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Research Article

Phenolic composition and antibacterial activity of crude methanolic *Calendula officinalis* flower extract against plant pathogenic bacteria

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Abstract

Plant-derived compounds comprise diverse biological activities with different mechanisms of actions. The aim of this study was to comparatively evaluate antibacterial activities of methanol and aqueous flower and leaf extracts of *Calendula officinalis* (pot marigold) on plant-borne pathogens, with total phenolic contents and analysis of phenolics. Flower methanol and aqueous extracts had activity against 4 and 5 strains, respectively out of 11 phytopathogens tested. The highest activity was obtained with methanol extract of the flower against E. *amylovara* and C. *michiganensis* in all strains tested with 256 and 512 µg/mL minimum inhibitory concentrations, respectively. Total phenolic content of flower extracts were higher than leaf extracts, and the methanol extract of the flower had the highest total phenols among four extracts obtained. Two flower extracts with antibacterial activity were tested for phenolic content. Chlorogenic acid, caffeic acid, rutin, and salicylic acid were common in both methanol and aqueous flower extracts. Gallic acid was only present in methanol extract, whereas vanillic acid was present in the aqueous extract. The Higher antibacterial activity of the methanol extract may be correlated to the considerably higher relative rutin amount. Considering eco-safety and effectiveness, antibacterial activities of plant extracts would be important in phytopathogen control.

Keywords: Marigold, phytopathogenic bacteria, antibacterial activity, phenol content
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Calendula officinalis çiçek özütlerinin fenolik bileşenlerinin ve bitki kaynaklı patojen bakterilere karşı antibakteriyel etkilerinin incelenmesi

Özet

Bitki türevi bileşikler, çeşitli etki mekanizmaları ile biyolojik aktivite gösterebilirler. Bu çalışmanın amacı, Calendula officinalis (aynısafa) türüne ait bireylerin çiçek ve yapraklarından metanol ve su ile elde edilen özütlerin fenolik bileşen analizleri ile bitki kaynaklı patojenler üzerine antibakteriyel etkilerinin karşılaştırmalı olarak değerlendirilmesidir. Çiçeklerden metanol elde edilen özüt 11 test fitopatojenden 4'üne, su ile elde edilen özüt ise bu fitopatojenlerin 5'ine karşı antibakteriyel etki gösterdi. Çiçek metanol özütü 256 ve 512 µg/mL minimum inhibisyon konsantrasyonları ile tüm test suşlarından E. amylovara ve C. michiganensis suşları üzerinde en yüksek etkiye sahipti. Çiçek özütlerinin toplam

fenolik madde içeriği yaprak özütlerinden daha fazla idi. Çiçeklerden metanol ile elde edilen özüt, tüm özütler arasında en yüksek toplam fenolik madde içeriğine sahipti. Antibakteriyel etki gösteren her iki çiçek özütü de fenolik içerik açısından test edildi ve iki özütte de ortak klorojenik asit, kafeik asit, rutin ve salisilik asit tespit edildi. Test edilen fenolik bileşiklerden gallik asit sadece metanol özütünde bulunurken, vanilik asit ise sadece su özütünde belirlendi. Metanol ile elde edilen çiçek özütündeki rutin miktarının su ile elde edilen özüte göre çok daha fazla olduğu ve bu özütün yüksek antibakteriyel aktivitesinin rutin içeriği ile ilişkilendirilebilir. Ekolojik açıdan güvenli bitki özütlerinin antibakteriyel etkinliğinin saptanması fitopatojen kontrolü açısından önemlidir.

Anahtar Kelimeler: Kadife çiçeği, fitopatojenik bakteri, antibakteriyel aktivite, fenol bileşeni

Introduction

Calendula officinalis is commonly known as pot marigold, and it is widely cultivated outdoors in warm temperate regions of the world. It is an annual herb in the genus *Calendula* of the family Asteraceae.

