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Flavonoid Constituents and Antimicrobial Activity of Date (*Phoenix dactylifera* L.) Seeds Growing in Egypt

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ABSTRACT

The methanolic extract of date palm (*Phoenix dactylifera* L.) seeds cultivated in El-Dakhla Oases, Egypt, was investigated for their phenolics and antimicrobial activity. Seven flavonoids were isolated and identified as isoquercetrin (1), luteolin 7-*O*-β-D-neohesperopyranoside 3′-*O*-methylether (2), luteolin 7-*O*-β-D-neohesperopyranoside (3), acacetin 7-*O*-β-D-neohesperopyranoside (4), apigenin 7-*O*-α-D-apiofuranoside (5), apigenin 7-*O*-α-D-apiofuranosyl-(1→2)-*O*-β-D-glucopyranoside (6) and genistein 8-*C*-β-D-glucopyranoside (7). The structures were determined mainly by spectroscopic methods (UV, ^{1}H-, ^{13}C-NMR, 2D-NMR). Results of an antimicrobial test showed that the methanolic extract of the *P. dactylifera* seeds moderately inhibited the growth of Gram-positive and Gram-negative bacteria.

Keywords: bacteria, fungi, methanolic extract, Palmae, phenolics, *Phoenix dactylifera*, yeasts

INTRODUCTION

In nature there are a large number of different types of antimicrobial compounds. Food contamination and spoilage by microorganisms have attracted increased attention because they are problems that have not yet been brought under adequate control despite all the preservation techniques available.

The fruits of date palm (*Phoenix dactylifera* L., family Palmae) are consumed throughout the world and have always played an important role in the economic and social lives of the people of the Middle East region. The fruit of the date palm is composed of a fleshy pericarp and seed (Besbes *et al.* 2004; Hong *et al.* 2006).

Dates have high tannin content and are used medicinally as an emollient and astringent in intestinal troubles. As an infusion, decoction, syrup, or paste, compounds and extract from dates may be administered for sore throat, colds, bronchial catarrh, and taken to relieve fever and a number of other complaints. One traditional belief is that dates can counteract alcohol intoxication (Boulos 1983; Kirtikar and Basu 1984; Rizk and Al-Nowaihi 1989). The seed powder is also used in some traditional medicines. A gum that exudes from the wounded trunk is employed in India for treating diarrhea and genitourinary ailments. The roots are used against toothache. The pollen yields an estrogenic principle, estrone, and has a gonadotropic effect on young rats (Boulos 1983; Kirtikar and Basu 1984; Rizk and Al-Nowaihi 1989).

Date seeds are soaked and ground for animal feed. Their oil is suitable for use in soap and cosmetics. The seeds are also burned to make charcoal for silversmiths, and can be strung in necklaces. Date seeds are also ground and used in the manner of coffee beans, or as an additive to coffee. Pits of date palm (seed) are a waste product of many industries, after technological transformation of the date fruits (Khatchadourian *et al.* 1983; Youssif 1990; Youssif *et al.* 1996; Al-Hooti *et al.* 1997; Hobani 1998; Youssif and Alghamdi 1999).

Flavonoids constitute a large group of secondary plant metabolites. Moreover, dietary flavonoids have attracted much interest recently because *in vitro* and *in vivo* studies suggest that they have a variety of beneficial biological properties, which may play an important role in the maintenance of human health (Middleton and Kandaswami 1994; Hertog *et al.* 1995). Phenolic compounds may affect growth and metabolism of bacteria that can exhibit activating or inhibiting effects on microbial growth according to their constitution and concentration (Reguant *et al.* 2000; Alberto *et al.* 2001, 2002).

Of the few works that have been published on the chemistry of date seeds mostly describe the presence of such constituents as protein, fat, mineral and carbohydrate (El-Shurafa *et al.* 1982; Al- Al-Showimann 1990; Devshony *et al.* 1992; Al-Hooti *et al.* 1998; Hamada *et al.* 2002), but little is known about phenolics in date seeds. Thus, the aim of this study was to describe the isolation and structure elucidation of seven flavonoids from date seeds and to evaluate the antimicrobial action of the investigated date seed extracts.

