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Antibacterial activity of monoacetylated alkyl gallates against *Xanthomonas citri* subsp. *citri*

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Keywords: Citrus canker, gallic acid, cell division, membrane disruption

Running title: Acetylated Alkyl Gallates target *X. citri* membrane

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22 **Abstract**

23 Asiatic Citrus Canker (ACC) is an incurable disease of citrus plants caused by the Gram-negative
24 bacterium *Xanthomonas citri* subsp. *citri* (*X. citri*). It affects all the commercially important citrus
25 varieties in the major orange producing areas around the world. Control of the pathogen requires recurrent
26 sprays of copper formulations that accumulate in soil and water reservoirs. Here, we describe the
27 improvement of the alkyl gallates, which are potent anti-*X. citri* compounds, intended to be used as
28 alternatives to copper in the control of ACC. Acetylation of alkyl gallates increased their lipophilicity,
29 which resulted in potentiation of the antibacterial activity. *X. citri* exposed to the acetylated compounds
30 exhibited increased cell length that is consistent with the disruption of the cell division apparatus. Finally,
31 we show that inhibition of cell division is an indirect effect that seemed to be caused by membrane
32 permeabilization, which is apparently the primary target of the acetylated alkyl gallates.
33

34 **Introduction**

35 *Xanthomonas citri* subsp. *citri* is the etiological agent of Asiatic Citrus Canker, a severe disease that
36 affects orange trees, and for which no healing process is known (Brunings and Gabriel 2003). The host
37 range of this pathogen consists of a wide diversity of *Citrus* spp. of economic importance around the
38 world. Symptomatic plants exhibit brownish eruptive lesions on their aerial parts, which may be
39 surrounded by chlorotic halos. Untreated infections may lead to premature fruit-drop, stem dieback and
40 defoliation, which is responsible for major economic losses to citriculture (Gottwald et al. 2002). *X. citri*
41 can be introduced to new areas by the movement of infected citrus fruits and seedlings. Upon infection,
42 the bacterium is rapidly disseminated by rainwater and wind passing over the surfaces of lesions and
43 splashing onto uninfected nearby trees (Bock et al. 2005; Gottwald et al. 2002).

44 The control of citrus canker in the major orange producer area in the world, the state of São
45 Paulo, Brazil, was satisfactorily achieved by the plant eradication program that took place between the
46 years 1999-2009 (Belasque Jr and Behlau 2011; Belasque Jr. et al. 2009). During that period,
47 symptomatic plants and the neighboring ones had to be eliminated to refrain the spread of the bacterium.
48 The drawback of eradication was the high cost of visual inspections, and the enormous number of plants
49 that had to be eliminated over the course of the years. Pressures from different sectors of the orange
50 producing chain culminated in the current scenario in which control is exerted by the plantation of less
51 susceptible cultivars of citrus, the use of wind-breaks to avoid bacterial lateral spreading by the combined

52 action of wind and rain, and the use of cupric formulations as bactericides. According to the current
53 legislation, the state of São Paulo was declared as an area of Risk Mitigation System from 2017, and the
54 control of citrus canker is now similar to what is already performed in the Southern states of Brazil
55 (Behlau et al. 2008).

56 Concerns have now been raised about the massive use of copper as the only bactericide to
57 control the spread of citrus canker. Copper sprays have to be applied repeatedly for effectiveness,
58 especially after a new leaf flush, thereby control by mitigation will increase the chemical residuals left on
59 fruits, soil, and water reservoirs. Copper can be bio-cumulative and it is a toxic metal (Brunetto et al.
60 2016; Cornu et al. 2017; Fones and Preston 2013). Besides, the emergence of bacterial strains resistant to
61 copper is a fact (Behlau et al. 2012; Canteros 1999). Altogether, citriculture requires new formulations as
62 alternatives to copper in order to combat bacterial and fungal infections.