In Europe folk medicine, the leaves are considered resolvent and diaphoretic while the flowers are used as a stimulant, antispasmodic and emmenagogue. In India, the flowers are used for treating wounds, herpes, ulcers, frostbite, skin damage, scars and blood purification (reviewed in Muley et al. 2009). Phytopharmacological studies of different *Calendula* extracts have shown anti-inflammatory properties (Della Logia et al. 1994; Akihisa et al. 1996), anti-viral activity, anti-HIV properties (Kalvatchev et al. 1997), anti-genotoxic properties (Perez-Carreon et al. 2002), antioxidant (Albulescu et al. 2004), cytotoxic effect on tumor cell lines *in vitro* and its anticancer efficacy *in vivo* (Boucaud-Maitre et al. 1988), and wound healing activity (Preethi and Kuttan, 2011). *Calendula* was also highly efficacious in clinical studies in the prevention of acute dermatitis in breast cancer patients undergoing irradiation (Pommier et al. 2004).

Screening of plants which are used as traditional remedies increases the chance of finding new medicinal plants and bioactive formulations. There is limited availability of bactericidal agents in plant disease control. We have previously, identified high antibacterial activity of *Urtica* spp. seed extracts on both food and plant pathogenic bacteria (Körpe et al. 2013), screened different parts of *Nasturtium officinale* for biological activity (Iseri et al. 2014), and determined cytotoxic and genotoxic potential of *Corchorus olitorius* extracts on human multiple myloma cell line (Iseri et al. 2013), In the light of our previous findings, we investigated antibacterial effects of flower and leaf extracts of *Calendula officinalis* against phytopathogenic bacteria in the present study. Aqueous and methanol extracts were also evaluated for their total phenolics and phenol content.

Materials and methods

Plant material and extraction

Calendula officinalis were cultured from commercial seeds (Vilmorin, France) in the Greenhouse of Institute of Transplantation and Gene Sciences, Baskent University (Kazan-Ankara, Turkey) in between May and September (2012). Flowers and leaves were collected, and dried at room temperature (dark). Dried flowers and leaves were powdered by using a coffee blender. Powder (10 g) was mixed with 100 mL of pure Methanol (MetOH; Merck, Darmstadt, Germany), and incubated for 24 h at room temperature (dark) with continuous shaking. For aqueous extraction, 5 g of dried material was mixed with 100 mL of distilled water (dw), and incubated for 1 h at 70°C water bath (dark) with shaking. Solutions were filtered (Whatman No. 40), and the filtrates were lyophilized by using a freeze-dryer at -50°C, 0.50hPa (LyoPro 3000, Thermo Scientific Heto, Allerod, Denmark). Extracts were stored at -20°C. Dry material was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) and dw for MetOH and aqueous extracts, respectively.

Antibacterial activities

Test strains

Standard and isolated strains of plant bacteria used to test antibacterial activities of the extracts are given in Table 1.

Table 1. Plant pathogenic strains tested

Plant-borne pathogens	**Strain**
Pseudomonas tomato	*Erd-Pst; L. esculentum* isolate; Erdemli, Mersin; Çukurova University, Department of Plant Protection
Pseudomonas corrugata	National Collection of Plant Pathogenic Bacteria (NCPPB) No. 2445
Pseudomonas viridiflava	NCPPB No. 1382
Xanthomonas vesicotoria	*Krs-Xav; C. annum* isolate; Karaisalı, Adana; Çukurova University, Department of Plant Protection
Xanthomonas perforans	NCPPB No.4321
Xanthomonas gardneri	NCPPB No.4323
Erwinia amylovara	Poz-Ea; *P. communis* L. isolate Pozantı, Adana; Çukurova University, Department of Plant Protection
Erwinia caratovora	*Khs-Ecc; L. esculentum* isolate; Kocahasanlı, Mersin; Çukurova University, Department of Plant Protection
Erwinia persicinus	NCPPB No.3774
Clavibacter michiganensis subsp. michiganensis	*Erd-Cmm; L. esculentum* isolate; Erdemli, Mersin; Çukurova University, Department of Plant Protection
Agrobacterium tumefaciens	NCPPB No. 2437:

Bacteria were obtained from National Collection of Plant Pathogenic Bacteria (NCPPB, UK), and Department of Plant Protection, Çukurova University, Adana, Turkey. In addition to the plant pathogenic strain, *Pseudomonas aeroginosa* (American Type Culture Collection 27853) was also tested for extract activity. Before the assays, cultures were incubated at 27°C for 48 h (except for the *P. aeroginosa* which was incubated 37°C for 24 h) in Mueller-Hinton (MH) broth.

