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Membrane-Interactive Compounds from *Pistacia lentiscus* L. Thwart *Pseudomonas aeruginosa* Virulence

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Running Title: *Pistacia lentiscus* L. fruit prevents *Pseudomonas aeruginosa* virulence

30 **ABSTRACT**

31 *Pseudomonas aeruginosa* is capable to deploy a collection of virulence factors that are not

32 only essential for host infection and persistence, but also to escape from the host immune

33 system and to become more resistant to drug therapies. Thus, developing anti-virulence agents

34 that may directly counteract with specific virulence factors or disturb higher regulatory

35 pathways controlling the production of virulence armories are urgently needed. In this regard,

36 this study reports that *Pistacia lentiscus* L. fruit cyclohexane extract (PLFE1) thwarts *P.*

37 *aeruginosa* virulence by targeting mainly the pyocyanin pigment production by interfering with

38 4-hydroxy-2-alkylquinolines molecules production. Importantly, the anti-virulence activity of

39 PLFE1 appears to be associated with membrane homeostasis alteration through the

40 modulation of SigX, an extracytoplasmic function sigma factor involved in cell wall stress

41 response. A thorough chemical analysis of PLFE1 allowed us to identify the ginkgolic acid

42 (C17:1) and hydroginkgolic acid (C15:0) as the main bioactive membrane-interactive

43 compounds responsible for the observed increased membrane stiffness and anti-virulence

44 activity against *P. aeruginosa*. This study delivers a promising perspective for the potential

45 future use of PLFE1 or ginkgolic acid molecules as an adjuvant therapy to fight against *P.*

46 *aeruginosa* infections.

47

INTRODUCTION

Bacterial infections still constitute a serious public health threat even though their prevention and treatment have been improved over the last decades. The effects of common antibiotics are no longer effective against microbial threats including *Enterococcus faecium*, *Staphylococcus aureus*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species, also known as the "ESKAPE" pathogens group.[1] In a recent report published by the World Health Organization (WHO), *P. aeruginosa* was categorized as one of the "critical priority pathogens" for which there is an urgent need for the discovery of alternative and innovative new therapies.[2]

P. aeruginosa is predominantly responsible for different life-threatening infections in humans, including the respiratory system, burn and wound, urinary tract as well as medical implant devices.[3] This notorious multidrug resistant opportunistic Gram-negative bacterium deploys a wide variety of virulence factors and host-degrading enzymes as well as multiple secondary metabolites.[4] Pyocyanin is an important virulence factor produced and secreted abundantly by nearly 95% of *P. aeruginosa* isolates.[5] This phenazine-derived pigment, blue-green in color, confers a greenish hue to the sputum of cystic fibrosis (CF) individuals suffering *P. aeruginosa* chronic lung infection.[6] Moreover, pyocyanin is a highly diffusible redox-active secondary metabolite which plays an important role in several physiological processes[6,7] making it a good target. Therefore, pyocyanin production hindrance may have consequences regarding the cytotoxic effects and the full virulence of *P. aeruginosa* during infections related to airways in CF.

4

71 The sophisticated quorum sensing (QS) circuitry in *P. aeruginosa* strongly controls the

72 biosynthesis of pyocyanin. This process starts with the synthesis of the *N*-acyl-L-homoserine

73 lactone (AHL) type signal molecules followed by the *Pseudomonas* quinolone signaling (PQS).

74 Next, PQS regulates the expression of *phzA-G* operons resulting in the production of

75 phenazine-1-carboxylic acid (PCA) which is then modified to produce predominantly pyocyanin

76 *via* the action of the enzymes encoded by *phzM and phzS*.[8] In addition, pyocyanin biosynthesis

77 and regulation have been also linked to SigX, an extracytoplasmic function sigma factor

78 (ECFσ) that plays an essential role in the cell wall stress response network.[9–12] In *P.*

79 *aeruginosa*, the ECFσ SigX is a global regulator that modulates the expression of more than

80 300 genes, including several genes involved in pyocyanin biosynthetic pathways.[9,11,13] More

81 importantly, SigX has been reported to be involved in the regulation of fatty acid biosynthesis,

82 which triggers changes in cell membrane phospholipid composition that affect membrane

83 fluidity and envelope integrity.[11,14–16] SigX is also involved in physiological processes as

84 important as iron uptake, virulence, motility, attachment, biofilm formation, and antibiotic

85 resistance and susceptibility *via* direct and/or indirect governance.[9–11,17]

86

87 In view of this, developing anti-virulence agents that may precisely hinder the production,

88 secretion, or function of virulence determinants or interfere with their regulation has emerged

89 as an alternative promising strategy to fight against bacterial pathogens.[18,19] Plants have long

90 been used in traditional medicine to prevent or treat infectious diseases in many countries.[20]

91 Hydrophilic and lipophilic extracts from plants were reported to contain abundant and diverse

92 range of bioactive compounds with anti-virulence properties.[19,21,22] *Pistacia lentiscus* L., a plant

93 commonly known as mastic tree or lentisc, is an evergreen shrub of the family of

94 *Anacardiaceae* widespread all around the Mediterranean area where it grows wild in a variety

95 of ecosystems.[23,24] Medicinal uses of the fruit, galls, resin, and leaves of *P. lentiscus* L. are

96 described since antiquity. However, they may differ either in the therapeutic indication or in the

97 plant part used for medicinal purpose, depending of the geographical area.[25,26]

98

99 Currently, there are no research, which have explored the anti-virulence potential of *P.*

100 *lentiscus* L. fruit, or the molecular mechanism of action, which underpins its major bioactive

101 compounds against *P. aeruginosa*. Herein, we report that *P. lentiscus* L. fruit cyclohexane

102 extract (PLFE1) can function as a potent pyocyanin inhibitor while being devoid of any

103 antibacterial activity as judged by cell growth and viability assays. We show that PLFE1

104 interferes with 4-hydroxy-2-alkylquinolines molecules production, which might explain its anti-

105 pyocyanin activity. We also demonstrate that PLFE1 is able to increase significantly membrane

106 stiffness in *P. aeruginosa*. Interestingly, the cell wall stress response ECFσ SigX is found to be

107 a key regulatory element in this phenomenon, which might respond to the presence of

108 envelope-interactive compounds in PLFE1. Furthermore, comprehensive chemical analyses of

109 PLFE1 and its derived fractions allowed us to identify the ginkgolic acid (C17:1) and

110 hydroginkgolic acid (C15:0) as the major bioactive compounds and we confirmed that they are

111 responsible of the main anti-virulence activity against the pathogen *P. aeruginosa*.