63 Our research team is focused on the development of environmental friendly compounds able to
64 combat *X. citri*. We described the use of esters of gallic acid, the alkyl gallates, as potent cell division
65 inhibitors of *X. citri* (Król et al. 2015; Silva et al. 2013). Moreover, alkyl gallates were able to preclude
66 the ability of *X. citri* to infect citrus plants. Finally, alkyl gallates are safer than copper, and even exhibit
67 chemo-preventive action reducing the mutagenicity caused by agents that induce chromosomal damage
68 (e.g. compounds that generate Reactive Oxygen Species) (Silva et al. 2017). A downside of their
69 application in the field would be the possible broad anti-bacterial spectrum of the compounds, which may
70 be circumvented, at least in part, by the preparation of formulations able to attach specifically to citrus
71 leaves. In addition to this, compounds can be modified for increased potency, therefore minimizing the
72 dose necessary for effectiveness and the need for recurrent applications in the field.

73 One of the strategies used to modify and perhaps improve the action of lead compounds is the
74 optimization of physicochemical properties by the conversion of some of their functional groups. The
75 ester group is the main alternative to the carboxyl and hydroxyl polar groups, due to the increase of
76 lipophilicity and thus the biomembrane permeability (Beaumont et al. 2003; Rautio et al. 2008). Previous
77 studies performed by Sardi et al. (2017) demonstrated that an acetylated derivative of curcumin, a natural
78 polyphenolic compound, was more potent than its natural prototype against *Staphylococcus aureus*
79 strains, showing the importance of converting hydroxyl to ester groups for antibacterial activity. Here we
80 demonstrate that acetylation of some of the previously described alkyl gallates increased 100% their
81 potency against *X. citri*. Compounds stimulated morphological alterations in *X. citri*, which is consistent

82 with a disruption of the bacterial cell division process. However, our data support the view that the action
83 on division is indirect and a consequence of breakage of the cell transmembrane potential, which is
84 required for the correct assembly/positioning of the divisome.
85

86 **Materials and methods**

87 **Synthesis and ^{1}H NMR spectrum data of monoacetylated alkyl gallates**

88 Monoacetylated alkyl gallates were synthesized by the acetylation of alkyl gallates according to
89 Changtam et al. (2010) with minor modifications. First, alkyl gallates with side chains varying from five
90 to eight carbons were synthetized as described in Silva et al. (2013). Next, acetic anhydride (10 mL) was
91 added to the solutions containing the alkyl gallates (1 mmol) in pyridine (10 mL), mixtures were stirred at
92 100 °C for 7 days, and monitored by successive TLC analyses. When reactions were finished, residues
93 were poured into crushed ice. The resulting solutions were partitioned with ethyl acetate and the organic
94 phase dried at room temperature. The crude products were purified over silica gel column eluted with
95 mixtures of hexane and ethyl acetate, furnishing monoacetylated alkyl gallates (**8a−11a**). Compound
96 numbers were chosen to keep in line with our previous reports (Krol et al. 2015; Silva et al. 2013). NMR
97 spectra were recorded at 600 MHz for ^{1}H nucleus on a Bruker Avance III spectrometer at 25 °C.

98

99 **Bacterial strains and media**

100 The *Xanthomonas citri* subsp. *citri* used was the sequenced strain 306 (IBSBF-1594) (da Silva et al. 2002;
101 Schaad et al. 2006). *X. citri amy*::pPM2a-*zapA*, expressing GFP-ZapA (Martins et al. 2010), was used to
102 monitor the possible action of test compounds on the bacterial divisional septa. Cells were cultivated at 30
103 °C under rotation (200 rpm) in NYG/NYG agar medium (peptone 5 g/L, yeast extract 3 g/L and glycerol
104 20 g/L). Kanamycin and ampicillin were used at 20 μg/mL.