Disc-diffusion assay

The stock solutions of extracts were diluted to a final concentration of 22.5 mg/mL with distilled water, and filter sterilized by 0.45 µm Millipore filters. Disc-diffusion method (Murray et al. 1995) was followed by spreading 100 µL of 0.5 McFarland standard turbid cell suspensions on MH agar. The discs of 6 mm in diameter were impregnated with 450 µg of extracts per disc (20 µL of 22.5 $mg.mL^{-1}$ stock extract solutions). The DMSO concentrations in the 22.5 mg/mL diluted extract solutions were below 10% (v/v) for all of the extracts. So, 10% (v/v) DMSO was tested as a solvent control. Ampicillin (Amp; 10 µg per disc), tetracycline (Tet; 30 µg per disc), and sulbactam+cefoperazone (Sul+Cef; 50+50 µg per disc) were used as positive reference standards. The plates were incubated at 27°C for 48 h. Antibacterial activity was evaluated by measuring the zone of inhibition (IZ) in mm for each strain. All assays were performed in quadrates.

Micro-well dilution assay

The minimal inhibition concentrations (MIC) were studied for the extracts and strains which had 7 mm ≤ IZ according to disc-diffusion assays. Two hundred microliters of extracts in MH broth were dispensed into the 3rd column of 96-well plates, and serial 100 µL 2-fold dilutions were performed (extract concentrations were tested in 8-4096 $\mu g.mL^{-1}$ range). Tetracycline (0.0625-32 $\mu g.mL^{-1}$ range) served as positive control for the assay. First two columns served as medium and cell

controls. Plates were inoculated with 100 µL of cell suspension per well containing 5 µL of 0.5 McFarland standard turbid fresh bacterial cultures. Plates were incubated at previously mentioned conditions, and optical density was measured at 600 nm with an ELISA reader (Biotek Instrument ELx800, VT, USA). The MIC was defined as the lowest concentration to inhibit 90% of bacterial growth. MIC was confirmed by inoculating 5 µL samples from clear wells on MH agar.

Analysis of total phenols (TP) in the extracts

The Folin-Ciocalteu method was used to assay total phenols (Folin and Ciocalteu 1927; Slinkard and Singleton 1977). Two microliters of sample (0.05 $g.mL^{-1}$), 50 µL Folin's Reagent (Sigma-Aldrich), and 300 µL 10% (w/v) sodium carbonate (Sigma-Aldrich) were sequentially added to 1 mL assay mixture, and the mixture was incubated at 40°C water bath for 30 min. The absorbance was measured at 765 nm, and the total phenols were represented as mg gallic acid (Sigma-Aldrich) equivalents (GAE) per g extract using gallic acid calibration curve (R^2>0.9). Assays were performed as triplicate experiments.

Qualitative Analysis of Phenolic Compounds

Qualitative analysis of the phenolic compounds in the flower extracts were determined by high performance liquid chromatography coupled with the mass spectrometer (HPLC-TOF-MS; Agilent Technologies 1260 Infinity LC, 6210 TOF-MS) at the Department of Chemistry, Çankırı University (Çankırı, Turkey). In brief, 10 µL 100 ppm samples were injected to ZORBAX SB-C18 column (4.6x100 mm, 3.5 µm) through a 0.45 µm filter at 35°C column temperature. Qualitative determination of the extracts was performed by mass to charge ratio ($m.z^{-1}$) and retention time analysis of the extract components in comparison with the standard phenolics (salicylic acid, quercetin, kafeoiltartarik acid, gentisic acid, protocatechuic acid, 4-Hydroxybenzoic acid, caffeic acid, vanillic acid, 4-hydroxybenzaldehyde, chcoric acid, p-coumaric acid, ferulic acid, apigenin 7-glucoside, rosmarinic acid, protocatechuic acid ethyl ester, cinnamic acid, naringenin, kamperol, gallic acid, chlorogenic acid, catechol, catechin, rutin, hesperidin, coumarin).

Results

Antibacterial activities

Flower methanol and water extracts had activity against 4 and 5 strains, respectively out of 11 phytopathogens tested (Table 2).

Leaf methanol extract had activity only against *P. aeroginosa*, and did not have antibacterial activity against pyhtopathogenic bacteria tested. Similarly, aqueous leaf extract was only effective against *X. gardneri* with high MIC (4096 $\mu g.mL^{-1}$ < MIC; Table 3).

