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Terpenoids and phenol derivatives from *Malva silvestris*

Francesca Cutillo [a], Brigida D'Abrosca [b], Marina DellaGreca [a,*], Antonio Fiorentino [b], Armando Zarrelli [a]

[a] *Dipartimento di Chimica Organica e Biochimica, Università Federico II, via Cynthia 4, I-80126 Napoli, Italy*
[b] *Dipartimento di Scienze della Vita – Seconda Università di Napoli, via Vivaldi 43, I-81100 Caserta, Italy*

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Abstract

A sesquiterpene and a tetrahydroxylated acyclic diterpene as well as two known monoterpenes, 6 C_{13} *nor*-terpenes and 11 aromatic compounds were isolated from the water extract of *Malva silvestris*. The structures of the compounds were determined by spectroscopic NMR and MS analysis. Effects of these compounds on germination and growth of dicotyledon *Lactuca sativa* L. (lettuce) were studied in the 10^{-4}–10^{-7} M concentration range.

Keywords: *Malva silvestris*; *Lactuca sativa*; Phytotoxic activity; Spectroscopic analysis; Terpenes; Phenols

1. Introduction

Malva silvestris is a species widely distributed in Italy and used in traditional phytotherapy (Guarrera, 2005) and cosmetic treatments (Paufique, 2000). Fluidextract of *M. silvestris* flowers and leaves are used as a valuable remedy for cough and inflammatory diseases of mucous membranes (Farina et al., 1995). The chemical composition of a water extract of *M. silvestris* has been investigated, and resulted in the isolation and structure elucidation of a novel sesquiterpene and a new tetrahydroxylated linear diterpene as well as two monoterpenes, six C_{13} *nor*-terpenes and eleven aromatic compounds.

2. Results and discussion

Fresh plants of *M. silvestris* were extracted with water using a Naviglio extractor (Naviglio, 2003). This extractor is based on the suction effect, generated by the compression of the solvent used for extraction on solids at a pressure of about 8–9 bars for a particular time period, followed by immediate decompression at atmospheric pressure. The rapid release of the liquid used for extraction from the inside of a solid matrix, because of pressure gradient, transports the extractable compounds within the solid matrix towards the outside. After extraction of the aqueous portion shaken with EtOAc, the organic fraction was chromatographed on a silica gel column, the fractions were purified by preparative thin layer chromatography and HPLC yielding 21 compounds. A test of extraction conducted using conventional procedure (Cutillo et al., 2005) resulted in similar amount of extract.

The compounds were identified as 4-hydroxybenzoic acid (**1**), 4-methoxybenzoic acid (**2**), 4-hydroxy-3-methoxybenzoic acid (**3**), 2-hydroxybenzoic acid (**4**), and 4-hydroxy-2-methoxybenzoic acid (**5**), compounds **6** and **9** as 4-hydroxybenzyl alcohol and tyrosol, compounds **7** and **8** as 4-hydroxydihydrocinnamic acid and 4-hydroxy-3-methoxydihydrocinnamic acid, and **10** and **11** as 4-hydroxycinnamic acid and ferulic acid by comparison of their spectral data with those of authentic samples.

Monoterpenes **12** and **13** were linalool and linalool-1-oic acid (Nicoletti et al., 1989).

The EIMS of compound **14** showed a molecular ion peak at m/z 262, and prominent peaks at m/z 245 $[M - OH]^+$, 234 $[M - CO]^+$, and 219 $[M - C_3H_7]^+$. The molecular formula

* Corresponding author. Tel.: +39 081 674162; fax: +39 081 674393.
E-mail address: dellagre@unina.it (M. DellaGreca).