105

106 **Compound susceptibility test**

107 The antibacterial action of the acetylated alkyl gallates was measured by the resazurin microtiter assay
108 (REMA) described in Silva et al. (2013). Stock solutions of compounds at 10 mg/mL were prepared by

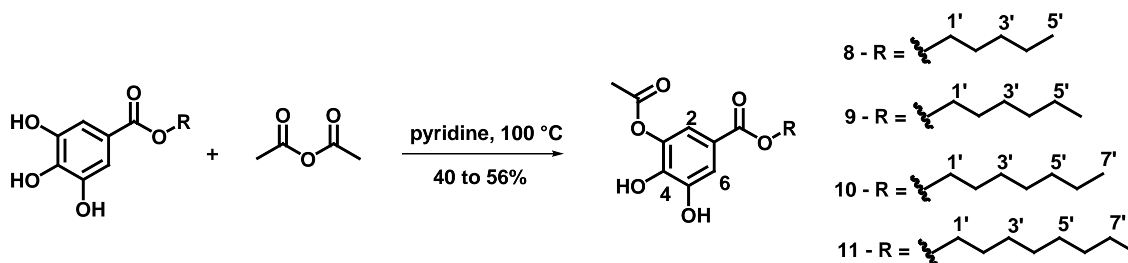
109 dissolving the acetylated alkyl gallates (dried-powder samples) in 100% dimethyl sulfoxide (DMSO;
110 SIGMA 276855). Test suspensions of acetylated alkyl gallates were prepared straight into 96-microtiter
111 wells by diluting the stock solutions with NYG medium using a two-fold serial dilution scheme. The
112 initial test concentration of a given compound was 100 µg/mL and 1% DMSO, and each well contained a
113 total volume of 100 µL. Cell inoculum was prepared by diluting an overnight culture of *X. citri* in NYG
114 medium to make a suspension at 10^7 CFU/mL. Ten microliters of this cell suspension was distributed into
115 the wells of the above-mentioned 96-microtiter plate so to give a final inoculum concentration of 10^5
116 cells/well. The negative control consisted of NYG medium and the bacterial inoculum. Kanamycin (20
117 µg/mL) and 1% DMSO were used as positive and vehicle control, respectively. After the tests assembly,
118 plates were incubated for 4 hours at 30 ºC. In order to develop the assay, 15 µL of a 0.01% resazurin
119 (SIGMA R7017) were added to each well followed by a further incubation period of 2 hours at 30 °C.
120 Viable cells were determined by their ability to reduce the blue resazurin dye to the pink fluorescent
121 compound resorufin, which was detected using a fluorescence scanner Synergy H1MFD (BioTek), with
122 excitation and emission wavelengths set to 530 and 590 nm, respectively. Three independent experiments
123 were conducted, and the data were used to construct plots of chemical concentration versus cell growth
124 inhibition in order to determine the MIC90 and MIC50 values (the concentration of a given compound
125 able to inhibit 90% and 50% of the cells in a culture, respectively). To investigate if the acetylated alkyl
126 gallates had bactericidal or bacteriostatic activities, we plated samples (~10 µL) of the cell suspensions
127 exposed to the compounds in REMA just before adding resazurin. Plating was done on solid NYG
128 medium containing ampicillin (20 µg/mL) using a 96-replica plater (8 X 12; SIGMA). Plates were
129 incubated at 30 °C for 48h, and experiments were performed in triplicates. The bacteriostatic action was
130 defined by the ability of a compound, at a specific concentration, to preclude bacterial respiration as
131 measured in the REMA assay, but cells can still grow after cultured in the absence of the compound. The
132 concentration of a given compound was considered bactericidal when bacterial growth was not observed
133 after plating on NYG-agar.

134

135 **Cell morphology and septum disruption analyses**

136 Overnight cultures of *X. citri* and the mutant strain *X. citri amy*::pPM2a-*zapA* were diluted 1:100 into
137 fresh NYG medium and cultivated at 30 ºC and 200 rpm until the OD600nm of ~ 0.7. One milliliter of
138 culture was treated with the compounds at MIC50 or 1% DMSO for 6 hours at 30 ºC. Cells were

