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Crescent Journal of Medical and Biological Sciences
Vol. 4, No. 1, January 2017, 17–22
eISSN 2148-9696

The Comparison of Composition and Biological Activities in Wild and Cultivated of *Thymus kotschyanus* Essential Oils and Methanolic Extracts From East Azarbayjan, Iran

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Abstract

Objective: The gathering of wild type *Thymus kotschyanus* as medicinal plant is common in Iran because of their several biological properties which caused destruction of them. The aim of this research was to compare the chemical composition, antioxidant and antibacterial properties of wild and cultivated type of *T. kotschyanus* collected from East Azarbayjan, Iran.

Material and Methods: The essential oils (EOs) from aerial parts of wild and cultivated *T. kotschyanus* were investigated by gas chromatography/mass spectrometry (GC/MS). Antibacterial activity of (EOs) and methanol extracts were tested against bacteria by disc diffusion method and determining of their minimum inhibitory concentration (MIC) values by agar dilution method and antioxidant activity by DPPH and FRAP assays.

Results: Thirty-five components were identified representing more than 90% of the total oil constituents. The oils were dominated by monoterpene hydrocarbons (50.2%) for wild type and oxygenated monoterpenes (63.38%) for cultivated type. The major components in the oils of the wild type were; thymol (29.96%), p-cymene (21.35%) and α-pinene (12.72%) and for the cultivated type were; thymol (47.48%) and α-pinene (5.49%). The MIC values of bacterial strains, which were sensitive to the EO of *T. kotschyanus*, were in the range of 2-128 µg/mL in wild type and 2-16 µg/mL in cultivated type.

Conclusion: Our data shows that, cultivation significantly affects the EOs' chemical composition and antioxidant potential of *T. kotschyanus*. They signify a reasonable source of natural antibacterial substances that exhibited potential as a drug for use in pathogenic bacteria.

Keywords: Antimicrobial activity, Cultivation, Essential oil composition, *Thymus kotschyanus*

Introduction

During the past decade not only emergence of multi-drug resistant (MDR) gram-negative and gram-positive bacteria is increased in community and hospital setting (1) but also the spread of mobile carbapenemases like bla_{NDM-1} resulting in superbugs phenotypes which leaving very few treatment options available for clinicians (2). So this problem recommended to investigate the new drugs with antibacterial and bioactivity effects. The plants especially medicinal plants is a main alternative compounds because of their effective biological activity (3). On the other hand, the plant of *Thymus* genus is one of the most popular plants throughout the world especially prevalent in the Mediterranean area and recognized as medicinal plants by showing several biological and pharmacological properties (4). Essential oils (EOs) of all species of *Thymus* genus contain phenolic compounds which are with antibacterial potential (5).The biosynthesis of these compounds controlled not only genetically but also by environmental factors like locality and population of them (6).

The main picking off medicinal plants methods could be destructed wild type of them which led to their subtraction and demolition (7,8) but the cultivation of these plants could be as an attractive ideal to surmount their high exploitation from the wild environment (7,9). This strategy is widely accepted in Iran for many medicinal and aromatic plants which create this hypothesis to beg the difference in biological compound and their activity. The aim of the present study was to compare the chemical composition of the EOs of *T. kotschyanus* plants collected from wild and cultivated populations in East Azarbayjan, Iran; also evaluation of the antimicrobial and antioxidant activates of EOs and methanol extracts (MEs) of wild and cultivated samples.

Materials and Methods

Fresh aerial parts of wild *T. kotschyanus* were collected in May-April 2013 at full flowering stage from 1300 m alti-

Received 14 February 2016, **Accepted** 27 August 2016, **Available online** 13 September 2016