The highest activity was obtained with methanol extract of the flower against *E. amylovara* and *C. michiganensis* in all strains tested. MIC of methanol extract of flower for *E. amylovara* and *C. michiganensis* growth were 256 and 512 $\mu g.mL^{-1}$, respectively (Table 3). Furthermore, both flower extracts were effective only on these strains in common, though the MICs for aqueous extracts were high indicating a lower antibacterial activity. DMSO had no effect on the strains tested at its solvent concentration. MIC of methanol flower extract was 1024 $\mu g.mL^{-1}$ for *X. vesicotoria.*

Total phenols of the extracts

Total phenols of the extracts were calculated regarding mg gallic acid equivalents per g extract. Total phenolic content of flower extracts were 17.6±2.3 and 12.3±0.1 mg $GAE.g^{-1}$ extract for methanol and water extractions, respectively. Total phenolic content of leaf extracts were lower than flower extracts (i.e. MetOH: 7.5±0.3 mg $GAE.g^{-1}$ extract, and dw: 9.6±0.7 mg $GAE.g^{-1}$ extract). Methanol extract of the flower had the highest total phenols among four extracts obtained.

Phenolics of the flower extracts

The phenolic content of the flower extracts is given in Table 4.

Chlorogenic acid, caffeic acid, rutin, and salicylic acid were common in both methanol and aqueous flower extracts. On the other hand, gallic acid was only present in methanol extract, whereas vanillic acid was present

in the aqueous extract. In addition, when chromatograms of the two extracts were overlapped and comparatively analyzed for 9-11 min acquisition time range (Fig. 1), three common peaks were determined. Considering retention times and $m.z^{-1}$ of the standards, the middle peak (Fig. 1) was determined as rutin with 9.9 min retention time and 609.1461 $m.z^{-1}$ (Table 1). Relative rutin amount in the methanol extract was considerably higher than the one found in the aqueous extract (Fig. 1).

Table 2. Results of the disc diffusion assay

	C. officinalis (IZ; mm)				Controls (IZ; mm)			
	Flower		Leaf		Amp (10 mg)	Tet (30 mg)	Sul+Cef (50+50 µg)	10% DMSO
	Met	dw	Met	dw				
P. aeroginosa	10.8	-	11.3	-	18.5	33.5	28.8	-
P. tomato	10.3	-	-	-	-	47	21.7	-
P. corrugata	-	-	-	-	-	37.3	17.8	-
P. viridiflava	-	7	-	-	-	34.3	12.5	-
X. vesicotoria	10	-	-	-	47.5	53	53	-
X. perforans	-	-	-	-	-	41.8	36	-
X. gardneri	-	7	-	8	8	45.8	39.3	-
E. amylovara	11.3	8	-	-	-	41.8	27.5	-
E. caratovora	-	-	-	-	-	45	22.8	-
E. persicinus	-	-	-	-	-	46.5	19	-
C. michiganensis	11.8	7.8	-	-	21.8	59.8	38.5	-
A. tumefaciens	-	7	-	-	-	32.3	19	-

Table 3. Results of the micro-well dilution assay. ND: MIC was not determined in the 8-4096 $\mu g.mL^{-1}$ extract concentration range.

	C. officinalis (MIC; µg.mL-1)				
	Flower		Leaf		Tet (MIC; µg.mL-1)
	Met	dw	Met	dw	
P. aeroginosa	ND	-	ND	-	64
P. tomato	ND	-	-	-	0.25
P. corrugata	-	-	-	-	2
P. viridiflava	-	ND	-	-	1
X. vesicotoria	1024	-	-	-	2
X. perforans	-	-	-	-	2
X. gardneri	-	ND	-	ND	0.5
E. amylovara	256	ND	-	-	1
E. caratovora	-	-	-	-	0.25
E. persicinus	-	-	-	-	<0.0625
C. michiganensis	512	ND	-	-	1
A. tumefaciens	-	ND	-	-	2

Table 4. Phenolics of the flower extracts

Flower extract	Retention time (min)	Compound	Molecular Formula	Mass/charge ($m.z^{-1}$)
MetOH	2.51	Gallic acid	$C_7H_6O_5$	169.0142
	6.27	Chlorogenic acid	$C_{16}H_{18}O_9$	353.0778
	7.60	Caffeic acid	$C_9H_8O_4$	179.0399
	9.92	Rutin	$C_{27}H_{30}O_{16}$	609.1461
	13.76	Salicylic acid	$C_7H_6O_5$	137.0244
dw	6.28	Chlorogenic acid	$C_{16}H_{18}O_9$	353.0778
	7.66	Caffeic acid	$C_9H_8O_4$	179.0399
	7.71	Vanillic acid	$C_8H_8O_4$	167.0350
	9.92	Rutin	$C_{27}H_{30}O_{16}$	609.1461
	13.74	Salicylic acid	$C_7H_6O_5$	137.0244