112

RESULTS

113

Pyocyanin production inhibition by *P. lentiscus* L. fruit extracts

114

115 Planktonic cultures of wild-type *P. aeruginosa* H103 were exposed to cyclohexane, ethyl

116 acetate, methanol, and water *P. lentiscus* L. fruit extracts at concentrations of 100, 50, 25, 12.5,

117 6.25, 3.12, 1.6 and 0.8 µg mL^{-1} and assayed for interference with pyocyanin production (Fig.

118 1A). Pyocyanin levels from H103 strain cultures untreated with *P. lentiscus* L. fruit extracts and

119 treated with 1% v/v DMSO were also measured as negative control and defined as 100%

120   pyocyanin production. The PLFE1 (cyclohexane extract) showed statistically significant

121   inhibition of pyocyanin biosynthesis at the all concentration tested. The PLFE1 at 100 µg mL^{-1}

122   concentration caused the maximum percent reduction in pyocyanin production (82%;

123    $P<0.0001$) over the untreated control with an IC$_{50}$ value of 4.9 µg mL^{-1}. In addition, PLFE1

124   inhibitory activity was shown to be dose-dependent. The PLFE2 (ethyl acetate extract) was

125   also capable of inhibiting pyocyanin biosynthesis following the same pattern as observed for

126   PLFE1 but not in a drastic manner where approximately 50% of inhibition of this pigment

127   production was observed at 100 µg mL^{-1} ($P<0.001$). There was no significant or slight variation

128   in pyocyanin production by H103 strain when exposed to PLFE3 (methanol extract) or PLFE4

129   (water extract). Of all the *P. lentiscus* L. fruit extracts tested for pyocyanin inhibition, PLFE1

130   cyclohexane extract showed remarkably better inhibitory activity and was therefore selected

131   for all further sets of experiments. Further, the inhibitory activity of PLFE1 on phenazine

132   biosynthetic pathway was investigated at the level of gene expression. In the presence of

133   PLFE1 at 100 µg mL^{-1}, RT-qPCR analyses results showed significant down-regulation of the

134   *phzA* gene involved in the production of phenazine-1-carboxylic acid (PCA), *phzM*, and *phzS*

135   which converts PCA into the derivative pyocyanin (Fig. 1B). However, the expression levels of

136   the *phzH* gene were not significantly different in H103 strain treated with PLFE1 as compared

137   to untreated H103 (Fig. 1B). In summary, the repression of the expression levels of genes

138   involved in phenazine biosynthesis correlate with pyocyanin production inhibition indicating that

139   PLFE1 has the ability to function as a strong inhibitor of the pyocyanin virulence factor in *P.*

140   *aeruginosa* H103 strain.

141

P. aeruginosa virulence attenuation by PLFE1

143   We next sought to evaluate anti-virulence effect of PLFE1 on *P. aeruginosa* strain H103 using

144   human lung A549 cells and *Caenorhabditis elegans* infection models. As depicted in Fig. 2A,

145   the results showed lower LDH release (20%; $P \leq 0.05$) in A549 cells after their infection with

146   H103 treated with PLFE1 (100 µg mL^{-1}) for 20h incubation. A549 cells infected with non-treated

147   strain H103 were used as a control. This result revealed that PLFE1 exhibited significant *P.*

148   *aeruginosa* anti-virulence effect in human lung A549 cells. In addition, the impact of PLFE1 on

149   *in vivo* virulence of *P. aeruginosa* strain H103 was assessed using a *C. elegans* fast-killing

150   infection assay. When *C. elegans* worms were placed on a lawn of H103 strain, the percentage

151   of nematodes survival drastically decreased (65%; $P<0.0001$) after 24h incubation as

152   compared to *C. elegans* fed with *E. coli* OP50 (Fig. 2B). Interestingly, the presence of PLFE1

153   (100 µg mL^{-1}) significantly protected *C. elegans* from killing by *P. aeruginosa* (27%; $P<0.0001$)

154   as compared to *C. elegans* when applied to lawns of untreated H103 (Fig. 2B). Altogether,

155   these data indicate that PLFE1 attenuated the virulence of *P. aeruginosa* strain H103 in human

156   lung A549 cells and *C. elegans* infection models.

157

158   **Absence of effect of PLFE1 on *P. aeruginosa* growth and cell viability**

159   The impact of PLFE1 (cyclohexane extract) on the cell growth of *P. aeruginosa* H103 was

160   monitored at 37 ºC over the course of 24 h. None of the concentrations assayed (100, 50, 25,

161   12.5, 6.25, 3.12, 1.6 and 0.8 µg mL^{-1}) had an impact on planktonic cell growth of H103 strain

162   as compared to the untreated culture (H103 strain grown in presence of 1 % v/v DMSO)

163   (Supplementary Fig. S1A). Moreover, the effect of PFLE1 on H103 cell viability was evaluated

164   by flow cytometry using *Bac*Light live/dead stain. Similarly, the results showed that at all the

165   concentrations tested, PLFE1 did not affect the cell viability according to normalized events

166   counting of live, injured, and dead cells when compared to the control condition

167   (Supplementary Table S1 and Fig. S1B). Based on these results, it can be concluded that the

168   inhibition of pyocyanin pigment production by *P. lentiscus* L. fruit extract (PLFE1) was achieved

169   without affecting the growth of the bacteria and cell viability, at least *in vitro*.

170

171 **PLFE1 modulates the production of 4-hydroxy-2-alkylquinolines (HAQs)**

172 **molecules**

173 We next sought to assess whether the inhibition of pyocyanin production was directly due to

174 PLFE1 on Pqs QS system that is known to tightly regulate pyocyanin biosynthesis. *P.*

175 *aeruginosa* H103 cultures were exposed to different concentrations of PLFE1 (100, 50, 25,

176 12.5, 6.25, 3.12, 1.6 and 0.8 µg mL^{-1}) and then HAQs molecules were extracted twice by ethyl

177 acetate. The production of HAQs was determined using a PAO1 ΔpqsA CTX-*pqsA::lux*

178 biosensor strain which does not produce HAQs molecules and shows response to exogenous

179 HAQs. The H103 cultures treated with 100, 50, and 25 µg mL^{-1} of PLFE1 showed significant

180 reduced HAQs production (Fig. 3A). Additionally, we measured the expression levels of *pqsA*,

181 *pqsH*, *pqsL*, and *pqsR* genes representative of Pqs QS system by using RT-qPCR. The assays

182 were performed after 24h of growth of H103 untreated or treated with 100, 25, and 6.25 µg mL^{-1}

183 PLFE1 (Fig. 3B). The *pqsA*, *pqsH* and *pqsL* genes involved in the biosynthesis of the major

184 molecules from the HAQs family, namely 3,4-dihydroxy-2-heptylquinoline [termed the

185 *Pseudomonas* quinolone signal (PQS)], its precursor 4-hydroxy-2-heptylquinoline (HHQ) and

186 4-hydroxy-2-heptylquinoline *N*-oxide (HQNO) were all significantly repressed upon exposure

187 to PLFE1 at 100 and 25 µg mL^{-1}. However, the expression of *pqsR* (*mvfR*) encoding for the

188 cognate receptor/regulator of HHQ and PQS molecules did not change in the presence of 100

189 and 25 µg mL^{-1} PLFE1. Taken together, these data indicate that PLFE1 extract represses gene

190 expression levels of the Pqs QS system that correlates with the reduction in HAQs molecules

191 production, suggesting that PLFE1 exhibits its anti-pyocyanin production effect possibly

192 through an anti-QS activity.