139 immobilized on 1% agarose (0.9% NaCl)-covered slides and observed using a fluorescence microscope
140 BX-61 (Olympus) equipped with a monochromatic camera OrcaFlash 2.8 (Hamamatsu). Image
141 documentation and processing were conducted using the software Cell-Sens version 11 (Olympus).
142
143 **Membrane permeability assay**
144 Overnight cultures of *X. citri* were diluted 1:100 into fresh NYG medium and cultivated at 30 ºC and 200
145 rpm until the OD600nm of ~0.7. Approximately 1 mL of cell suspension was exposed to the compounds
146 at MIC50 or the vehicle control 1% DMSO for 60 minutes at 30 ºC. A positive control for membrane
147 permeability was performed using heat shock at 55 ºC for 2 min. Cell samples were concentrated by
148 centrifugation for 30 seconds at 11.000 x g and the pellets were dissolved in 70 µL of 0.9% NaCl. The
149 membrane integrity was assessed using the Live/Dead BacLight bacterial viability kit (Invitrogen)
150 according to the manufacturer's instructions. After treatment, cells were concentrated by centrifugation,
151 and the pellets were dissolved in 1 mL of 0.9 % NaCl prior to microscope observation.
152
153 **Data analyses**
154 Dose-response curves were generated using data from three independent REMA experiments. The
155 minimal inhibitory concentration (MIC) values were determined using the regression curves generated by
156 the best-fit method available in the software package GraphPad-Prism 6. Statistical analyses of cell length
157 were performed using one-way analysis of variance (ANOVA) followed by a Tukey posttest ($P < 0.05$).
158
159 **Results**
160 **Synthesis and ^{1}H NMR spectrum data of monoacetylated alkyl gallates**
161 The monoacetylated alkyl gallates carrying the alkyl radicals pentyl, hexyl, heptyl and octyl (compounds
162 **8a**, **9a**, **10a** and **11a**, respectively) were synthesized with yields ranging from 40 to 56 % (Scheme 1).



163
164

165 **Scheme 1.** Synthesis of monoacetylated alkyl gallates (**8a−11a**)

166 The signals that certify the achievements of **8a−11a** correspond to the singlet in 2.4 ppm, relative to the
167 hydrogens of the acetyl group and two doublets relating to hydrogens H-2 and H-6, which indicate loss of
168 chemical equivalence due to the insertion of the acetyl group. For all compounds, NMR parameters
169 corresponded with the proposed structures.

170 **Monoacetylated pentyl gallate (8a): pentyl 3-acetoxy-4,5-dihydroxybenzoate. White solid. 40 %**
171 **yield. ^{1}H NMR (600 MHz; $CDCl_3$) δ_H (mult.; *J* in Hz):** 7.55 (d; 1.8; H-2), 7.39 (d; 1.8; H-6), 4,29 (t;
172 6.0; H-1'), 2.40 (s; 3-OCOC**H**$_3$), 1.74 (m; H-2'), 1.41 to 1.30 (m; H-3' and H-4'), 0.90 (t; 7.2; H-5').

173 **Monoacetylated hexyl gallate (9a): hexyl 3-acetoxy-4,5-dihydroxybenzoate. White solid. 53 % yield.**
174 **^{1}H NMR (600 MHz; $CDCl_3$) δ_H (mult.; *J* in Hz):** 7.52 (d; 2.4; H-2), 7.40 (d; 2.4; H-6), 4.29 (t; 6.6; H-
175 1'), 2.41 (s; 3-OCOC**H**$_3$), 1.75 (m; H-2'), 1.44 to 1.32 (m, H-3' to H-5'), 0.92 (t; 7.2; H-7').

176 **Monoacetylated hepyl gallate (10a): heptyl 3-acetoxy-4,5-dihydroxybenzoate. White solid. 49 %**
177 **yield. ^{1}H NMR (600 MHz; $CDCl_3$) δ_H (mult.; *J* in Hz):** 7.50 (d; 1.8; H-2), 7.38 (d; 1.8; H-6), 4.28 (t;
178 6.6; H-1'), 2.40 (s; 3-OCOC**H**$_3$), 1.76 (m; H-2'), 1.44 to 1.30 (m; H-3' to H-6'), 0.91 (t; 6.6; H-7').