MATERIALS AND METHODS

General

The NMR (^{1}H and ^{13}C) spectra were recorded at 400, 500 (^{1}H) and 100 (^{13}C) MHz, on Varian Inova-500, and Vnmrs-400 spectrometer apparatus at the University of Texas at Austin using TMS (trimethyl silane, Sigma-Aldrich Chemical Co., St. Louis, USA) as internal standard. The δ-values are reported as ppm relative to TMS in DMSO-d_6 and *J*-values are in Hz. UV analyses for pure samples were recorded separately in methanol with different diagnostic UV shift reagents on a Shimadzu UV 240 spectrophotometer (Mabry *et al.* 1970).

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Original Research Paper

Plant material

Phoenix dactylifera (cv. 'Balah Meghal') seeds were obtained from El-Dakhla Oases, Egypt. Voucher specimens (Reg. No.: V-20) were deposited in the herbarium of the Pharmacognosy Department, National Research Centre, Egypt.

Extraction and isolation

Air-dried *P. dactylifera* seeds (1 Kg) were extracted with pet. ether for 11 h, then with aqueous methanol (70%) for 18 h in a continuous extraction apparatus (Soxhlet) until exhaustion. The aqueous extract was concentrated to a small volume and partitioned successively with chloroform and *n*-butanol. The *n*-butanol extract was then fractionated on a silica gel 60 (63-200 mesh) column chromatography (ϕ3.0 × 80 cm, 30 g) and eluted with chloroform followed by step-wise addition of methanol to afford four fractions. Each fraction was subjected to Sephadex LH-20 and eluted with a mixture of methanol and water where seven compounds were isolated. All separation processes were followed by comp. PC using Whatman No. 1 paper with (S_1) *n*-butanol-HOAc-H_2O (4:1:5, top layer) and (S_2) 15% aqueous HOAc.

Acid hydrolysis

A solution of each compound (except 7) in 6% aqueous HCl was refluxed for 2 h. The reaction mixture was diluted with water and then extracted with ether. All aglycones and free sugars were identified by comp. PC with authentic samples using convenient solvent systems and specific spray reagents (aniline hydrogen phethalate) (Harborne *et al*. 1975).

Antimicrobial activity

1. Microbial strains

Gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*), Gram-negative bacteria (*Escherichia coli*, *Pseudomonas fluorescens*), a yeast strain (*Candida albicans*) and fungal strains (*Fusarium oxysporium*, *Aspergillus niger*) were obtained from the Microbial Chemistry Department, National Research Centre, Egypt.

2. Media

The culture media used was Lauri-Bertani medium (Maniatis *et al.* 1982), yeast extract peptone medium (Dillon *et al.* 1985) and potato dextrose agar medium (Subba Rao 1977) for bacteria, yeast and fungi, respectively. The media were sterilized by autoclaving for 20 min at 121°C and 15 psi.

3. Test assay for antimicrobial activity

The screening of antimicrobial activity of aqueous methanolic extract of the seed of *P. dactylifera* against Gram+ and Gram– bacteria, yeast and fungi were carried out by using the paper-disc antibiotic assay method (Gnanamanickam and Mansfield 1981; Govindarajan and Gnanamanickam 1981). Ampicillin trihydrate was used as a standard antibacterial agent and clotrimazole was used as a standard antifungal agent. The discs (8 mm) were loaded with 100 µg of the 70% methanolic extract and standards. They were placed on the surface of the specified medium in Petri dishes seeded with the tested organisms and incubated at 37, 30 or 28°C for bacteria, yeast or fungi, respectively. Each inhibition zone diameter was measured in mm, after an incubation period of 24-48 h for bacteria and yeasts or 48-72 h for fungi. Each test was carried out in triplicate. Further dilutions were then performed using the same method. The final concentrations of the tested extract were 10, 7.5, 5, 2.5 and 1% in absolute methanol. Absolute methanol without seed extract was used as a blank.

Statistical analyses

All values were expressed as the mean of three measurements for each treatment. Data were subjected to paired samples *t*-test. Probabilities of less than 0.05 were considered significant.