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tude in Arasbaran mountains, East Azarbayjan, and the cultivated samples were obtained from Saeed Abad station of agricultural research center (37° 58′ N and 46° 33′ E, average height; 1880-1900 m), East Azarbaijan province, Iran. Texture of the soil was nearly level and gently sloping. It typically consists of pale brown, calcareous, moderately alkaline fine sandy loam. The collected part of plants were air-dried at room temperature (~25°C) in the shade and powdered using a clean laboratory blender. Fifty grams of each sample mixed with 500 mL of distilled water and subjected to hydro-distillation, using clevenger-type apparatus for 3 hours until total recovery of EOs. The preparation of EOs was performed two times and oils obtained were dried with sodium sulfate, weighed and stored at 4°C until use. Plant material (100 g) of each sample soaked in methanol for two days at room temperature. The mixture was filtered through a membrane and the solvent was separated from samples with vacuum evaporator to obtain MEs. A gas chromatograph mass spectrometer (Shimadzu-17A-QP505, Japan) were used for analyzing of the EOs. The gas chromatography column was a super CP-Sil 5CB capillary column (50 m × 0.32 mm ID, 0.25 µm film thickness). The temperature of column was regulated by following methods: 1) for 1 minute at 70°C with a rate of 1.5°C/min, up to 100°C. 2) at a rate of 4°C/min, up to 180°C and left for 1 minute. 3) at a rate of 10°C/min, up to 200°C 4) at a rate of 2.5°C/min, up to 250°C and left for 5 minutes. The 280°C and 300°C were applied as injection and detection temperatures, respectively. The ionization energy was scanned for 1 second, with 70 eV. The mass range selected from 40 to 300 amu.

The retention indices and mass spectral data of wild and cultivated *T. kotschyanus* EOs were compared with standard compounds for identifying of their available compounds. The results of identification were matched by NIST NBS54K Library in computer and fragmentation patterns of mass spectra were compared by other reports (10,11).

DPPH radical scavenging capacity was assessed according to Wettasinghe et al, (12) with some modification as the report of Dehghan et al (13). Different concentrations of extracts and EOs were added to 2 mL of DPPH solution (0.1 mM in methanol) and reduction of DPPH absorbance was followed by monitoring at 517 nm (A_S). The absorbance of 2 mL DPPH solution was determined at 517 nm (A_C) as a blank control. The percentage of radical scavenging activity (RSA %) was calculated according equation 1:

$$\text{RSA \%} = \frac{100\ (A_C - A_S)}{A_C} \quad (1)$$

To quantify the results IC_{50} value, the effective concentration that could scavenge 50% of the DPPH radicals, were calculated (12). Ferric reducing antioxidant power (FRAP) method were done according to the method of Benzie and Strain by some modifications (13). The antioxidant activity of a sample measured at 593 nm in spectrophotometer because of reducing ferric (Fe^{3+}-TPTZ) to a ferrous form (Fe^{2+}) in this method. The incubation temperature was selected 37°C during the monitoring period. The standard calibration curve were regulated by using different concentrations of $FeSO_4.7H_2O$ and FRAP values were determined for studied EOs.

The antibacterial activity of EOs and MEs were tested against the following bacteria: *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC25952), *Staphylococcus aureus* (ATCC33591), *Staphylococcus aureus* (ATCC29213), *Streptococcus sanguis* (PTCC1449), *Enterobacter aerogenes* (ATCC13048), *Klebsiella pneumoniae* (ATCC700603), *Proteus mirabilis* (ATCC43071), and *E. coli* O157:H7 (Razi Institute, Tehran, Iran). These strains kept at -70°C in tryptic soy broth (TSB) with 20% of glycerol. They were inoculated in blood agar (BA) and incubated overnight at 35°C. Subsequently, one colony from each culture was inoculated in TSB and incubated at 35°C for 24 hours with shaking (100 rpm) in order to obtain freshly cultured microbial suspension (10^8 CFU/mL) for tests and antimicrobial activity of the EOs was determined with agar disc diffusion method (15). Briefly, a suspension of tested microorganisms (10^8 CFU/mL-1) in log phase was spread on Mueller Hinton agar (MHA) using sterile cotton swabs. Subsequently, filter paper discs (6 mm in diameter, Mast) were impregnated with 10 µL of EOs of each wild and cultivated *T. kotschyanus* and placed on the surface of inoculated plates. For antibacterial effect of MEs, various concentrations of wild and cultivated *T. kotschyanus* (0.05 g/mL, 0.1 g/ mL, 0.2 g/ mL and 0.4 g/ mL) were prepared. They were filtered by 0.22 µm sterile filters and 10 µL of each, impregnated on paper discs as well as described above. Tetracycline (30 µg/disk) and kanamycin (30 µg/disk) (HiMedia) were used as control. The Petri dishes incubated at 37°C for 24 hours. The tests were done duplicate for each strain. Antibacterial activity was evaluated by measuring the diameter of inhibition zone to the nearest millimeter. Determination of minimum inhibitory concentration (MIC) of EOs and MEs of wild and cultivated *T. kotschyanus* were determined by using agar dilution method as recommended by the Clinical & Laboratory Standards Institute (CLSI) guidelines (16). Briefly, the twofold serial broth dilution of MEs and EOs were prepared. Petri dishes containing MHA plates (Merck, Germany) in 45°C and EOs or MEs of wild and cultivated *T. kotschyanus* achieved concentration ranging from 512 µg/mL to 0.5 µg/mL for EOs and 2048 µg/mL to 2 µg/mL for MEs. The bacterial strains were inoculated in TBS and incubated for 24 hours in 37°C and standardized suspension of studied bacteria at 0.5 on the McFarland scale (10^8 CFU/mL). The bacterial suspension equal 10^6 CFU/mL was inoculated in each plate (12 plates for each series) similar in a plate without EOs and MEs as a positive growth control. They were incubated at 35°C for 24 hours. The microorganisms that are sensitive to the concentration of MEs or EOs contained in any given agar do not produce a circle of growth at the inoculum site where those that are resistant appeared as circular colonies.