179 **Monoacetylated octyl gallate (11a): octyl 3-acetoxy-4,5-dihydroxybenzoate. White solid. 56 % yield.**
180 **^{1}H NMR (600 MHz; $CDCl_3$) δ_H (mult.; *J* in Hz):** 7.52 (d; 1.8; H-2), 7.40 (d; 1.8; H-6), 4.29 (t; 6.6; H-
181 1'), 2.41 (s; 3-OCOC**H**$_3$), 1.76 (m; H-2'), 1.44 to 1.31 (m; H-3' to H-7'), 0.91 (t; 7.2; H-8').

182

183 **Acetylated alkyl gallates inhibit growth of *X. citri***

184 The antibacterial potential of the acetylated alkyl gallates was evaluated using REMA, a method that
185 allows the measurement of the bacterial cell respiratory activity. All of the compounds tested exhibited
186 strong inhibition of *X. citri* with minimum inhibitory concentration (MIC) values ranging from

approximately 28 to 46 µg/mL, which are nearer to the value of the positive control kanamycin (20 µg/mL) (Table 1). The anti-*X. citri* activity of the monoacetylated alkyl gallates (**8a−11a**) indicated a correlation among MIC values and the length of alkyl side chains. Note that the MIC values decreased with the increase of the alkyl chain: pentyl (MIC 45.73 µg/mL) < hexyl (MIC 34.65 µg/mL) < heptyl (MIC 31.97 µg/mL) < octyl (MIC 27.92 µg/mL). The same correlation was observed in the activity of the non-acetylated alkyl gallates (**8−11**) (Silva et al. 2013). However, when we compare the MIC values of the non-acetylated alkyl gallates (**8, 9, 10** and **11** exhibited MICs of ~60 µg/mL) with the acetylated alkyl gallates (~28 - 46 µg/mL) we detect a clear increase of potency. Here, the antibacterial activities of the acetylated compounds increased 36% for **8a**, 80% for **9a**, 95% for **10a**, and 123% for **11a** (Table 1).

As lipophilicity is a central parameter for the development of novel bioactive compounds we determined the theoretical lipophilicity (C log P) of the acetylated alkyl gallates (Table 1). As expected, the acetylation of alkyl gallates led to an increase of lipophilicity in the order of ~22%. The non-acetylated alkyl gallates **8-11** (Silva et al. 2013) displayed C log P values ranging from 2.53 to 3.72, while the acetylated forms **8a-11a** started with a C log P value of 3.12 and ended in 4.51 (Table 1). Taken together, our results indicate that the increase in lipophilicity induced by the esterification of hydroxyl groups resulted in increased potency of the acetylated derivatives against *X. citri*.

Finally, we determined the minimal bactericidal concentration (MBC) of the acetylated alkyl gallates. The MBC is defined as the lowest concentration capable of inhibiting growth of 99.99% of the bacterial inoculum (NCCLS 2003). *X. citri* was exposed to a concentration range of the compounds varying from 12.5 to 100 µg/mL following the same procedure used in REMA. After treatment, cell suspensions were plated on NYG-agar and incubated for up to 48h to score for colony development. In our evaluation, compound **8a** had only bacteriostatic action, where the highest dose used (100 µg/mL) was not enough to prevent cell growth on plate (Table 2). Note that the dose of 100 µg/mL is twice as much as the dose that led to a growth halt in REMA (45.73 µg/mL; Table 1). For the three remaining compounds (**9a**, **10a**, and **11a**), the bactericidal dose was related to the size of carbon side chain (Table 2). Compound **9a,** with the shortest carbon side chain of the three, exhibited a MBC between 50-100 µg/mL (the exact concentration was not determined); the concentration of 50 µg/mL for compound **9a** was therefore considered bacteriostatic. Compounds **10a** and **11a** displayed MBC values in the ranges of 25-50 µg/mL and 12.5-25 µg/mL, respectively, with the concentrations of 25 µg/mL and 12.5 µg/mL being considered bacteriostatic (Table 2).