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Table 1
NMR spectral data of compound **14** in $CDCl_3$

Position	δ_H[a]	NOESY	δ_C	HMBC[b]
1			125.1 (*q*)[c]	
2			142.9 (*q*)	
3			144.0 (*q*)	
4			127.0 (*q*)	
5	6.69 *s*	12, 13, 15	115.8 (*t*)	1, 3, 7
6			139.5 (*q*)	
7			74.7 (*q*)	
8	2.12 *m*, 1.30 *m*		27.3 (*s*)	6, 7, 9
9	1.97 *m*, 1.49 *m*		23.6 (*s*)	1
10	3.45 *m*		27.8 (*t*)	2, 6
11	2.43 *q* (6.8)		31.2 (*t*)	6, 7, 12, 13
12	1.14 *d* (6.8)	5	18.5 (*p*)	7, 11
13	1.13 *d* (6.8)	5	17.8 (*p*)	7, 11
14	4.00 *dd* (8.3, 2.4), 3.45 *m*		67.9 (*s*)	1, 7, 9, 10
15	2.32 *s*	5, 12, 13, OMe	16.1 (*p*)	3, 4, 5
OMe	3.81 *s*	15	60.7 (*p*)	3

[a] ^1H chemical shift values (δ ppm from $SiMe_4$) followed by multiplicity and then the coupling constants (*J* in Hz).
[b] HMBC correlations from H to C.
[c] Letters, *p*, *s*, *t* and *q*, in parentheses indicate, respectively, the primary, secondary, tertiary and quaternary carbons, assigned by DEPT.

was determined to be $C_{16}H_{22}O_3$ by HREIMS. Its IR spectrum showed absorption bands of hydroxyl group (3300 cm^{-1}) and phenyl group (1600 cm^{-1}). The structure of compound **14** was established using ^1H NMR and ^{13}C NMR including COSY, NOESY, HMQC, and HMBC experiments (Table 1). The ^1H–^1H COSY experiment showed a correlation series beginning with signal of a methine at δ 2.43 assigned to H-11 and coupled to two methyls at δ 1.14 and 1.13. The signals at δ 4.00 and 3.45 assigned to methylene H-14 were correlated with H-10 at δ 3.45, which in turn was coupled to the two H-9 protons at δ 1.97 and 1.49. These were correlated with H-8 at δ 2.12 and 1.30. Present also, were three singlets attributed to two methyls and a methine. The ^{13}C NMR spectrum of **14** showed 16 carbon signals due to four methyls, three methylenes, and three methines. An HMQC experiment allowed to assign the protons to the corresponding carbons. In the HMBC spectrum H-5 was correlated with C-1, C-3, and C-7, while the methoxyl group at δ 3.81 and the methyl at δ 2.32 were correlated with the C-3. The multiplet at δ 3.45 (H-10) was correlated with C-2 and C-6. While H-14 was correlated with C-1, C-7, C-9, and C-10, thus completely dating the structure of **14**. The analysis of NOESY spectrum evidenced NOEs of the methyls at δ 1.14 and 1.13 with H-5 methine, and the methyl at δ 2.32 with the methoxyl at δ 3.81 (Fig. 1).

Compounds **15** and **16** were identified as (6*R*,7*E*,9*S*)-9-hydroxy-4,7-megastigmadien-3-one and blumenol A (Cutillo et al., 2005) and compound **17** as (+)-dehydrovomifoliol (Mori, 1974) on the basis of their spectral data.

Compounds **18**–**20** had spectral data identical with those reported for (3*R*,7*E*)-3-hydroxy-5,7-megastigmadien-9-one, (3*S*,5*R*,6*S*,7*E*,9*R*)-5,6-epoxy-3,9-dihydroxy-7-megastigmene and (3*S*,5*R*,6*R*,7*E*,9*R*)-3,5,6,9-tetrahydroxy-7-megastigmene isolated from *Chenopodium album* and *Cestrum parqui* (DellaGreca et al., 2004; D'Abrosca et al., 2004a).

Compound **21** showed a molecular ion peak at *m*/*z* 340, and peaks at *m*/*z* 325 $[M - CH_3]^+$, 322 $[M - H_2O]^+$, and 297 $[M - C_3H_7]^+$. The molecular formula was determined to be $C_{20}H_{36}O_4$ by HREIMS. The four oxygen functions were ascribed to two secondary hydroxyl groups and the remaining two were attributed to tertiary hydroxyl groups (Table 2). The structure of **21** was characterized by ^1H NMR and ^{13}C NMR including COSY, NOESY, HMQC, and HMBC experiments. Five singlet methyls, ten aliphatic protons of five methylenes, two methines bearing oxygen, five olefinic protons, two as broad triplets, and three as double doublets were present in the ^1H NMR spectrum of **21**. The ^{13}C NMR spectrum showed 19 carbon signals identified as five methyls, six methylenes, and five methines. All the carbons were correlated to the corresponding protons on the basis of an HMQC experiment. The tertiary hydroxyl groups were positioned at C-3 and C-15 on the basis of an HMBC experiment that showed correlations between the C-3 carbon with the H-1, H-2, H-4 protons, and C-15 with the H-14, H-16, and H-17 protons. Furthermore, NOESY correlations of H-8 with H-6, H-10, and CH_3-19, and H-14 with CH_3-16 and CH_3-17 confirmed the structure of diterpene **21** (Fig. 1).

