lathyrus odoratus* L., *Physiology and Biochemistry of Cultural Plants*, 36, 511-519 (2004) (in Ukrainian).
- Kasahara, Y., Yasukawa, K., Kitanaka, S., et al., Effect of methanol extract from flower petals of *Tagetes patula* L. on acute and chronic inflammation model, *Phytother Res.*, 16(3), 217-222 (2002).

- Kozyrovska, N.O., Korniiichuk, O.S., Voznyuk, T.M., et al., Microbial community in a precursory scenario of growing *Tagetes patula* L. in a lunar greenhouse, *Kosmichna Nauka i Technologiya (Space Science and Technology)*, 10, N5/6, 221-225 (2004).
- Kozyrovska, N.O., Korniiichuk, O.S., Voznyuk, T.M., et al., Growing pioneer plants for a lunar base, *Adv. Space Res.(in press)* (2005).
- Kozyrovska, N.O., Zaetz, I., Voznyuk, T.M., et al. , Rationally assembled microbial community for growing *Tagetes patula* L. in a lunar greenhouse, *Res. Microbiol. (in press)* (2005).
- Lee, S.-W., Glickmann, E., Cooksey, D.A, Chromosomal locus for cadmium resistance in *Pseudomonas putida* consisting of a cadmium-transporting ATPase and a MerR family response regulator, *Appl. Envir. Microbiol. , 67*, 1437-1444 (2001).
- Lychak, I. L., *Petrology of Korosten Pluton*, Naukova dumka, Kyiv, 1983, 248 p.
- Mashkovska, S.P., An accumulation and a role of the volatile oils in forming the allelopathic potential in marigold (*Tagetes* L.), *Dopovidi Natzionalnoi Akademii Nauk Ukrainy (Proc. Nat. Acad. Sci. Ukraine)*, 6, 167-170, 2003 (In Ukrainian).
- Mashkovska, S.P., Hryhoryuk, I.P., Marigolds – a source of effective drugs, *Phytotherapy*, 4, 41-47, 2003 (In Ukrainian).
- Mytrokhyn, O.V., Bogdanova, S.V., Shumlyansky, L.V., *Anorthosite rocks of Fedorivskyy suite (Korosten Pluton, Ukrainian Shield)*, In: Current problems of geological science. Eds. Kyiv State University, Kyiv, 2003, pp. 53–57.
- Natarajan, K.A., Modak, J.M., Anand, P., Some microbiological aspects of bauxite mineralization and beneficiation. *Minerals and Metallurgical Processing*, 14, 47-53 (1997).
- Nies, D. H., Microbial heavy-metal resistance, *Appl. Microbiol. Biotechnol. , 51*,730-750 (1999).
- Stoppel, R.-D., and Schlegel, H.D., Nickel-resistant bacteria from anthropogenically nickel-polluted and naturally nickel-percolated ecosystems, *Appl. Envir. Microbiol.*, 61, 2276-2285 (1995).
- Tibazarwa, C., Wuertz, S., Mergeay, M. et al., Regulation of the *cnr* Cobalt and Nickel Resistance Determinant of *Ralstonia eutropha* (*Alcaligenes eutrophus*) CH34, *J. Bacteriol.* 182, 1399-1409 (2000).
- Trajanovska, S., Britz, M.L., Bhave, M., Detection of heavy metal ion resistance genes in gram-positive and gram-negative bacteria isolated from a lead-contaminated site, *Biodegradation*, 8 , 113-124 (1997).
- Vasilenko, Yu., Bogdanov, A., Frolova, L., et al., Hepatoprotective properties of preparations from French marigold, *Chimiko-pharmatzevticheskii zhurnal (Chemical and Pharmaceutical Journal)*, N1, 53-56 (1990) (In Ukrainian).
- Yasukawa, K., Akihisa, T., Inoue, Y., et al., Inhibitory effect of the methanol extracts from compositae plants on 12-O-tetradecanoylphorbol-13-acetate-induced ear oedema in mice, *Phytotherapy Res.* 2, N7, 484 – 487 (1998).