193

194 **PLFE1 leads to increased membrane stiffness**

195 Further, we evaluated the effect of PLFE1 on *P. aeruginosa* strain H103 membrane fluidity

196 homeostasis. Planktonic cell growths of H103 exposed to PLFE1 at concentrations of 100, 50,

197 25, 12.5, 6.25, 3.12, 1.6 and 0.8 µg mL^{-1} were assayed for fluorescence anisotropy (FA) using

198 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescent probe (Fig. 4A). Anisotropy of H103 cells

199 increased in a concentration dependent-manner to achieve a maximum FA value of 0.223 ±

200 0.003 (*P*<0.001) at a concentration of 100 µg mL^{-1} of PLFE1 in comparison to the FA value of

201 untreated control (0.186 ± 0.002). This result reflects a significant decrease of approximately

202 20% of membrane fluidity (membrane rigidification) revealing physiological changes and

203 adaptations in the cellular envelope of H103 in response to PLFE1 exposure. Interestingly,

204 mRNA expression levels of *sigX* encoding the extracytoplasmic function sigma factor (ECFσ)

205 SigX that is required to maintain cell envelope integrity, and its known representative targets

206 *accA*, *accB*, and *fabY* that are involved in fatty acid biosynthesis were significantly decreased

207 upon exposure of H103 strain to PLFE1 at 100 and 25 µg mL^{-1} when compared to mRNA

208 expression levels in the control condition (untreated H103) (Fig. 4B). Further, *sigX* expression

209 was monitored during growth in the presence of PLFE1 at 100, 25 and 6.25 µg mL^{-1} by using

210 a transcriptional fusion construction where the *sigX* promoter region was fused to the promoter-

211 less *luxCDABE* cassette in the replicative pAB133 vector (pAB-P*sigX*) (Supplementary Fig.

212 S2). Remarkably, in the presence of PLFE1, the relative bioluminescence output from H103

213 harbouring pAB-P*sigX* increased in a dose dependent-manner reaching significant maximal

214 activity at 100 µg mL^{-1} during the transition from the late exponential phase to early stationary

215 phase (Fig. 4C; Supplementary Fig. S2A). However, bioluminescence activity of pAB-P*sigX*

216 decreased significantly in a concentration dependent-manner during the late stationary phase

217 achieving minimal activity at 100 µg mL^{-1} of PLFE1 (Fig. 4C; Supplementary Fig. S2A), in line

218 with the RT-qPCR results presented in Fig. 4B. These results reveal that *P. aeruginosa*

219   exposure to PLFE1 induced increased membrane stiffness suggesting alterations in envelope

220   homeostasis most likely through the activation of the cell wall stress ECFσ SigX. To further

221   validate this hypothesis, we used a ΔsigX deletion mutant[27] to determine the FA in the absence

222   or presence of various concentrations of PLFE1. The FA values appeared to be unaffected in

223   ΔsigX in response to PLFE1 exposure compared to the control condition (untreated ΔsigX)

224   (Supplementary Fig. S2A). Altogether, these data indicate that PLFE1 contains bioactive

225   membrane-interactive compounds that reduce membrane fluidity, in which the ECFσ SigX

226   seems to play a key role.

227

228   **Isolation and identification of major bioactive compounds of PLFE1**

229   To isolate and identify the major constituents of PLFE1, multiple chemical analyses were

230   performed. The fractionation of PLFE1 by medium pressure liquid chromatography (MPLC)

231   allowed to recover a total of 13 fractions that we labelled as PLFE1-(1-13) (Supplementary

232   Table S2). Thin layer chromatography (TLC) examination showed that the major compounds

233   of PLFE1 were present in the fractions PLFE1-(2-4). These last were then selected for further

234   analyses in order to identify the main compounds present in each fraction. For PLFE1-2, ^{1}H-

235   NMR spectra, proton signals between 7.36 and 6.78 ppm showed the presence of a tri-

236   substituted aromatic ring. The signal at 5.36 ppm [t J = 4.6 Hz, 2H] accounted for an olefinic

237   double bond. The major derivative ginkgolic acid (C17:1) with a molecular formula of $C_{24}H_{38}O_3$

238   (m/z 373.1 [M-H]$^-$) displayed one unsaturation in its aliphatic chain. Ozonolysis reaction of

239   PLFE1-2 allowed to localize the double bond of ginkgolic acid (C17:1) between positions C-8'

240   and C-9' (Fig. 5). LC-ESI-MS analyses corroborated the presence of ginkgolic acid (C17:1) in

241   PLFE1-2 and showed the occurrence of hydroginkgolic acid (C15:0) derivative and a mixture

242   of both ginkgolic acids (C17:1/C15:0) in PLFE1-3 and PLFE1-4, respectively (Fig. 5).

243   Altogether, these results indicate that ginkgolic acid (C17:1) and hydroginkgolic acid (C15:0)

244 are the main metabolites in PLFE1 and might be responsible for *P. aeruginosa* virulence

245 attenuation.

246

247 **Ginkgolic acid-enriched fractions from PLFE1 are involved in virulence**

248 **attenuation of *P. aeruginosa***

249 To further assay whether GA (C17:1), GA (15:1) or a mixture of both GA (C17:1/C15:0) are the

250 main bioactive compounds involved in the anti-virulence activity against *P. aeruginosa*, their

251 effect on pyocyanin production and virulence attenuation in human lung A549 infection model

252 was evaluated. Consistent with the results of pyocyanin inhibition by the crude extract PLFE1,

253 H103 strain treated with GA-enriched fractions showed a dose-dependent reduction of

254 pyocyanin production when compared to untreated H103 (Fig. 6A). Interestingly, the fractions

255 enriched in GA (C17:1) or GA mix of (C17:1)/(C15:0) showed approximately 90% pyocyanin

256 inhibition activity at 100 µg mL^{-1}. The GA (C17:1) and GA (C15:0) displayed an IC$_{50}$ of 6.3 µg

257 mL^{-1} (16.75 µM) and 12.5 µg mL^{-1} (35.75 µM), respectively (Supplementary Fig. S3). Looking

258 at virulence attenuation, H103 strain exposed to GA-enriched fractions showed significant

259 reduced LDH release (20%) as compared to the control condition (H103 untreated) revealing

260 that GA-enriched fractions were able to protect the human lung A549 line cells (Fig. 6B). Taken

261 together, these results provide important insights into the involvement of GA-enriched fractions

262 on the anti-virulence activity against *P. aeruginosa*.