RESULTS AND DISCUSSION

The aqueous extract of date seeds was partitioned successively with chloroform and *n*-butanol. Further isolation of compounds from the *n*-butanol fraction was carried out using silica gel and Sephadex LH-20 column chromatography. This led to isolation of seven flavonoids, whose structures were determined to be isoquercetrin (**1**), luteolin 7-*O*-β-D-neohesperopyranoside 3′-*O*-methylether (**2**), luteolin 7-*O*-β-D-neohesperopyranoside (**3**), acacetin 7-*O*-β-D-neohesperopyranoside (**4**), apigenin 7-*O*-α-D-apiofuranoside (**5**), apigenin 7-*O*-α-D-apiofuranosyl-(1→2)-*O*-β-D-glucopyranoside (**6**) and genistein 8-*C*-β-D-glucopyranoside (**7**) by detailed spectroscopic analysis and comparing with data in the literature. Many phenolic compounds have been isolated from *P. dactylifera* fruits and leaves (Williams *et al.* 1971; Williams and Harborne 1973; Rizk and Al-Nowaihi 1989; Hong 2006). However, compounds **1-7** were isolated from date seeds for the first time.

Quercetin 3-*O*-β-D-glucopyranoside (1)

Yellow powder, Rf: 0.24 (S_1), 0.28 (S_2).

UV/Vis λ_{max} (MeOH) nm: 257, 265 (sh), 275 (sh), 357, (NaOMe): 272, 328, 410, (NaOAc): 270, 321, 396, (NaOAc+H_3BO_3): 261, 298, 378, ($AlCl_3$): 274, 303, 421, ($AlCl_3$+HCl): 269, 298, 300, 359, 402.

^{1}H NMR (500 MHz, DMSO-d_6): 7.66 (2H, m, H-2',6'), 6.88 (1H, d, *J* = 8.18 Hz, H-5'), 6.46 (1H, d, *J* = 2 Hz, H-8), 6.22 (1H, d, *J* = 2 Hz, H-6), 5.59 (1H, d, *J* = 7.6 Hz, H-1"), 4.0-3.1 (m, remaining sugar protons).

Luteolin 7-*O*-β-D-neohesperopyranoside 3′-*O*-methylether (2)

Yellow powder, Rf: 0.23 (S_1), 0.38 (S_2).

UV/Vis λ_{max} (MeOH) nm: 255, 268, 348, (NaOMe): 272, 330, 412, (NaOAc): 268, 348, (NaOAc+H_3BO_3): 269, 348, ($AlCl_3$): 261, 280, 298, 362, 393, ($AlCl_3$+HCl): 261, 280, 300, 362, 392.

^{1}H NMR (500 MHz, DMSO-d_6): 7.56 (1H, d, *J* = 8.8 Hz, H-6'), 7.54 (1H, brs, H-2'), 6.97 (1H, s, H-3), 6.92 (1H, d, *J* = 8.8 Hz, H-5'), 6.8 (1H, d, *J* = 2 Hz, H-8), 6.35 (1H, d, *J* = 2 Hz, H-6), 5.2 (1H, d, *J* = 7.2 Hz, H-1"), 5.11 (1H, brs, H-1'"), 3.86 (3H, s, CH_3O), 3.8-3.1 (m, remaining sugar protons), 1.18 (3H, d, *J* = 6 Hz, -CH_3).

Luteolin 7-*O*-β-D-neohesperopyranoside (3)

Yellow powder, Rf: 0.23 (S_1), 0.38 (S_2).

UV/Vis λ_{max} (MeOH) nm: 255, 268, 348, (NaOMe): 267, 405, (NaOAc): 252, 268, 352, (NaOAc+H_3BO_3): 256, 372, ($AlCl_3$): 270, 298, 325, 423, ($AlCl_3$+HCl): 263, 358, 385.