Results

Chemical compositions of the EOs' yield state of the distinct variation between 2 types of plant oils. Our data show that cultivation did not significant effect in EO production. Hydro distillation of dried and ground aerial parts of wild and cultivated *T. kotschyanus* yielded 1.55% and 1.70% of V/W of a greenish yellow color oil. Analysis of the oils led to the identification of 35 compounds (Table 1) which accounted for 99.57% and 99.67% of the total oils. The majority of compounds in oils were monoterpene hydrocarbons (50.2%) for wild type and oxygenated monoterpenes (63.38%) for cultivated type. The chemical analyses carried out revealed the richness and variability of wild and cultivated *T. kotschyanus* EOs. Thymol was found as a predominant component in both of the EOs, together with p-cymene and α-pinene for wild type and with α-pinene for cultivated *T. kotschyanus*. In the oil obtained from cultivated *T. kotschyanus*, we observed a decrease in the percentage of α-pinene and m-cymene against increase in thymol (Table 1).

The antioxidant activity of wild and cultivated *T. kotschyanus* EOs and MEs were screened by DPPH free radical scavenging assay and ferric reducing antioxidant potential (FRAP). As shown in Table 2, both EOs and MEs obtained from wild and cultivated *Thymus* showed antioxidant activity in both assays. The cultivation of our studied medicinal plant were affected the antioxidant activity of the *T. kotschyanus*. For the DPPH assay, the most potent oil was obtained from wild type (IC_{50}; 2.74 µg/mL) and highest ferric reducing capacity resided with cultivated type (IC_{50}; 1.56 µg/mL). The antioxidant capacity of the extracts was better than that of EOs in two assay systems as shown in Table 2.

The results of the antimicrobial assays by disc diffusion method with EOs and MEs of *T. kotschyanus* are summarized in Table 3. Both EOs obtained from wild and cultivated studies of *Thymus* showed that the inhibitory activity on the tested microorganisms but MEs of them had not inhibitory activity against most of microorganisms except *Streptococcus sanguis* PTCC1449, *Staphylococcus aureus* ATCC33591 and *S. aureus* ATCC29213

The MIC of EOs and MEs of studies of the species are shown in Table 4. In present study, the MIC of *T. kotschyanus* EOs ranged from 2-128 µg/mL in wild type and 2-16 µg/mL in cultivated type for tested bacteria.