263

264 **Cytotoxicity of PLFE1 and GA-enriched fractions**

265 Our last goal for this study was to investigate the potential of PLFE1 and its GA-enriched

266 fractions to induce cytotoxicity in the human lung A549 cells. As depicted in Fig. 7A, no

267 significant differences in cytotoxicity levels were found between A549 cells treated at various

268 concentrations of crude extract PLFE1 after 1 h incubation as compared to the control condition

269　(untreated A549 cells). However, a moderate cytotoxic effect was observed when A549 cells

270　were incubated for more than 3 h in the presence of PLFE1 at concentrations above 12.5 µg

271　mL^{-1}. Remarkably, when A549 cells were treated with PLFE1 at IC$_{50}$ value of pyocyanin activity

272　inhibition (4.9 µg mL^{-1}) there was no differences in cytotoxicity at different time points incubation

273　assayed (1 h, 3 h, 6 h, and 24 h) (Fig. 7B). Moreover, similar interesting trend results were

274　obtained for the GA-enriched fraction mix of both GA (C17:1/C15:0) when tested at 5.4 µg mL^{-1}

275　IC$_{50}$ value of pyocyanin inhibition (Fig. 7B). On the other hand, after 3 h incubation, a slight

276　cytotoxicity increase was observed when A549 cells were exposed to enriched fractions of GA

277　(C17:1) and GA (C15:0) separately at their IC$_{50}$ values (6.3 µg mL^{-1} and 12.5 µg mL^{-1},

278　respectively) (Fig. 7B). However, this cytotoxicity increased by about 3-fold after 24 h exposure

279　as compared to the control condition (A549 cells treated with DMSO). In summary, these

280　results indicate that PLFE1 and its complex GA-enriched fraction (C17:1/C15:0) displayed no

281　significant cytotoxicity in the human lung A549 line cells when tested at their IC$_{50}$ pyocyanin

282　inhibition values.

283

284

DISCUSSION

Antibiotic-resistant pathogens are threatening individuals and public health over the world to a such extent that new therapeutic strategies need to be developed urgently.[2,18] The anti-virulence approaches represent an attracting alternative to the unmet clinical need in the treatment of bacterial infections.[28,29] These new therapies target essentially the prevention of virulence factors production by pathogens, rather than their survival, and they also aim to thwart the regulatory mechanisms controlling their expression.[19,30] In addition, by targeting essential virulence determinants, the emergence and spread of bacterial resistance will be reduced due to decreased selective pressure and the natural microbiota will be preserved. In line with this scenario, the current study was designed to explore new anti-virulence agents to fight against the multidrug-resistant *P. aeruginosa* that is responsible for many different infections. Thus, we based our research on the traditional medicine reported to treat several infectious diseases. In particular, the fruit of *P. lentiscus* L. is used for the treatment of respiratory tracts infections, and in ointments for articular pain, burn wounds, and ulcer among other traditional uses.[25,26]

Our findings show that *P. lentiscus* L. fruit cyclohexane extract (PLFE1) contains potent inhibitory compounds that restrict *P. aeruginosa* virulence by abolishing pyocyanin pigment production, which is known to facilitate colonization of the host and subsequent infection.[6,7] This anti-virulence activity does not perturb growth and cell viability, which can minimize selective pressures towards the development of resistance compared to conventional antibiotics.[28] Nonetheless, additional investigations need to be undertaken to assess whether *P. aeruginosa* can develop resistance towards PLFE1 upon repeated exposure. Further, by exploring the mechanistic behind the anti-pyocyanin activity, we have shown that PLFE1 interferes with the HAQs QS-molecules production. Since for *P. aeruginosa* infections QS is

309 the master regulator involved in the expression of many virulence factors such as phenazines,

310 exoproteases (elastase, alkaline protease), siderophores, and toxins among others, its

311 inhibition represents a valuable adjuvant therapy that might be used to potentiate the activity

312 of the available antibiotics applied to handle early *P. aeruginosa* infections.[19,30] In the last five

313 years there was a large amount of published investigations reporting natural products

314 highlighting their potential in targeting bacterial virulence factors.[21] Several plant-derived

315 compounds that belong to alkaloids, organosulfurs, coumarins, flavonoids, phenolic acids,

316 phenylpropanoids, terpenoids among other chemical classes have been found to be active

317 against pyocyanin pigment production.[21] However, there is still a significant lack on the specific

318 underlying molecular mechanisms of action.

319

320 One important finding of the present study was that membrane stiffness in *P. aeruginosa* has

321 significantly increased upon exposure to PLFE1. Furthermore, we demonstrate that PLFE1

322 decreases the expression of the ECFσ SigX, which is involved in the regulation of membrane

323 lipid composition,[11,15] and thus, triggers modifications on membrane fluidity and homeostasis.

324 Accordingly, our data show a decreased expression levels of *accA* and *accB* encoding subunits

325 of the biotin-dependent enzyme acetyl-CoA carboxylase complex (ACC) that catalyzes the first

326 step in fatty acid biosynthesis in *P. aeruginosa*.[31] In addition, *fabY* (PA5174) encoding the β-

327 keto acyl synthase (FabY) involved in fatty acid biosynthesis, is another gene that showed

328 decreased expression in presence of the PLFE1. Interestingly, these results mirror those

329 observed in *P. aeruginosa sigX* mutant and over-expressing strains when compared to the

330 wild-type strain.[11,15,16] Moreover, Δ*sigX* cells display reduced membrane fluidity (membrane

331 rigidification), HAQs QS-molecules production, pyocyanin pigment production, and virulence

332 towards a *C. elegans* model.[9,16] Overall, these data provide evidences that PLFE1 most likely

333 contain membrane-interactive compounds, as it is the case for many plant extracts.[32,33] PLFE1

334   interaction with membrane lipid bilayers seems to be involved in the increased membrane

335   stiffness, which might trigger the modulation of the ECFσ SigX. Thus, our discoveries propose

336   that PLFE1 exerts its anti-virulence activity through a new potential mechanism that at least

337   involves the ECFσ SigX since no impact of PLFE1 was observed in membrane fluidity of the

338   Δ*sigX* mutant strain.