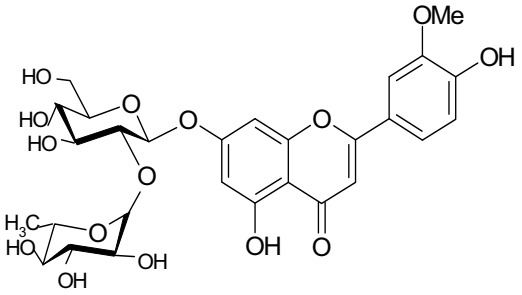
^{1}H NMR (500 MHz, DMSO-d_6): 7.53 (1H, d, *J* = 8.5 Hz, H-6'), 7.48 (1H, d, *J* = 2 Hz, H-2'), 6.84 (1H, d, *J* = 8.5 Hz, H-5'), 6.81 (1H, s, H-3), 6.77 (1H, d, *J* = 2 Hz, H-8), 6.35 (1H, d, *J* = 2 Hz, H-6), 5.21 (1H, d, *J* = 7.0 Hz, H-1"), 5.12 (1H, brs, H-1'"), 3.8-3.1 (m, remaining sugar protons), 1.1 (3H, d, *J* = 6.5 Hz, -CH_3).

Acacetin 7-*O*-β-D-neohesperopyranoside (4)

Yellow powder, Rf: 0.25 (S_1), 0.34 (S_2).

UV/Vis λ_{max} (MeOH) nm: 266, 328, (NaOMe): 278, 357, (NaOAc): 267, 331, (NaOAc+H_3BO_3): 269, 334, ($AlCl_3$): 274, 300, 345, 384, ($AlCl_3$+HCl): 274, 299, 338, 381.

^{1}H NMR (500 MHz, DMSO-d_6): 7.89 (2H, d, *J* = 9.0 Hz, H-2',6'), 6.89 (2H, d, *J* = 9.0 Hz, H-3',5'), 6.88 (1H, s, H-3), , 6.79 (1H, d, *J* = 2.0 Hz, H-8), 6.34 (1H, d, *J* = 2.0 Hz, H-6), 5.2 (1H, d, *J* = 7.0 Hz, H-1"), 5.12 (1H, brs, H-1'"), 4.0-3.1 (m, remaining sugar protons), 3.84 (3H, s, OCH_3), 1.1(3H, d, *J* = 6.5 Hz, -CH_3).



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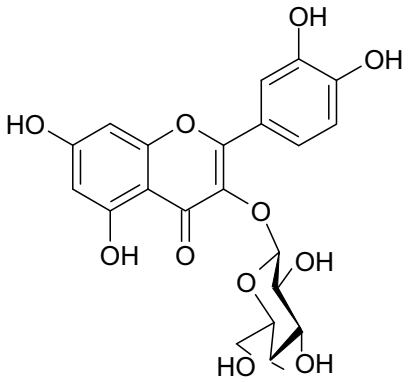

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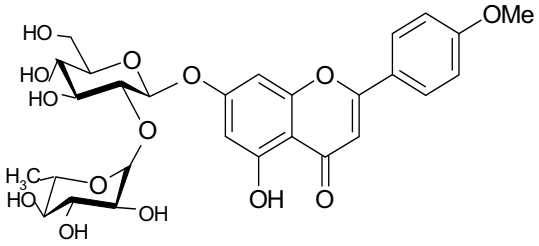

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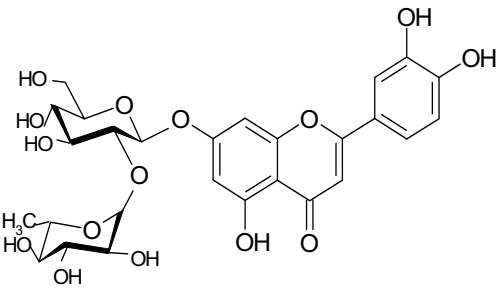

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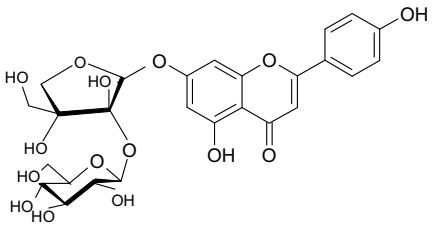

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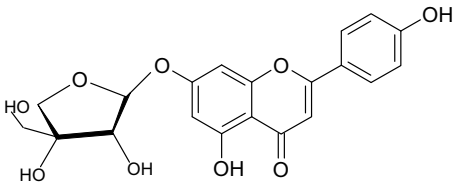

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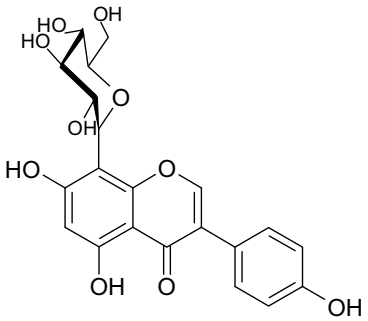

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Apigenin 7-*O*-α-D-apiofuranoside (5)

Yellow powder, Rf: 0.69 (S_1), 0.38 (S_2).