217

218 **Acetylated alkyl gallates induce morphological changes in *X. citri***

219 In our previous work with the non-acetylated versions of the alkyl gallates, we showed that these
220 compounds induce filamentation in *B. subtilis* and increased cell size in *X. citri*, which may reflect
221 interference with the bacterial cell division process (Król et al. 2015; Silva et al. 2013). To investigate if
222 the acetylated alkyl gallates had the same mechanism of action, we examined the morphology of *X. citri*
223 exposed to these compounds. Wild-type cells of *X. citri* were exposed to the compounds for 6 hours, and
224 after analyzed under the microscope. First, the average cell length determined for the untreated cells was
225 1.7 ± 0.36 μm (Table 3). Treatment with compounds **10a** and **11a** led to a significant increase of cell size,
226 which now reached 1.94 ± 0.39 and 2.07 ± 0.40 μm, respectively. The average cell length in cultures
227 exposed to the compounds **8a** and **9a** did not differ significantly from the control (untreated). Overall, the
228 acetylated alkyl gallates of longer carbon side chains (compounds **10a** and **11a**) induced morphological
229 alterations in *X. citri.*

230

231 **Septum disruption in *X. citri***

232 The cell elongation phenotype induced in *B. subtilis* and *X. citri* by the alkyl gallates **8-11** was in part
233 explained by the direct interaction of these compounds with the cell division protein FtsZ (Król et al.
234 2015; Silva et al. 2013). In order to evaluate if the acetylated derivatives could target the divisome as
235 well, we monitored the integrity of the divisional septum of *X. citri* treated with the compounds. This was
236 possible by the use of a *X. citri* mutant strain (*X. citri amy*::pPM2a-*zapA*; (Martins et al. 2010))
237 expressing the FtsZ accessory protein ZapA, as GFP-ZapA, which labels the Z-ring. A normal septum can
238 be observed in dividing cells of *X. citri amy*::pPM2a-*zapA* as a fluorescent bar perpendicular to the long
239 axis of the rod (Fig. 1A; white arrow). Treatment with the vehicle DMSO did not interfere with the Z-ring
240 (Fig. 1B). However, the exposure of the cells to the compounds **10a** and **11a** for 10 min at MIC50
241 dissolved the septa and the GFP-ZapA fluorescence is now scattered within the rods (Fig. 1C, 1D). The
242 compounds **8a** and **9a** had no noticeable effect on the septa, which displayed a normal microscopic
243 pattern (data not shown). Taken together, results indicate that the acetylation of compounds **10a** and **11a**
244 kept their ability to disrupt the Z-ring of *X. citri*; however, the acetylation of compounds **8a** and **9a**

245 apparently abolished this property that was observed before in *B. subtilis* and *X. citri* (Król et al. 2015;
246 Silva et al. 2013).

247

248 **Membrane integrity is affected by acetylated alkyl gallates**

249 After observing that compounds **10a** and **11a** could disrupt the divisional septum of *X. citri*, we wondered
250 if they were capable of targeting FtsZ directly. To evaluate for that we monitored if compounds **10a** and
251 **11a** could interfere with the polymerization/associated GTPase activity of FtsZ. Purified *B. subtilis* FtsZ
252 was combined with various concentrations of the compounds in a pre-polymerization buffer (without
253 nucleotides), and the reaction was initiated by adding 1 mM GTP. The GTP hydrolysis rate was
254 determined by the generation of Pi as described in Król et al. (2015). Surprisingly, we did not observe any
255 effect on the GTPase activity of purified *B. subtilis* FtsZ in the presence of compounds **10a** and **11a** (data
256 not shown), which raised the possibility that the compounds may perturb the divisome indirectly.