339

340   Herein, the fractionation of PLFE1 led to the isolation and identification of fractions mainly

341   enriched in C17:1 and C15:0 ginkgolic acids (GA) or a mix of both GA (C17:1/C15:0). To the

342   best of our knowledge, the presence of these chemical constituents in *P. lentiscus* L. fruit has

343   never been previously reported. However, according to the literature, GA has already been

344   isolated from *Ginkgo biloba* that has long been used in traditional Chinese medicine.[34] The GA

345   are a mixture of several 2-hydroxy-6-alkybenzoic acid congeners that differ in carbon alkyl

346   group length and unsaturation and are structurally similar to salicylic acid, which is reported to

347   impact negatively the pathogenicity of *P. aeruginosa* PA14 strain by repressing pyocyanin,

348   elastase, and protease production.[35] A number of pharmacological effects have been attributed

349   to GA, such as anti-bacterial, anti-fungal, insecticidal, anti-tumour, and neuroprotective effects

350   among others.[36–39] Moreover, GA are also proven to be effective as anti-biofilm molecules

351   against bacterial pathogens such as *Escherichia coli* O157:H7 strain, *Staphylococcus aureus*,

352   *Streptococcus mutans*, *Salmonella spp*., and *Listeria spp*.[40–42] In line with our results,

353   antibacterial activity studies show that GA did not affect Gram-negative bacteria survival,

354   however, they exhibit strong anti-microbial activity against Gram-positive bacteria.[39,40,43,44]

355   Interestingly, our results indicate that PLFE1 anti-virulence properties can be mostly attributed

356   to the identified GA-enriched fractions (C17:1, C15:0, C17:1/C15:0) since they are able to

357   function as pyocyanin production inhibitors without negative effect on bacterial growth.

358   Moreover, these GA-enriched fractions are capable of inducing *P. aeruginosa* membrane

359   rigidification (*data not shown*). This result is supported by the fact that the lipid soluble

360   components of the cell wall of Gram-negative bacteria is shown to intercept GA compounds.[43]

361

362   Following demonstration of PLFE1 and GA-enriched fractions for their *in vitro* anti-virulence

363   properties against *P. aeruginosa*, we assayed them for *in vivo* efficacy using the human lung

364   A549 cells and the nematode *C. elegans* as models host. In both infection models, the virulence

365   of *P. aeruginosa* upon exposure to PLFE1 at a dose of 100 µg/mL is shown to be significantly

366   attenuated. Further, the evaluation of the cytotoxicity of PLFE1 and GA molecules using A549

367   lung human cells indicated no or slight cytotoxicity at IC_{50} values. Taken together, these studies

368   deliver a promising perspective for the potential future development of PLFE1 or GA molecules

369   as an adjuvant agent to fight against *P. aeruginosa* infections. Nonetheless, it remains to be

370   verified whether PLFE1 or GA molecules have an antibiotic-potentiating activity, both *in vitro*

371   and *in vivo*, against the pathogen *P. aeruginosa*.

372

CONCLUSION

374   Overall, the data of this study suggest that the anti-virulence efficacy of PLFE1 and GA-

375   enriched fractions might possibly be attributed to their interaction with the lipid bilayer

376   membranes of *P. aeruginosa* resulting in the modification of membrane fluidity. Therefore, the

377   increased membrane stiffness in presence of PLFE1 and GA-enriched fractions appears to be

378   mediated through the modulation of the ECFσ SigX, which is known to be involved in the

379   regulation of *P. aeruginosa* virulence. These findings need further experimental evidences, not

380   least in terms of identifying the specific molecular mechanisms of action leading to impact the

381   ECFσ SigX as being a potential molecular target to alter the expression of virulence in *P.*

382   *aeruginosa*.

383 **METHODS**

384 **Bacterial strains, media and growth conditions.** The *P. aeruginosa* H103, H103-pAB-P*sigX*,

385 and H103-Δ*sigX*[27] used in this study are all derivatives of *P. aeruginosa* wild-type PAO1.

386 Planktonic cultures were grown aerobically at 37 ºC in LB broth on a rotary shaker (180 rpm)

387 from an initial inoculum adjusted to an OD at 580 nm of 0.08. The antibiotics stock solutions

388 used in this study were sterilized by filtration through 0.22-µm filters, aliquoted into daily-use

389 volumes and kept at -20 ºC.

390

391 **Collection and preparation of *P. lentiscus* L. fruit extracts.** Fruits of *P. lentiscus* L. were

392 collected within the wilaya of Jijel, Algeria, in September 2016, with the collect agreement

393 (48/MC/DGCE/DSPEC/2016) delivered by the Research Centre on Analytical Chemistry

394 (CRAPC). A voucher herbarium specimen is deposited in the herbarium of PNAS laboratory,

395 Université Paris Descartes (France). A sample of 8.98 g of fruits was subjected to a pressurized

396 solvent extraction (PSE) using a Speed Extractor E-914 (Büchi) equipped with four cells (120

397 mL) and a collector with four flat bottom vials (220 mL) successively with cyclohexane, ethyl

398 acetate, methanol, and water. Maximum pressure and temperature were adjusted to 100 bar

399 and to 50 °C, respectively. Two extraction cycles with a hold-on time of 15 min were performed

400 in each case. Solvents were removed under reduced pressure. A total of 4 extracts were

401 obtained (Supplementary Table S3). Solutions at 10 mg mL^{-1} were prepared in DMSO of each

402 extract and stored at 4 °C until use.

403

404 **Pyocyanin quantification assay.**To perform pyocyanin pigment quantification assay, *P.*

405 *aeruginosa* H103 cells untreated (grown in presence of 1% DMSO) and treated with 100, 50,

406 25, 12.5, 6.25, 3.12, 1.6 and 0.8 µg mL^{-1} of *P. lentiscus* L. fruit extracts (PLFE1-4) or ginkgolic

407   acid-enriched fractions (PLFE1-(2-4)) were grown on 96-well microtiter plate for 24 h at 37 ºC

408   on a rotary shaker (180 rpm). Then, supernatants samples were collected by centrifugation

409   and extracted with chloroform. The chloroform layer (blue layer) was acidified by adding 0.5 M

410   HCl. The absorbance of the HCl layer (pink layer) was recorded at 520nm using the Spark 20M

411   multimode Microplate Reader controlled by SparkControl™ software Version 2.1 (Tecan Group

412   Ltd.) and the data were normalized for bacterial cell density (OD_{580nm}).

413

414   **Virulence attenuation of *P. aeruginosa* using human lung A549 line cells.**The human lung

415   A549 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Lonza,

416   BioWhittaker®) supplemented with 4.5 g L^{-1} glucose, L-Glutamine, 10% heat-inactivated (30

417   min, 56 ºC) fetal bovine serum (FBS), and 100 Units mL^{-1} each of penicillin and streptomycin

418   antibiotics. Cells were grown at 37 ºC under the atmosphere of 5% CO2 and 95% air with