UV/Vis λ_{max} (MeOH) nm266, 333, (NaOMe): 269, 301(sh), 386, (NaOAc): 267, 355, 387, ($NaOAc+H_3BO_3$): 267, 340, ($AlCl_3$): 276, 300, 348, 386, ($AlCl_3$+HCl): 277, 299, 341, 380.

^{1}H NMR (400 MHz, DMSO-d_6): 7.96 (2H, d, J = 9 Hz, H-2',6'), 6.93 (2H, d, J = 9 Hz, H-3',5'), 6.86 (1H, s, H-3), , 6.79 (1H, d, J = 2.5 Hz, H-8), 6.34 (1H, d, J = 2.5 Hz, H-6), 5.6 (1H, s, H-1"), 3.8-3.1 (m, remaining sugar protons).

^{13}C NMR (100 MHz, DMSO-d_6): 182 (C-4), 165 (C-7), 160 (C-2), 159 (C-4'), 157 (C-5), 155 (C-9), 130 (C-2',6'), 120 (C-1'), 115 (C-3',5'), 107 (C-1"), 105(C-10), 103 (C-3), 99 (C-6), 95 (C-8), 79.4 (C-3"), 76 (C-2"), 74 (C-4"), 64 (C-5").

Apigenin7-*O*-α-D-apiofuranosyl-(1→2)-β-D-glucopyranoside (6)

Yellow powder, Rf: 0.45 (S_1), 0.48 (S_2).

UV/Vis λ_{max} (MeOH) nm: 266, 333, (NaOMe): 267, 300, 386, (NaOAc): 267, 354, 387, (NaOAc+H_3BO_3): 267, 341, ($AlCl_3$): 275, 300, 348, 382, ($AlCl_3$+HCl): 276, 300, 341, 380.

^{1}H NMR (500 MHz, DMSO-d_6): 7.94 (2H, d, J = 8.8 Hz, H-2', 6'), 6.89 (2H, d, J = 8.8 Hz, H-3',5'), 6.84 (1H, s, H-3), , 6.78 (1H, d, J = 2.4 Hz, H-8), 6.39 (1H, d, J = 2.4 Hz, H-6), 5.58 (1H, brs, H-1"), 5.26 (1H, d, J = 8.4 Hz, H-1'").

^{13}C NMR (100 MHz, DMSO-d_6): 182 (C-4), 164.3 (C-2), 164 (C-7), 160 (C-4'), 159 (C-5), 157 (C-9), 129 (C-2',6'), 122 (C-1'), 116.3 (C-3',5'), 109 (C-1'"), 104.5 (C-10), 103 (C-3), 100 (C-1"), 98 (C-6), 95 (C-8), 80.4 (C-3'"), 78.4 (C-5"), 78 (C-2'"), 76.6 (C-3"), 75.8 (C-2"), 75 (C-4'"), 72 (C-4"), 65 (C-5'"), 62 (C-6").

Genistein 8-C-glucopyranoside (7)

Yellow powder, Rf: 0.34 (S_1), 0.29 (S_2).

UV/Vis λ_{max} (MeOH) nm: 260, 336 (NaOMe): 276, 335, (NaOAc): 271, 338, (NaOAc+H_3BO_3): 262, 339, ($AlCl_3$): 272, 306, 371, ($AlCl_3$+HCl): 273, 307, 371.

^{1}H NMR (500 MHz, DMSO-d_6): 8.26 (1H, s, H-2), 7.4 (2H, d, J = 8.5 Hz, H-2',6'), 6.8 (1H, d, J = 8.5 Hz, H-3',5'), 6.11 (1H, s, H-6), 4.61 (1H, d, J = 10 Hz, H-1"), 4.0-3.1 (m, remaining sugar protons).

Natural products have been a particularly rich source of anti-infective agents. Flavonoids possess antimicrobial activity, and quercetin and other related compounds act essentially by enzyme inhibition of DNA gyrase (Cushnie and Lamb 2005).