Table 1. Chemical Composition of EOs From Aerial Part of Wild and Cultivated *T. kotschyanus*

Compound	RI[a]	Wild (%)	Cultivated (%)
α-Thujene	928	1.29	0.36
α-Pinene	931	12.72	5.49
Camphene	943	1.83	-
Sabinen	964	t	-
β-Pinene	970	0.76	0.48
β-Myrcene	979	1.51	0.80
3-Octanone	993	0.18	-
α-Phellandrene	997	0.43	0.19
α-Terpinen	1008	1.62	1.80
p-Cymene	1011	21.35	-
m-Cymene	1013	8.87	-
1,8-Cineole	1023	4.57	4.79
γ-Terpinen	1047	8.01	4.00
1,3-Dimethyl-2-vinylbenzene	1060	-[b]	1.41
o-Cymol	1076	-	1.16
Terpinolen	1078	0.43	-
Camphor	1121	0.89	0.34
3,4-Dimethylcyclohexanol	1126	-	0.51
Verbenene	1128	0.71	-
Isoborneol	1138	-	0.21
Terpinen-4-ol	1160	-	0.82
4-Terpineol	1161	2.19	-
α-Terpineol	1172	1.08	0.92
Thymol methyl ether	1215	2.44	2.10
Carvacrol methyl ether	1244	-	4.14
Thymol	1266	29.96	47.48
Carvacrol	1278	3.79	0.62
3-Allyl-6-methoxyphenol	1362	-	1.96
Copaene	1397	-	1.51
β-Bourbonene	1408	0.15	3.30
Caryophyllene	1424	1.27	2.92
Aromadendrene	1439	0.10	0.85
Humulene	1456	-	0.27
GermacreneD	1480	0.17	-
γ-Murolene	1494	0.15	-
Bicyclogemacrene	1496	0.25	-
β-Bisabolene	1500	0.11	0.17
γ-Cadinene	1505	-	0.60
δ-Candinene	1514	0.20	0.47
α-Bisabolene	1518	0.40	0.50
Nerolidol	1545	-	0.19
Caryophylleneoxide	1576	0.38	0.23
Varidiflorene	1590	0.39	-
Monoterpene hydrocarbons	-	50.2	24.38
Oxygenated monoterpenes	-	46.01	63.38
Sesquiterpene hydrocarbons	-	2.80	10.98
Oxygenated Sesquiterpene	-	0.38	0.42
Others	-	0.18	0.51
Total	-	99.57	99.67

[a]Retention indexes measured relative to n-alkanes (C-9 to C-24) on the non-polar HP-5 column. t, traces (<0.1%). [b] Compound not detected in the oil.

Discussion

There are some reports about high variability on biological activity and chemical composition of EOs of wild type thyme species depending on gathering site, harvesting period and the studied population. For example, Nejad Ebrahemi and et al identified 31-37 constituents in different parts of *T. caramanicus*' oil from Kerman, Iran which oxygenated monoterpenes are the major portion of all samples (17). These results are in agreement with previously published data by Hazzit et al, (18) and Sarikurkcu et al (3). In the study of Mazooji et al on *T. kotschyanus* from Firouzkouh, Iran 10 compounds and thymol (89.08%) as main constituents were obtained (19). The chemical compositions of *T. kotschyanus* EOs of East-Azarbayjan were compared with other reports about *Thymus* species

Table 2. Radical Scavenging and FRAP Values (mM F^{2+}/mg Extract) of Wild and Cultivated *T. kotschyanus*

Test	EO		ME	
	Wild	Cultivated	Wild	Cultivated
DPPH (IC_{50} µg/mL)	2.74	3.24	2.36	1.82
FRAP value (mM Fe^{2+}/mg extract)	1.83	1.56	1.27	1.53

Abbreviations: EO: essential oil; ME, methanol extract.

in Iran. There are shown some differences and similarities between these reports. For example, Kalvandi et al, showed that thymol (42.8%), linalool (11.1%), γ-terpinene (6.0%), 1,8-cineole (5.6%), borneol (3.4%), and α-terpineol (1.8%) in *T. eriocalyx* collected from Markazi province of Iran, before flowering stage. and in full flowering stage these compounds were changed to 43.1%, 4.0%, 6.3%, 3.3%, 4.9% and 7.1%, respectively (20). However, in another study in Lorestan province on *T. eriocalyx* from Iran is reported thymol and 1-borneol (66.3% and 10.5%), respectively (4).