The absolute configurations at the C-8 and C-14 secondary carbinol carbons have been established by Mosher's method (Dale and Mosher, 1973) by converting compound **21** into the diasteromeric MTPA diesters. The chemical shift differences of protons, at β position of C-8 and C-14 chiral carbons, were assigned by a ^1H–^1H COSY experiment (Ohtani et al., 1991). The chemical shifts comparison of the signals due to H-9 and H-6/H-19 protons in both the (*R*) and the (*S*) MPTA derivatives and the calculation of the corresponding differences, expressed as $\Delta\delta_{R-S}$, were in agreement with the *S* configuration for C-8. For the C-14

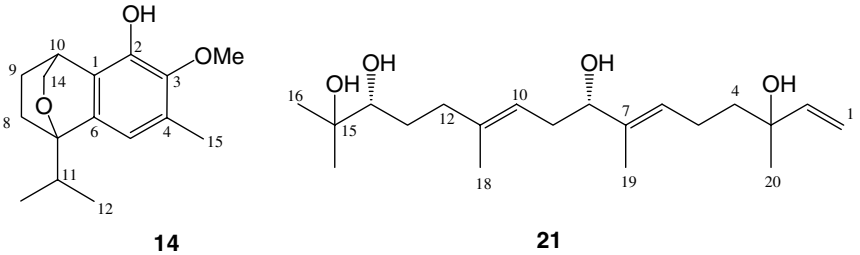

Fig. 1. Structure of terpenes isolated from *M. silvestris*.

Table 2
NMR spectral data of compound **21** in CD_3OD

Position	δ_H[a]	NOESY	δ_C	HMBC[b]
1	5.19 *dd*, 5.03 *dd* (17.0, 10.5, 1.5)		112.6 (*s*)[c]	2, 3
2	5.94 *dd* (17.0, 10.5)		146.7 (*t*)	3
3			74.3 (*q*)	
4	1.52 *dd* (8.0, 7.6)		43.5 (*s*)	2, 3, 5, 6, 20
5	2.08 *ddd* (8.0, 7.6, 7.0)		23.8 (*s*)	4, 6, 7
6	5.34 *brt* (7.0)	4, 8	127.9 (*t*)	4, 5, 8, 19
7			138.4* (*q*)	
8	3.93 *t* (6.8)	6, 10, 19	79.2 (*t*)	6, 7, 9, 10, 19
9	2.24 *m*		35.2 (*s*)	7, 8, 10, 11
10	5.16 *brt* (7.0)	8, 12	122.4 (*t*)	12, 18
11			138.2* (*q*)	
12	2.24 *m*, 2.05 *m*		38.5 (*s*)	10, 11, 13, 18
13	1.70 *m*, 1.34 *m*		31.2 (*s*)	12
14	3.23 *dd* (10.4, 2.0)	12, 16, 17	79.5 (*t*)	12
15			74.3 (*q*)	
16	1.16 *s*	14	25.5 (*p*)	14, 15, 17
17	1.15 *s*	14	26.1 (*p*)	14, 15, 16
18	1.63 *s*		16.9 (*p*)	10, 12
19	1.60 *s*	8	11.9 (*p*)	6, 7, 8
20	1.25 *s*		28.1 (*p*)	2, 3, 4

[a] ^1H chemical shift values (δ ppm from $SiMe_4$) followed by multiplicity and then the coupling constants (J in Hz).
[b] HMBC correlations from H to C.
[c] Letters, *p*, *s*, *t* and *q*, in parentheses indicate, respectively, the primary, secondary, tertiary and quaternary carbons, assigned by DEPT.
* Assignments may be interchanged.

carbon, the positive $\Delta\delta_{R-S}$ for the H-16/H-17 and negative value for H-13 were found, indicating *R* configuration for C-14. Therefore, the structure of **21** was deduced to be (6*E*,8*S*,10*E*,14*R*)-3,7,11,15-tetramethylhexadeca-1,6,10-trien-3,8,14,15-tetraol.