This is the first report on the *in vitro* antimicrobial activity of the aqueous methanolic extract of the seeds of *P. dactylifera*. The screening of antimicrobial activities of the aqueous methanolic extract of the seeds of *P. dactylifera* were performed after Soxhlet extraction and compared with certain standard antibiotics (**Table 1**). These results indicate that the aqueous methanolic extract moderately inhibited all bacteria but was ineffective against tested yeast and fungi. Inhibition against bacterial strains by different extract concentrations is presented in **Table 2** and **Fig. 1**. The mean of the inhibition zones for all bacteria increased with an increase in concentration following the sequence 10 > 7.5 > 5 > 2.5 > 1%. *E. coli* showed the highest sensitivity against *P. dactylifera* aqueous methanolic extracts. Since the aqueous methanolic extract of the seeds used in this study produced inhibition zones against Gram+ and Gram- bacteria, the inhibitory activity could be attributed to the flavonoids present in the methanolic extract.

Table 1 The mean of inhibition zones (mm) of aqueous methanolic extract (100 µg) of *P. dactylifera* Seeds

Microorganism	Standard ± SEM[a]	Extract ± SEM[b]
Bacillus subtilis	20 ± 0.17	8 ± 0.29*
Staphylococcus aureus	29 ± 0.58	13 ± 0.50*
Escherichia coli	25 ± 0.07	14.5 ± 0.50*
Pseudomonas fluorescens	20 ± 0.07	7 ± 0.23*
Aspergillus niger	25 ± 0.16	0.0
Fusarium oxysporum	20 ± 0.08	0.0
Candida albicans	25 ± 0.16	0.0

[a] Standards: Ampicillin hydrate for bacteria; Clotrimazole for fungi
[b] Extract: aqueous methanolic extract (100 µg) of *P. dactylifera*
* Significant change from standard is denoted by $p < 0.05$ (Paired samples *t*-test)

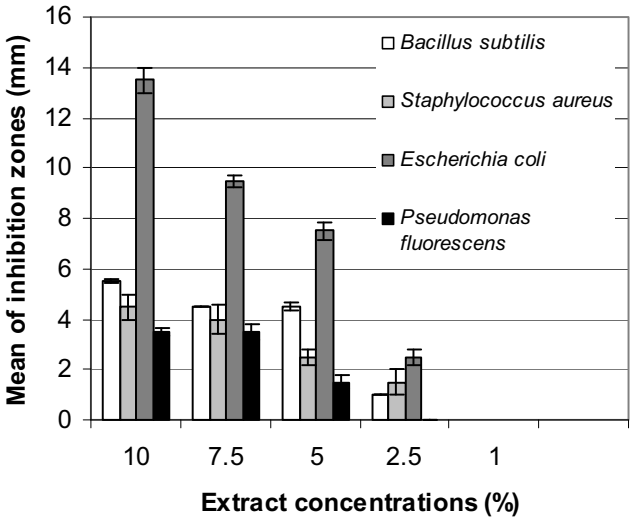

Fig. 1 The mean of inhibition zones (mm) of the methanolic extracts (100 µg) of *P. dactylifera* seeds at different concentrations (%).

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Table 2 The mean of inhibition zones (mm) of the methanolic extracts (100 µg) of *P. dactylifera* seeds at different concentrations (%).

Microorganism	Extract concentration (%)				
	10 ± SEM	7.5 ± SEM	5 ± SEM	2.5 ± SEM	1 ± SEM
Bacillus subtilis	5.5 ± 0.1*	4.5 ± 0.03*	4.5 ± 0.17*	1.0 ± 0.0*	0.0
Staphylococcus aureus	4.5 ± 0.5*	4.0 ± 0.58*	2.5 ± 0.29*	1.5 ± 0.5*	0.0
Escherichia coli	13.5 ± 0.5*	9.5 ± 0.23*	7.5 ± 0.34*	2.5 ± 0.29*	0.0
Pseudomonas fluorescens	3.5 ± 0.15*	3.5 ± 0.29*	1.5 ± 0.29*	0.0	0.0

* Significant change from standard is denoted by $p < 0.05$ (Paired samples *t*-test)

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