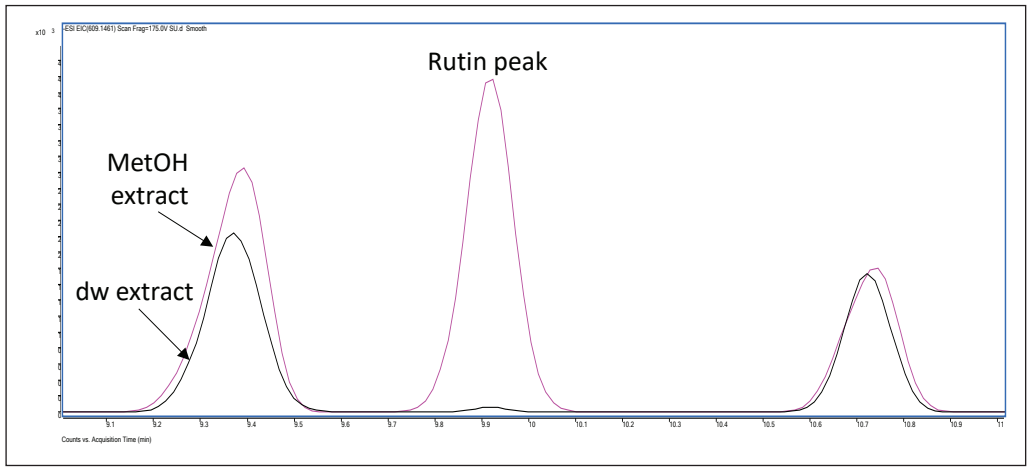

Figure 1 Chromatogram overlap of metanol (MetOH; pink) and aqueous (dw; black) flower extracts in 9-11 min acquisition time range.

Discussion

The control of bacterial diseases of crops is a considerable agricultural problem due to the limited availability of bactericidal agents. Use of antibiotic and copper compounds is very restricted in many countries, for either human and animal health or the environment (Lo Cantore et al. 2004). Secondly, phytopathogenic bacteria spread at long distances by contaminated and infected seeds. In addition, resistant populations of *E. amylovora*, *Pseudomonas* spp., and *Xanthomonas campestris* have been determined (McManus et al. 2002).

The plant originated antibacterial compounds can be a non-toxic eco-friendly alternative approach to plant disease management. Antibacterial activity of several plant parts such as fruits, vegetables, flowers, leaves, and roots have been identified so far. Aligiannis et al. (2001) have suggested a classification of antibacterial activity of plant extracts according

to MIC values; i.e. strong inhibition: MIC<500 μg.mL^{-1}, moderate inhibition: 600 μg.mL^{-1}<MIC<1500 μg.mL^{-1}, and low inhibition: 1600 μg.mL^{-1}<MIC. Flower extracts of *C. officinalis* had antibacterial activity against 7 phytopathogenic bacteria among 11 strains tested (Table 2). Methanol extracts of flowers yielded 10-12 mm inhibition zone by disc diffusion assays. We have determined minimum inhibitory concentrations of methanol flower extract against these bacteria (Table 3). Results of the micro-well dilution assay demonstrated that the extract had strong inhibition against *E. amylovara* and *C. michiganensis* with MIC values of 256 μg.mL^{-1} (strong inhibition) and 512 μg.mL^{-1} (moderate inhibition), respectively. Methanol extract of the flowers exerted moderate inhibition against *X. vesicotoria* with a MIC of 1024 μg.mL^{-1}. Though aqueous flower extract had antibacterial activity against 5 phytopathogenic bacteria tested, inhibition zones were very low i.e. IZ≤8. In concordance, we could not be able to detect MIC values in the concentrations ranges we studied (i.e. 4096 μg.mL^{-1} as the highest concentration). On the other hand, leaf extracts had no antibacterial activity except for the low activity of aqueous leaf extract against *X. gardneri*. Potential use of the plant derived extracts may be as seed protectants and disinfectants, particularly for the seed-borne and -transmitted bacterial diseases. *E. amylovora* (Gram negative) is the causal pathogen of fire blight which affects apples, pears, and some other members of the family *Rosaceae*. *C. michiganensis* (Gram positive) is the causal agent of bacterial canker disease of tomato. It causes considerable crop loss, and is persistent on seeds. *X. vesicatoria* (Gram negative) is the causal agent of bacterial spot disease, and has a wide range of hosts especially of Solanaceaous plants including tomato and pepper. According to a study of Kotan et al. (2010), *S. spicigera* essential oil, carvacrol and thymol were more effective than streptomycin sulfate according to seed disinfection assays conducted with *P. tomato*, *X. vesicotoria* and *C. michiganensis* infected tomato and pepper seeds. In addition, Balestra et al. (2009) performed *in vivo* antibacterial tests of *A. sativum* and *F. carica* extracts with *P. tomato*, *X. vesicotoria,* and *C. michiganensis* inoculated tomato plants and observed disease control at 15 days post-inoculation.