The phytotoxicity of compounds **1**, **3**, **7**–**10**, and **15**–**20** on the seeds of *Lactuca sativa* was previously reported (DellaGreca et al., 2004; D'Abrosca et al., 2004a,b). Compounds **2**, **5**, and **11**–**14** were tested for their activities on the seeds of *L. sativa*. Aqueous solutions, ranging between 10^{-4} and 10^{-7} M, were tested on germination, root length and shoot length of treated lettuce seeds (Fig. 2). Compound **2** and sesquiterpene **14** reduced the germination by 20% at 10^{-4} M respect to the control. Compounds **5**, **11**, and **13** were inactive at all tested concentrations. Linalool showed about 80% inhibition on germination at the higher concentration tested, while no effects were observed on root and shoot length. Among compounds tested, only **2** reduced root length significantly. Activities of 15–30% on the shoot length at a concentration of 10^{-4} M have been observed for compounds **2**, **5**, and **11**–**13** as compared to the control.

3. Experimental

3.1. General experiment procedures

^1H and ^{13}C NMR spectra were run on a Varian INOVA 500 NMR spectrometer at 500 and 125 MHz, respectively, in $CDCl_3$ or CD_3OD at 25 °C. MS spectra were obtained with a HP 6890 spectrometer equipped with a MS 5973 N detector. IR spectra were recorded on a Jasco FT/IR-430 instrument. UV–Vis spectra were recorded in $CHCl_3$ or MeOH on a Perkin–Elmer Lambda 7 spectrophotometer. HPLC was performed on an Agilent 1100 by using an UV detector. Silica gel 60 (230–400 mesh, Merck) was used for CC, and preparative TLC was performed on silica gel (UV-254 precoated) plates with 0.5 and 1.0 mm thickness (Merck). Preparative HPLC was performed using RP-18 (LiChrospher 10 μm, 250 × 10 mm i.d., Merck) column.

3.2. Plant material

Aerial parts of *M. silvestris* were collected near Caserta (Italy) in the spring of 2003 and identified by Professor Antonino Pollio of the Dipartimento di Biologia Vegetale of University of Naples. Voucher specimens (HERB-NAQA650) are deposited at the Dipartimento di Biologia Vegetale of University Federico II of Naples.

3.3. Extraction and isolation

Fresh leaves (10.0 kg) of the plant were extracted with H_2O at room temperature using the Naviglio extractor. The water was reduced in volume and partitioned between EtOAc and H_2O. The organic extract (19 g) was subjected to silica gel column chromatography, by using $CHCl_3$ and successively increasing the EtOAc concentration by 25%, 50% and 80% in $CHCl_3$. Fractions of 200 ml were collected and fractions with similar TLC profiles were combined. The first fraction eluted with 100% $CHCl_3$ was purified by flash silica gel column chromatography with hexane–ethyl ether (1:1) to give fractions containing compounds **14**–**20**. Fraction containing crude **14** was purified by reverse phase C-18 HPLC with MeOH–MeCN–H_2O [(1:6:3), 5 mg]: $[\alpha]_D^{25}$ −2.0° ($CHCl_3$; *c* 0.5), MS: *m*/*z* 262 $[M]^+$; HRE-IMS *m*/*z* 262.1498 (Calcd. for $C_{16}H_{22}O_3$, 262.1569); NMR data: see Table 1. Compounds **15** (10 mg), **17** (14 mg), and **18** (19 mg) were purified by preparative TLC with $CHCl_3$–Me_2CO (7:3). The fraction containing crude **20** (5 mg) was purified by preparative TLC with CH_2Cl_2–MeOH–H_2O (11:10:9). Compounds **16** (20 mg) and **19** (20 mg) were purified by preparative TLC with $CHCl_3$–MeOH (19:1).