257 It has been demonstrated that disruption of the membrane potential interferes with the
258 localization of proteins like FtsZ (Strahl and Hamoen 2010). Moreover, we reported recently that alkyl
259 gallates **10** and **11** target both purified FtsZ and the bacterial membrane of *B. subtilis* (Król et al. 2015).
260 To verify if the acetylated alkyl gallates **10a** and **11a** could produce similar effects, we monitored the *X.*
261 *citri* membrane integrity using the nucleic acid dyes SYTO 9 and Propidium Iodide (PI). PI penetrates the
262 cells with damaged membranes as can be seen after a heat shock treatment (HS; Fig. 2). Healthy
263 membranes are not permeable to PI, and in general, normally growing *X. citri* will have ~5% of cells with
264 compromised membranes (NC; Fig. 2). Exposure to the vehicle DMSO at 1% did not alter this pattern.
265 However, treatment with the four acetylated compounds **8a-11a** at MIC50 led to significant increases of
266 permeability (Fig. 2). Noteworthy, the extent of membrane damage seems correlated to either the
267 lipophilicity or the size of the carbon chain of these compounds. The longer the side chain the worse the
268 effect on *X. citri* membrane. Taken together, data support that the acetylated alkyl gallates may act
269 indirectly on the divisome via disruption of membrane integrity.

270

271 **Discussion**

272 Esters of gallic acid (the alkyl gallates) are potent growth inhibitors of *X. citri* and *Bacillus subtilis*,
273 displaying as mechanism of action a combined activity against the divisome and the bacterial membrane
274 (Król et al. 2015; Silva et al. 2013). Upon treatment with these compounds *X. citri* lost the ability to
275 colonize the host citrus and to produce disease symptoms (Silva et al. 2013). Here, we designed and
276 synthesized four alkyl gallate derivatives (the acetylated alkyl gallates **8a**–**11a**) identified as new
277 chemical entities, which also inhibited the phytopathogen *X. citri*, but exhibited greater potency than
278 their prototypes. Our data are in line with reports from other groups showing that acetylated
279 derivatives can improve potency when compared to their starting compounds (Biasutto et al. 2007;
280 Sardi et al. 2017; Vlachogianni et al. 2015). The conversion of functional groups, known as drug
281 latentiation, results in increased lipophilicity that may be related to greater capacity of penetration
282 into biomembranes (Ettmayer et al. 2004; Han and Amidon 2000). One of the reasons why we chose
283 to design the monoacetylated alkyl gallates instead of di- or tri-acetylated derivatives is associated to
284 the appropriate balance between lipophilicity and hydrophilicity. This balance ensures high
285 permeability through biological membranes and solubility in aqueous medium, which are determinant
286 factors for the success of bioactive compounds (Dahan et al. 2016; Lipinski 2000). In addition,
287 compounds with higher lipophilicity are correlated to the high environmental toxicity of some
288 pesticides, which is due to their tendency to be accumulated in plants and animals (Zhang et al. 2016).
289 Finally, the immediate advantage of the acetylated alkyl gallates would be the lower effective dose to be
290 used for bacterial control and a lower environmental impact in agriculture. We are developing
291 formulations containing gallates as alternatives to copper that is the bactericide currently in use to refrain
292 the spread of the bacterium in the orchards (Behlau et al. 2010).

293 Copper formulations have long been utilized in citriculture, as well as in other cultures, to
294 protect them against bacterial and fungal infections (Fones and Preston 2013; Leite Jr and Mohan 1990).
295 Despite its efficacy, several environmental toxicity problems can be associated to the excessive use of
296 copper as a crop defensive, which soon may call for a ban of its use worldwide (reviewed by Fones and
297 Preston in 2013). It is worth mentioning, it was reported that copper induces viable but nonculturable
298 state (VBNC) in *X. citri*, which consequently will lead to reduced protection irrespective of the dose and
299 frequency of copper application (del Campo et al. 2009). Another outcome of the long-term exposure to
300 this metal is the already documented emergence of copper-resistant strains of *X. citri* (Behlau et al. 2012;
301 Canteros 1999). So far, we were not able to isolate strains of *X. citri* resistant to the gallates in laboratory

302 controlled culture (data not shown), and this may be related to the fact that alkyl gallates have a suggested
303 multi-target mechanism of action (Król et al. 2015). Finally, the non-acetylated alkyl gallates were
304 evaluated in a set of *in vitro* experiments as non-cytotoxic, and non-genotoxic/mutagenic compounds;
305 moreover, they exhibit a desirable chemopreventive action being able to protect cells against chemically
306 induced chromosomal damage (Silva et al. 2017). These observations make the gallates a safer alternative
307 to copper to be adapted in citriculture.