Phenols are found in all parts of plants such as leaves, fruits, seeds, roots and bark (Mathew and Abraham 2006). Total phenolic content of flower extracts were higher than leaf extracts, and methanol extract of the flower had the highest total phenols among four extracts obtained. High antimicrobial effect of phenol compounds has been discussed in terms their ability to alter microbial cell permeability, membrane function, and interaction with membrane proteins, causing deformation in structure and functionality, although the exact detailed mechanism of action of many of them is not clear yet (Tiwari et al. 2009). We qualitatively analyzed phenolics of the flower extracts with antibacterial activity. Flavonoids and phenolic acids are the most important groups of secondary metabolite bioactive compounds in plants (Kim et al. 2003). Chlorogenic acid, caffeic acid, rutin, salicylic acid, and gallic acid were present in the methanol extract of flowers (Table 4). Among these, chlorogenic acid, caffeic acid, salicylic acid, and rutin were common in both of the extracts, and vanillic acid was only present in the aqueous extract of the flowers. However, relative rutin amount was considerably higher in methanol extracts in comparison to aqueous extracts (Fig. 1). Rutin (quercetin rutinoside) is a glycoside of the flavonoid quercetin. Previously, it was shown that rutin selectively promoted topoisomerase IV-dependent DNA cleavage and induced the SOS response leading to growth inhibition of *E. coli* (Bernard et al. 1997). In fact, the antibacterial activity of various flavonoids has been studied so far, and mechanisms of actions were reviewed as inhibition of nucleic acid synthesis, cytoplasmic membrane function, and energy metabolism by Cushnie and Lamb (2005). Gallic acid, on the other hand, was only present in methanol extract of the flowers. Gallic acid is a simple phenolic acid possessing a single aromatic ring. Kang et al. (2008) reported that gallic acid inhibited the growth of oral pathogens and *S. mutans* biofilm formation. In another study, different extracts of *Caesalpinia mimosoides* Lamk.

were tested against human pathogenic bacteria and fungal strains, and the bioactive substance, responsible for the antimicrobial property was assigned as gallic acid by chromatographic and spectroscopic analyses (Chanwitheesuka et al. 2007).

Crude extracts from plants with a history of use in folk medicine have been screened *in vitro* for antibacterial activity (reviewed in Cushine and Lamb 2005). A variety of phytochemicals such as terpenoids, flavonoids, coumarins, quinones, volatile oil, carotenoids and others have been previously reported to be present in different parts of this plant (reviewed in Muley et al. 2009; in Khalid and da Silva 2012). Methanol extract of flowers has previously tested for antibacterial activity against anaerobic and facultative aerobic periodontal bacteria, and the results showed inhibition against all tested microorganisms with MIC < 2048 $\mu g.mL^{-1}$ (Iauk et al. 2003). Extracts of stems, roots, and leaves prepared with different solvents were also tested for antimicrobial activity against human pathogenic bacteria (reviewed in Khalid and da Silva 2012). However, to our knowledge, there is not any report on screening of the antibacterial activity of the marigold extracts against phytopathogenic bacteria. Low MIC results obtained with the extracts against *E. amylovara* and *C. michiganensis* are of particular importance, and have a potential for further research and application on seed disinfection.

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