419   regularly medium change until a confluent monolayer was obtained. The anti-virulence effect

420   of PLFE1 or ginkgolic acid-enriched fractions (PLFE1-(2-4)) on *P. aeruginosa* H103 was

421   determined using an enzymatic assay (Pierce™ LDH Cytotoxicity Assay Kit, Thermo

422   Scientific™), which measures lactate dehydrogenase (LDH) released from the cytosol of

423   damaged A549 cells into the supernatant. After overnight incubation with *P. aeruginosa* H103

424   (10^8 CFU mL^{-1}) previously treated or untreated (control condition), the supernatants from

425   confluent A549 monolayers grown on 24-well tissue culture plates were collected and the

426   concentration of the LDH release was quantified. A549 cells exposed to 1X Lysis Buffer were

427   used as a positive control of maximal LDH release (100% lysis) as specified by the

428   manufacturer's recommendations. The background level (0% LDH release) was determined

429   with serum free culture medium. The LDH release assays were also used to determine the

430   cytotoxicity of PLFE1 or ginkgolic acid-enriched fractions (PLFE1-(2-4)) in A549 cells upon

431   direct exposure after 1-, 3-, 6-, and 24-h incubation.

432

433   *Caenorhabditis elegans* **fast-killing infection assay.** The *C. elegans* wild-type Bristol strain

434   N2 worms were grown at 22 °C on nematode growth medium (NGM) agar plates using

435   *Escherichia coli* OP50 as a food source. The *P. aeruginosa-C. elegans* fast-kill infection assay

436   was performed as described previously[45] with minor modifications. Briefly, cultures of *P.*

437   *aeruginosa* H103 strain untreated or treated with PLFE1 at 100 µg mL^{-1} were seeded on a 24-

438   well plate containing in each well 1 mL of Peptone-Glucose-Sorbitol (PGS) agar. Control wells

439   were seeded with 25 µL of *E. coli* OP50 from an overnight culture. The plate was then incubated

440   at 37 ºC for 24 h to make bacterial lawns and then shifted to 22 ºC for 4 h. For each assay, 15

441   to 20 L4-synchronized worms were added to the killing and control lawns and incubated at 22

442   °C. Worm survival was scored at 4-, 6-, 8-, 20-, and 24-h after the start of the assay, using an

443   Axiovert S100 optical microscope (Zeiss).

444

445   **Bacterial cell viability assays by flow cytometry.** Cell viability assays of *P. aeruginosa* H103

446   cells untreated and treated by different concentrations of *P. lentiscus* L. fruit extract (PLFE1)

447   were assessed by using the LIVE/DEAD™ BacLight™ Bacterial Viability and Counting Kit, for

448   flow cytometry (Invitrogen, Molecular Probes). Briefly, *P. aeruginosa* H103 untreated and

449   treated suspensions were diluted in filter-sterilized PBS to reach a final density of 1 x 10^6 CFU

450   mL^{-1}, then stained following the manufacturer's instructions. The data were acquired using

451   CytoFlex S flow cytometer (Beckman coulter Life science). An aliquot of cells was killed with

452   100% ethanol and used as a control of death cells. The SYTO9 stained cells were detected by

453   an excitation with 22 mW blue laser at 488 and with emission wavelength at 525 nm (green,

454   with band pass filter of 40 nm), while the PI (Propidium iodide) stained cells were detected by

455   690 nm (red, with band pass filter of 50 nm).

456

Extraction and quantification of HAQs. Planktonic cultures of *P. aeruginosa* H103 untreated (control DMSO 1%) and treated by 100, 50, 25, 12.5, 6.25, 3.12, 1.6 and 0.8 µg mL^{-1} of PLFE1 were subjected to HAQ molecules extraction following the technique described in a previous study.[46] HAQs were quantified by a combined spectrophotometer/luminometer microplate assay using the biosensor strain PAO1 *pqsA* CTX-*lux::pqsA*.[47] The HAQ biosensor strain was grown overnight and OD was measured and adjusted with fresh LB medium to achieve OD$_{580nm}$ of 1. For each test well, 5 µL of HAQs crude extracts were diluted in 100 µL of LB and added to 100 µL of 1 in 50 dilution of the HAQ biosensor strain. Further, bioluminescence and OD$_{580nm}$ were monitored in specialized white sided and clear bottom 96-well microtiter plate every 15 min for 24 h at 37 ºC using the Spark 20M multimode Microplate Reader controlled by SparkControl^{TM} software Version 2.1 (Tecan Group Ltd.). Both HHQ and PQS synthetic standards (Sigma-Aldrich) at a final concentration of 5 µM, used as positive controls, were added to 1 in 100 dilution of the HAQ biosensor strain, as both activate bioluminescence production. The recorded bioluminescence as relative light units (R.L.U) were normalized to OD$_{580nm}$ of cultures suspensions.

Membrane fluidity measurement by fluorescence anisotropy. Fluorescence anisotropy analysis of *P. aeruginosa* cells were performed as previously described.[16] Briefly, cell pellets of *P. aeruginosa* H103 untreated (control DMSO 1%) and treated by 100, 50, 25, 12.5, 6.25, 3.12, 1.6 and 0.8 µg mL^{-1} of PLFE1 were washed two times (7500 × g, 5 min, 25 °C) in 0.01 M MgSO$_4$ and resuspended in the same wash solution to reach an OD$_{580nm}$ of 0.1. One µL of a 4 mM of 1,6-diphenyl-1,3,5-hexatriene (DPH) stock solution (Sigma-Aldrich) in tetrahydrofuran was added to 1 mL aliquot of the resuspended cultures and incubated in the dark for 30 min at 37 °C to allow the probe to incorporate into the cytoplasmic membrane. Measurement of the fluorescence polarization was performed using the Spark 20M multimode Microplate Reader,

482 equipped with an active temperature regulation system (Te-Cool™, Tecan Group Ltd.).

483 Excitation and emission wavelengths were set to 365 nm and 425 nm, respectively, and the

484 Fluorescence Anisotropy (FA) was calculated according to Lakowicz.[48] Three measurements

485 were performed for each sample and data were recorded using SparkControl™ software

486 (Version 2.1, Tecan Group Ltd.). The relationship between fluorescence polarization and

487 membrane fluidity is an inverse one, where increasing anisotropy values correspond to a more

488 rigid membrane and vice versa. All values are reported as means of triplicate analyses for each

489 experimental variable.