According to El Bouzidi et al, the major compounds of wild and cultivated types of *T. broussonetii*, *T. maroccanus* and *T. satureioides* were determined carvacrol (43.4%, 60.8%, 70.1%, 71.6%, 26.5%, and 26.0%), respectively (8). In a study by Safaei-Ghomi et al, carvacrol content of *Thymus* species was 85.94%. Data obtained from this study, showed a noticeable difference in percentage of thymol (29.96% and 47.48%) in the oils of wild and cultivated types (4). The similarity in quantity of thymol has previously been published by Sarikurkcu et al, from wild type of *T. longicaulis* in Turkey (3). The percentage of carvacrol resulted in this study was comparable with *T. algeriensis* studied by Teixeira et al, in Portugal (21) but was not resemblance with the results obtained for *T. pallescens* in Algeria (18) and in *T. caramanicus* Jalas from Iran (4). In the study of Khoshsokhan et al, in 10 populations of *T. kotschyanus* oxygenated monoterpenes were the main group of constituents in all samples and many difference in thymol (2.45%-78.65%), carvacrol (1.84%-49.38%), α-terpineol (1.79%-17.1%), borneol (0.68%-3.8%), linalool (0.5%-39.05%), 1,8 cineole (0.53%- 8.39%), p-cymene (0.38%-7.74%) (6).

In some previous reports were shown the in vitro antioxidant activity in several *Thymus* species EOs (3,8,20). The excessive amount of flavonoid and phenolic compounds in MEs of medicinal plants could be participated in the free-radical-scavenging activity, because of more polar sub fraction of them (22).

The Roby et al and Sokmen et al were stated that a few *Thymus* species such as some varieties of *T. tosevii*, *T. daenensis* subsp *lancifolius* and *T. longidens* could be used as natural antioxidant sources compounds (23,24). Based on the results of this study *T. kotschyanus* can also be added to the above mentioned species which have antioxidant activity. The high content of thymol in wild and cultivated *T. kotschyanus* explains their antioxidant activity.

The antibacterial activity by presence of inhibition zone appeared from EOs in cultivated type on all tested microorganisms significantly higher than wild type which can be attributed to the presence of high concentration of thymol (47.48%) against wild type (29.96%), one of oxygenated monoterpenes with well documented antibacterial and antifungal potential (25-27). *T. kotschyanus* EOs and MEs showed strong antibacterial activity against *S. sanguis* which made them as alternative compound in maintenance oral health system. Antibacterial activity of different species of thyme were documented in various studies include: anti-*E. coli* activity of *T. vulgaris* EOs in Slovak, *T. broussonetii*, *T. maroccanus* and *T. satureioides* EOs in Morocco (8), and *T. caramanicus* EOs in Iran (17). Our observations are in accordance with those reported by Teixeira et al, (21), in thymol rich EO, Ebrahimi et al, (17) and El Bouzidi et al, (8) by carvacrol rich of *T. mastichina*, *T. caramanicus* and Moroccan thyme species, respectively. Anti–*Bacillus cereus* (8), EOs of *T. pallescens*, *T. algeriensis* and *T. dreatensis* in Algeria (18), *T. vulgaris* EOs (25,28).

Table 3. Antimicrobial Activity of the Studied EOs and MEs Using Disk Diffusion Agar

Test microorganisms	Inhibition Zone Diameter (mm)					
	EOs		MEs		CP[a]	K[b]
	Wild	Cultivated	Wild	Cultivated		
Enterococcus faecalis ATCC 29212	32.5±3.5	36.0±5.6	-	-	22.5±2.1	19.5±2.1
Staphylococcus aureus ATCC 25952	27.5±3.53	33.0±2.8	-	-	23.5±2.1	19.0±1.4
Streptococcus sanguis PTCC 1449	45.0±1.4	49.0±1.4	19.0±1.4	19.0±1.4	27.5±0.7	19.0±1.4
Staphylococcus aureus ATCC 33591	29.0±1.4	31.0±1.4	18.0±2.8	18.5±2.1	22.5±3.5	16.0±2.8
Staphylococcus aureus ATCC 29213	38.0±2.8	42.0±2.8	18.5±2.1	21.0±1.4	25.5±0.7	16.0±2.8
Enterobacter aerogenes ATCC13048	26.5±2.1	33.5±0.7	-	-	22.0±2.8	17.5±3.5
Klebsiella pneumoniae ATCC 700603	18.0±2.8	17.5±3.5	-	-	19.0±1.4	12.0±2.8
Esherichia coli ATCC 25922	18.0±2.8	25.5±0.7	-	-	28.0±2.8	18.0±2.8
Proteus mirabilis ATCC43071	37.0±1.4	38.5±3.5	-	-	27.5±3.5	19.0±2.8
E coli O157:H7	29.5±0.7	31.0±1.4	-	-	26.0±5.6	19.0±1.4

Abbreviations: EO: essential oil; ME, methanol extract.
[a]Ciprofloxacin, [b]Kanamycin.