308 The reported action of the gallates on the bacterial divisome was attributed to the inhibition of
309 the FtsZ function (Król et al. 2015). FtsZ is the bacterial tubulin that assembles into protofilaments and
310 organizes a ring-like structure (the Z-ring; divisional septum) in the middle of the cells to orchestrate the
311 recruitment of all the proteins necessary for cytokinesis and cell wall remodeling/synthesis (reviewed by
312 Erickson et al. in 2010). Conservation within the domain Bacteria and a rather dissimilarity with
313 eukaryotic tubulins make of FtsZ an interesting target for antimicrobials. Several of the compounds that
314 target FtsZ do so by inhibiting its GTPase activity, which consequently over-stabilizes the FtsZ
315 protofilaments and break its assembly/disassembly dynamics needed for proper cell division function
316 (Hurley et al. 2016). We showed previously that alkyl gallates **8-11** inhibited the GTPase activity of *B.*
317 *subtilis* FtsZ (Król et al. 2015). Although, strong binding to FtsZ was observed only for compounds **10**
318 and **11** (Kd values of 0.08 and 0.84 μM, respectively), while binding of **8** and **9** seems aspecific.
319 Consistent with our previous data, the derivatives **10a** and **11a** induced morphological alterations in *X.*
320 *citri*, documented as increased cell length, as well as disruption of the divisional septa (Fig. 1C, 1D).
321 However, **10a** and **11a** lost the ability to interact with FtsZ, since these compounds no longer inhibited the
322 GTPase activity of purified *B. subtilis* FtsZ. One possibility raised to explain how **10a** and **11a** dissolved
323 the bacterial septum was because they kept their ability to act on membranes (Fig. 2) (Król et al. 2015).
324 The increased lipophilicity of **10a** and **11a** may explain, in part, the higher potency if compared to the
325 prototypes, and their ability and/or preference to attack the bacterial membrane. Disruption of the cell
326 transmembrane potential, e.g. by membrane permeabilization, interferes with the localization of protein
327 factors necessary for the Z-ring mid-cell assembly (Strahl and Hamoen 2010). Although **8a** and **9a** did not
328 alter cell morphology and septum assembly, they kept the capacity to act on membranes, which probably
329 confer to these compounds a marginal/undetectable effect. Therefore, our data suggest that the increase of
330 lipophilicity in monoacetyl derivatives of alkyl gallates enhanced their anti-*X. citri* activity while

331 maintaining their ability to act on membranes. However, the conversion of a hydroxyl to an acetyl group
332 resulted in loss of the ability to interact with FtsZ.

333

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341

342 **Figures legends**

343 **Fig. 1. Acetylated alkyl gallates disrupt the divisional septum of *X. citri*.** The mutant strain *X. citri*
344 *amy*::pPM2a-*zapA*, expressing GFP-ZapA, was cultivated until the O.D.600nm of ~0.3, and then
345 subjected to the acetylated alkyl gallates at MIC50 for 10 min prior to microscope observation. A)
346 Untreated; B) cells exposed to 1% DMSO; C) **10a**, and D) **11a**. The divisional septum is marked with
347 white arrows in A and B. DIC: Differential Interference Contrast microscopy. Scale bars correspond to
348 2µm; magnification 100X.

349 **Fig. 2. Membrane integrity is affected by the acetylated alkyl gallates.** Cells of *X. citri* were incubated
350 for 1h with 1% DMSO and the compounds at MIC50. Following this period, membrane integrity was
351 assessed using the Live/Dead kit. NC, untreated; HS, Heat Shock for 2 minutes at 55 ºC (positive control
352 for membrane permeabilization); **8a, 9a, 10a**, and **11a** compounds as in Table 1. This experiment was
353 performed twice with n=250 of cells scored per treatment. Bars represent the average values for the
354 combined experiments/treatments; vertical lines indicate the standard deviation.

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