490

491 **Transcriptional fusion PsigX::*luxCDABE* construction and *sigX* promoter activity**

492 **analysis in response to PLFE1.** The promoter region of the *sigX* gene (P*sigX*) was amplified

493 by PCR with primers PsigX-*SacI*-F and PsigX-*SpeI*-R (Supplementary Table S4,) incorporating

494 SacI and SpeI linkers, respectively. The promoter region of *sigX* gene was fused to the

495 *luxCDABE* cassette in the promoterless pAB133 vector.[49] Upon amplification, DNA was

496 digested with the appropriate restriction enzymes, and cloned into pAB133 generating pAB-

497 P*sigX* vector. The pAB-P*sigX* plasmid was then transformed separately into one shot™ *E. coli*

498 TOP10 competent cells (Invitrogen). The constructions were confirmed by DNA sequencing

499 (Sanger sequencing services, Genewiz). Finally, the plasmids were transferred by

500 electroporation into *P. aeruginosa* H103 strain. Promoter activity was analysed by monitoring

501 bioluminescence during course time.

502

503 **Reverse transcription-quantitative PCR analyses (RT-qPCR).** Total RNAs from three

504 independent H103 untreated and treated cultures with PLFE1 at 100, 25, and 6.25 µg mL^{-1}

505 were isolated by the hot acid-phenol method[27] followed by treatment with Turbo DNA-*free*™ kit

506 (Invitrogen) according to the manufacturer's protocol. Synthesis of cDNAs and RT-qPCR were

507   achieved as previously described[50] using the oligonucleotides listed in Supplementary Table

508   S4. The mRNAs levels were calculated by comparing the threshold cycles (Ct) of target genes

509   with those of control sample groups and the relative quantification was measured using the

510    $2^{-\Delta\Delta Ct}$ method[51] using DataAssist^{TM} software (Applied Biosystems).

511

512   **PLFE1 fractionation.** Two hundred mg of PFLE1 were subjected to fractionation by medium

513   MPLC on 9.6 g of silice 60 M with cyclohexane/ethyl acetate as mobile phase solvent mixture

514   with increasing polarity. A total of 13 fractions were recovered and designated PLFE1-(1-13)

515   (Supplementary Table S2)

516

517   **Structure determination of compounds present in PLFE1-(2-4) fractions by NMR and**

518   **ozonolysis.** To determine the structure of the main compounds of PLFE1-(2-4), ^{1}D and ^{2}D

519   NMR experiments were conducted in a Bruker 400 MHz spectrometer apparatus and recorded

520   in deuterated chloroform ($CDCl_3$). To identify the double bond position of the main compound

521   identified in PLFE1-2, an ozonolysis reaction was carried out. Briefly, a solution of 10 mg (0.027

522   mmol, 1.0 equiv) of PLFE1-2 was put in a round bottom flask containing 20 mL of distilled

523   dichloromethane and a small amount of Sudan III. A stream of ozone (O_3) was bubbled into

524   the solution at -78 °C until the pink solution became colorless (10 min). Then, 200 µL of

525   dimethyl sulphide ((CH_3)$_2$S) (2.7 mmol, 100 equiv) were added to the reaction mixture and

526   stirred for 1 h at 20 °C. From the reaction mixture, 1 µl was directly analyzed by GC-MS.

527

528   **Gas chromatography-mass spectrometry (GC-MS) analyses.** The GC-MS analyses were

529   carried out in a Hewlett Packard GC 6890/MSD 5972 apparatus equipped with HP-5 (30 m x

530   0.25 mm x 0.25 µm). Carried gas was Ar with a flow of 0.9 mL min^{-1}, split 1:20, oven was

531   programmed increasing from 180°C to 270°C at 8°C min^{-1} with an initial and final hold of 1 and

532 65 min, respectively. The inlet and GC-MS interface temperature were kept at 240 °C. The

533 temperature of EI (electron impact) 70 eV was 220°C with full scan (80-500 m/z). The injection

534 volume was 1 μL. Identification of constituents was achieved by comparing their mass spectra

535 with those of the Mass Spectra Library (NIST 98) compounds.

536

537 **Statistical analyses.** Statistical significance was evaluated using R (https://www.r-

538 project.org).[52] The data were statistically analyzed using two-sample unpaired two-tailed *t* test

539 to calculate *P* values. The mean with standard error of the mean (SEM) of at least three

540 independent biological experiments were calculated and plotted. *C. elegans* survival curves

541 were prepared using R to perform a statistical log-rank (Mantel-Cox) test.

542

DATA AVAILABILITY

544 The authors declare that all relevant data supporting the findings of the study are available in

545 this article and its Supplementary Information files, or from the corresponding author upon

546 request.

547

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562

COMPETING INTERESTS

564 The authors declare no competing interests.

565

566 **AUTHOR CONTRIBUTIONS**

567 A.T., S.C., E.B., S.B., R.G., and O.L. conceived and designed the experiments; A.T., S.O.,

568 O.C.A., N.B., O.M., and D.T. conducted experiments and analyzed the data; A.T., S.C., P.C.,

569 E.B., S.O., M.K., S.C., S.M., M.F., and N.O. contributed to the writing of the manuscript. All

570 authors proofread the final draft and approved the final manuscript.

571

572 **FIGURE LEGENDS**

573 **Figure 1.** *P. lentiscus* L. fruit extract (PLFE) is a potent inhibitor of pyocyanin production in *P.*
574 *aeruginosa* strain H103. (**A**) Effect of various concentrations of *P. lentiscus* cyclohexane
575 (PLFE1), ethyl acetate (PLFE2), methanol (PLFE3), and water (PLFE4) extracts on pyocyanin
576 production. (**B**) Relative expression levels of representative genes from phenazine
577 biosynthesis pathway in H103 treated with PLFE1 at 100, 25, and 6.25 µg mL^{-1} compared to
578 the relative mRNA levels in the control condition (H103 untreated). Values represent the mean
579 (± SEM) of three independent assays. Statistics were achieved by a two-tailed *t* test: ****,
580 *P*<0.0001; ***, *P*=0.0001 to 0.001; **, *P*=0.001 to 0.01; *, *P*=0.01 to 0.05; NS (Not Significant), *P*≥0.05.

582 **Figure 2.** Virulence attenuation on *P. aeruginosa* by PLFE1. (**A**) Anti-virulence effects of
583 PLFE1 in human A549 lung cells infection model. The presence of PLFE1 (100 µg mL^{-1})
584 significantly protected A549 lung cells from lysis after 20h infection. Data are presented as the
585 mean ± SEM values of four independent experiments performed in duplicate. ** *P*=0.001 to
586 0.01 (two-tailed *t* test) versus untreated cells. (**B**) *P. aeruginosa* H103 virulence attenuation in
587 a *C. elegans* infection model by PLFE1. Sixty L4-stage nematodes per experimental group
588 were placed on lawns of *E. coli* OP50 (black) or H103 strain in the absence (red) or presence
589 of PLFE1 at 100 µg mL^{-1} (green). Alive nematodes were scored at 4-, 6-, 8-, 20- and 24-h after
590 the start of the assay. ****, *P*<0.0001 (log-rank [Mantel-Cox] test) versus untreated cells.