Table 4. MICs of tested bacteria towards EOs and ME of *T. kotschyanus*

Test Microorganisms	MIC (µg/mL)			
	EOs		MEs	
	Wild	Cultivated	Wild	Cultivated
Enterococcus faecalis ATCC 29212	128	16	>2048	2048
Staphylococcus aureus ATCC 25952	2	2	16	32
Streptococcus sanguis PTCC 1449	16	8	128	1024
Staphylococcus aureus ATCC 33591	16	2	64	1024
Staphylococcus aureus ATCC 29213	16	4	256	128
Enterobacter aerogenes ATCC13048	64	16	>2048	>2048
Klebsiella pneumoniae ATCC 700603	256	64	>2048	> 2048
Escherichia coli ATCC 25922	64	16	>2048	>2048
Proteus mirabilis ATCC43071	64	16	>2048	2048
E coli O157:H7	64	16	>2048	>2048

Abbreviations: EO: essential oil; ME, methanol extract; MIC, Minimum inhibitory concentration.

In comparing wild and cultivated *Thymus* species oils, it appears the cultivated type exhibit strong antimicrobial activity, but in extracts obtained from the same plant did not observe the same results. In MEs poor antimicrobial activity were shown on streptococci and staphylococci species. The results of our study were marked difference with the report of El Bouzidi et al, in which domestication had not effect on antimicrobial activity of studies of *Thymus* species in which EOs obtained from wild and cultivated thyme species showed an inhibitory activity (MIC) the same on all the microorganisms tested (8). The MIC of *T. vulgaris* EO ranged from 75- 1100 µg/mL (29), 0.12-1.78 mg/mL of *T. broussonetii*, *T. maroccanus* and *T. satureioides* EOs , 15.6->500 µg/mL of *T. kotschyanus* EOs in Iraq for tested bacteria (30).

The MIC values of *T. kotschyanus* in Mohammed et al (30) were similar with wild type EOs of Iranian thyme while by increasing in thymol concentration in cultivated type, reduced MIC's values were seen for all tested organisms. In Ahmadi et al, the MIC of *T. kotschyanus* EOs prepared from Estahban was lower than our study but the zone of inhibition was similar (31).

In conclusion, our results demonstrated that the variations in the qualitative and quantitative composition in the obtained oils from the wild and cultivated *Thymus* species. The environmental condition, geographic distribution or genotyping of medicinal plants can be explained these variation. Generally, the EOs of medicinal plants which contain a high percentage of phenolic compounds such as carvacrol and thymol possessing antibacterial activity is against pathogens. The results obtained in this study support that imagination that higher antimicrobial potential of Iranian *Thymus* oils especially against *Klebsiella pneumoniae* ATCC700603 (extended-spectrum β-lactamases producer bacteria, ESBLs), *S. aureus* ATCC 33591 (methicillin-resistant *S. aureus,* MRSA) and *S. sanguis* PTCC 1449 (normal flora of oral) is conferred by high thymol content. High antimicrobial effect of EOs of cultivated type had straight relation to thymol content. Therefore this study suggest that the possibility of using the oils of this *Thymus* species as natural antimicrobial component in toothpaste because of anti-streptococcal activity of that and as a food additive accompanied by antibiotics in life threaten infection such as ESBLs producing gram negative bacteria and MRSA associated infections. Present results show that the cultivation significantly affected the biological activity and may constitute an alternative solution for maintaining this medicinal plant.

Ethical Issues

This study was approved by ethical research committee, Tabriz Branch, Islamic Azad University.

Conflict of Interests

The authors declare no conflict of interests.

Financial Support

Islamic Azad University, Tabriz Branch, Tabriz, Iran.

Acknowledgments

The authors would like to thank Tabriz Branch, Islamic Azad University for the financial support of this research, which is based on a research project contract and Ali Hashemi for sincerely cooperation in supply standard bacterial strains.

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