The second fraction eluted with 100% $CHCl_3$ was extracted with 2 N NaOH. After neutralization this fraction was extracted with EtOAc to give 600 mg of residual material. Column chromatography on silica gel gave a fraction containing **4**, **8** and **13**. Compound **4** (20 mg) was purified by preparative TLC with CH_2Cl_2–MeOH–H_2O (33:30:35) lower layer. Compounds **8** (40 mg) and **13** (10 mg) were purified by C-18 HPLC with H_2O–MeCN–MeOH (7:2:1).

The fifth fraction eluted with 50% EtOAc was purified by flash column chromatography on Si gel using CH_2Cl_2 and successively increasing the Me_2CO concentration from 0% to 50% in CH_2Cl_2. Fractions eluted with 100% CH_2Cl_2

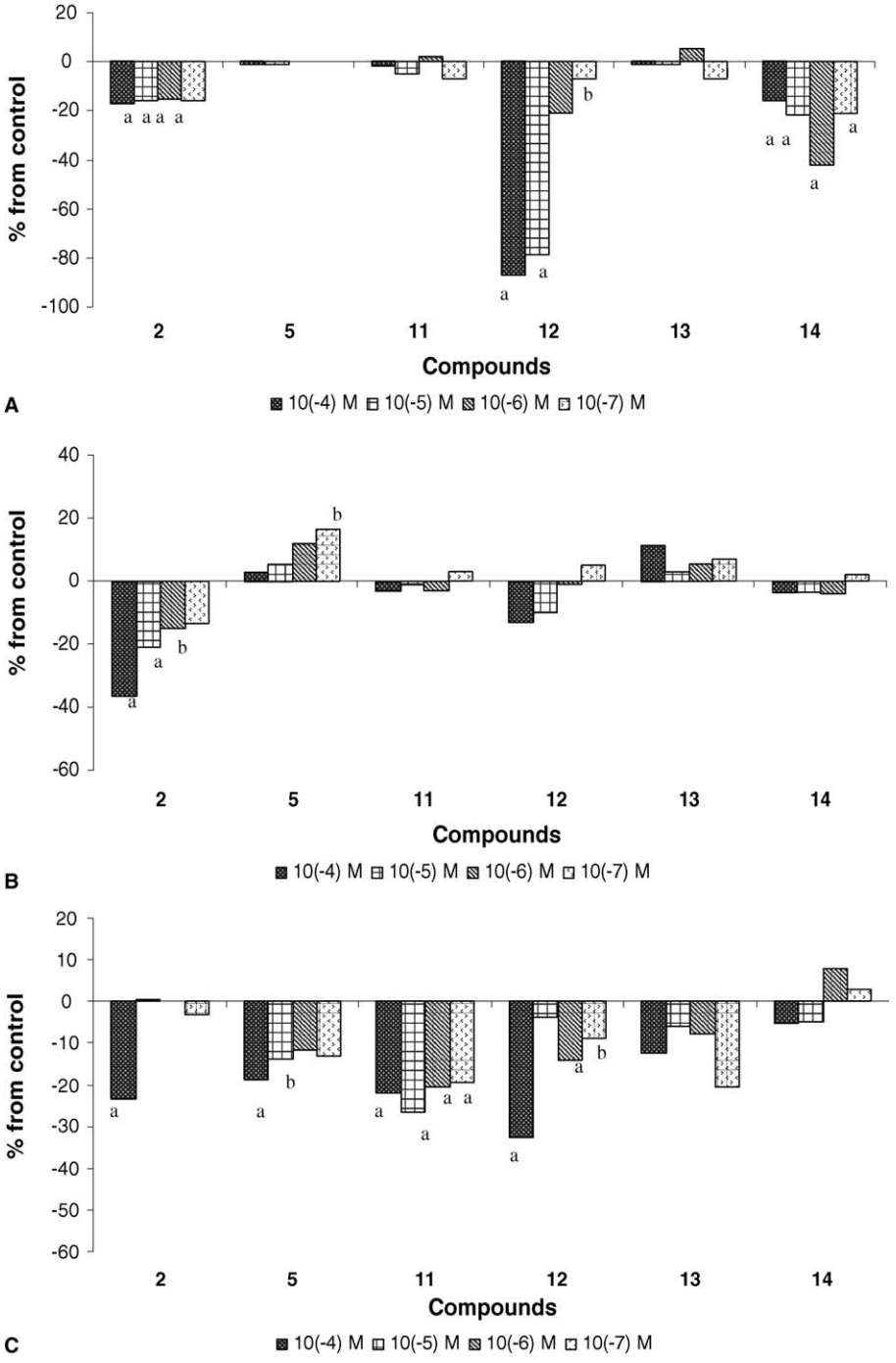

Fig. 2. (A) Effect of compounds **2**, **5**, and **11**–**14** on germination of *L. sativa* L. Value presented as percentage differences from control and are not significantly different with $P > 0.05$ for Student's t test: (a) $P < 0.01$; (b) $0.01 < P < 0.05$. (B) Effect of compounds **2**, **5**, and **11**–**14** on root length of *L. sativa* L. Value presented as percentage differences from control and are not significantly different with $P > 0.05$ for Student's t test: (a) $P < 0.01$; (b) $0.01 < P < 0.05$. (C) Effect of compounds **2**, **5**, and **11**–**14** on shoot length of *L. sativa* L. Value presented as percentage differences from control and are not significantly different with $P > 0.05$ for Student's t test: (a) $P < 0.01$; (b) $0.01 < P < 0.05$.

were rechromatographed on silica gel under the same conditions. The subfraction eluted with 10% Me_2CO was purified by C-18 HPLC with MeOH–H_2O (4:3) to give compounds **1** (5 mg), **2** (3 mg), **7** (20 mg), **10** (5 mg), and **11** (5 mg). The subfraction eluted with 15% Me_2CO was purified by C-18 HPLC with MeOH–H_2O (4:3) to give compounds **3** (2 mg), and **5** (2 mg). The subfraction eluted with 50% Me_2CO was purified by C-18 HPLC with MeOH–MeCN–H_2O (4:1:5) to give compound **6** (5 mg). The fraction eluted with 50% Me_2CO was rechromatographed on silica gel using CH_2Cl_2 and successively increasing the Me_2CO concentration by 20%, 40%, and 100% in CH_2Cl_2. The fraction eluted with 20% Me_2CO was purified by C-18 HPLC with MeOH–MeCN–H_2O