592 **Figure 3.** PLFE1 decreases HAQ-molecules production. (**A**) Normalized mean maximal
593 bioluminescence output from the HAQ-biosensor strain in the presence of crude ethyl acetate
594 HAQs extracts prepared from cultures of H103 treated with different concentrations of PLFE1
595 compared to positive control conditions (HHQ and PQS at 5 µM; HAQs crude ethyl acetate
596 extract from H103 untreated) and to negative control conditions (HAQs crude ethyl acetate
597 extract from HAQ-biosenor strain and LB medium). (**B**) Relative expression levels of
598 representative genes from HAQ QS system in H103 treated with PLFE1 at 100, 25, and 6.25
599 µg mL^{-1} compared to the relative mRNA levels in the control condition (H103 untreated). Values
600 represent the mean (± SEM) of three independent assays. Statistics were achieved by a two-
601 tailed *t* test: ***, *P*=0.0001 to 0.001; **, *P*=0.001 to 0.01; *, *P*=0.01 to 0.05; NS (Not Significant),
602 *P*≥0.05.

604 **Figure 4.** PLFE1 induces membrane stiffness. (**A**) Fluorescence anisotropy (membrane

605 fluidity) measurements in *P. aeruginosa* strain H103 exposed to various concentrations of

606 PLFE1 compared to the control condition (H103 untreated). (**B**) Relative expression levels of

607 *sigX* encoding the SigX extracytoplasmic function sigma factor (ECFσ) and its known target

608 genes (*accA*, *accB*, and *fabY*) involved in fatty acid biosynthesis in H103 treated with PLFE1

609 at 100, 25, and 6.25 µg mL^{-1} compared to the relative mRNA levels in the control condition

610 (H103 untreated). (**C**) Relative bioluminescence levels of H103 harboring the pAB-P*sigX*

611 plasmid (*sigX* promoter region) treated with PLFE1 at 100, 25, and 6.25 µg mL^{-1} compared to

612 the relative bioluminescence levels in the control condition (H103-pAB-P*sigX* untreated). Data

613 at both transition and stationary phases are displayed. Values represent the mean (± SEM) of

614 three independent assays. Statistics were achieved by a two-tailed *t* test: ****, *P*<0.0001; ***,

615 *P*=0.0001 to 0.001; **, *P*=0.001 to 0.01; *, *P*=0.01 to 0.05; NS (Not Significant), *P*≥0.05.

616

617 **Figure 5.** Chemical structures of ginkgolic acid derivatives identified in PLFE1-(2-4).

618

619 **Figure 6.** Effect of GA-enriched fractions from PLFE1 on *P. aeruginosa* virulence. (**A**)

620 Pyocyanin production upon exposure to different concentrations of GA-enriched fractions. (**B**)

621 Anti-virulence of GA-enriched in human A549 lung cells infection model. The presence of GA-

622 enriched fractions (100 µg mL^{-1}) significantly protected A549 lung cells from lysis after 20 h

623 infection. Data are presented as the mean ± SEM values of four independent experiments

624 performed in duplicate. Statistics were achieved by a two-tailed *t* test: **, *P*=0.001 to 0.01; *,

625 *P*=0.01 to 0.05.

626

627 **Figure 7.** Cytotoxic effect of PLFE1 and its GA-enriched fractions on human lung A549 line

628 cells. (**A**) PLFE1 was assayed at different concentrations. (**B**) Cytotoxicity of GA-enriched

629 fractions exhibiting pyocyanin production inhibition. LDH release was determined at 1 h, 3 h, 6

630 h, and 24 h. DMSO was used as a vehicle control. Data are displayed as the mean ± SEM

631 values of four independent experiments performed in duplicate. Statistics were achieved by a

632 two-tailed *t* test: ***, *P*=0.0001 to 0.001; **, *P*=0.001 to 0.01; *, *P*=0.01 to 0.05; NS (Not Significant),

633 *P*≥0.05.

634

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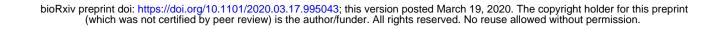
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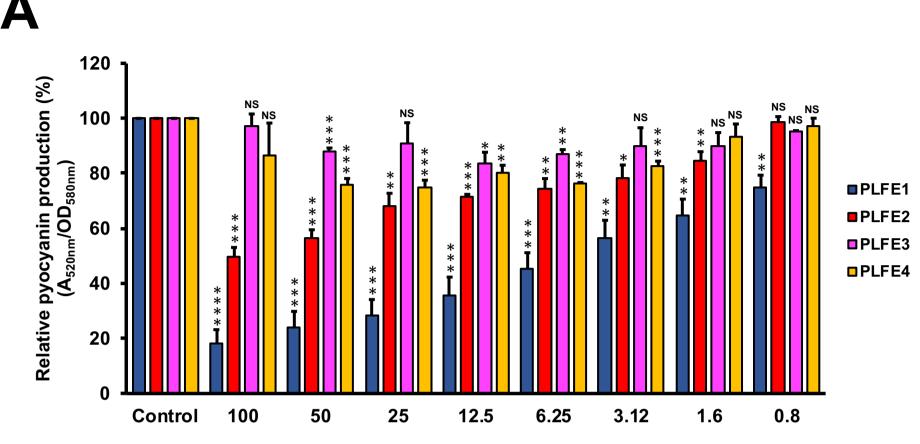
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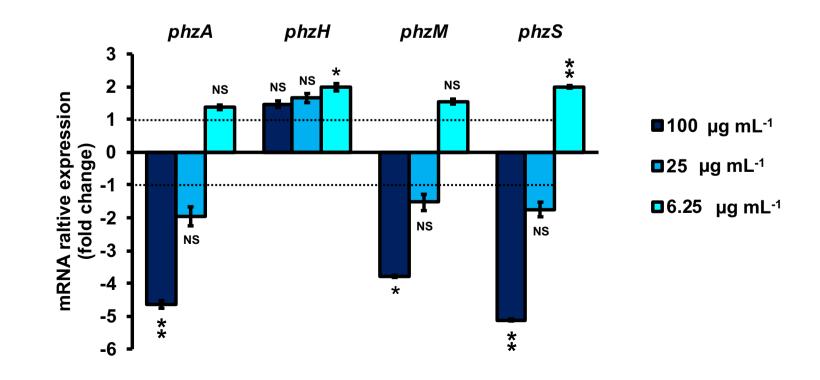
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Figure 1

Figure 2

A

B

Figure 3

Figure 4

Figure 5

Ginkgolic acid (C17:1)

Hydroginkgolic acid (C15:0)

Figure 6

Figure 7

A

B