(4:1:5) to give **12** (20 mg). The fraction eluted with 40% Me_2CO was purified by preparative TLC with EtOAc–Me_2CO (19:1) to give **9** (12 mg). The fraction eluted with 100% Me_2CO was purified by C-18 HPLC with MeOH–MeCN–H_2O (3:2:5) to give **21** (2 mg): $[\alpha]_D^{25}$ +21.0° (MeOH; *c* 0.05), MS: *m*/*z* 340 $[M]^+$; HREIMS *m*/*z* 340.2598 (Calcd. for $C_{20}H_{36}O_4$, 240.2614); NMR data: see Table 2.

3.4. Bioassays

Seeds of *L. sativa* L. (cv Napoli V.F.) collected during 2003, were obtained from Ingegnoli S.p.a. All undersized or damaged seeds were discarded and the assay seeds were selected for uniformity. Bioassays used Petri dishes (50 mm diameter) with one sheet of Whatman No. 1 filter paper as support. In four replicate experiments, germination and growth were conducted in aqueous solutions at controlled pH, using MES (2-[*N*-morpholino]ethanesulfonic acid, 10 mM, pH 6). Test solutions (10^{-4} M) were prepared in MES and the rest (10^{-5}–10^{-7} M) were obtained by dilution. Parallel controls were performed. After adding 25 seeds and 5 ml test solutions, Petri dishes were sealed with Parafilm® to ensure closed-system models. Seeds were placed in a growth chamber KBW Binder 240 at 25 °C in the dark. Germination percentage was determined daily for five days (no more germination occurred after this time). After growth, plants were frozen at −20 °C to avoid subsequent growth until the measurement process. Data are reported as percentage differences from control in the graphics and tables. Thus, zero represents the control; positive values represent stimulation of the control; positive values represent stimulation of the parameter studied and negative values represent inhibition.

3.5. Statistical treatment

The statistical significance of differences between groups was determined by a Student's *t* test, calculating mean values for every parameter (germination average, shoot and root elongation) and their population variance within a Petri dish. The level of significance was set at $P < 0.05$.

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