A novel inhibitor of plant pathogenic bacteria extracted from propolis residues

Yan Xu [1, 2], Sheng Sheng [1, 2], Wen-Miao Song [1], Dan Zhu [1], Jun Wang [1, 2], and Fu-An Wu [1, 2,*]

[1]School of Biotechnology, Jiangsu University of Science and Technology, Zhenjiang, Jiangsu 212018, China
[2] Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang, Jiangsu 212018, China
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Presenting author email: xuyan@just.edu.cn, fuword@163.com

Ralstonia solanacearum is a rod-shaped Gram-negative plant-pathogenic bacterium that causes bacterial wilt disease (Li et al., 2016). Nowadays, the bacterial wilt is one of the most widely infectious and damaging plant diseases, which affect approximately 300 species across 50 families in the world (Kim et al., 2016). It has been difficult to control bacterial wilt, and the conventionally chemical control methods such as 2% methanol and 5% whitewash, are harmful to the environment (Xu et al., 2017). Thus, it is highly desirable to find an environment-friendly novel inhibitor of plant pathogenic bacteria with high activity against *R. solanacearum*.

Phenolic compounds are one of the most diverse groups of secondary metabolites in nature (Cueva et al., 2010). Caffeic acid (CA) and its esters are a series of phenolic compounds widely distributed in the plant kingdom and carry strong physiological implications for biological materials and biochemical agents with commercial applications in agriculture, medicine, food, and cosmetics products (Ravn et al., 1989). Among these esters of CA, caffeic acid phenethyl ester (CAPE) is a high-value chemical compound firstly isolated from the honeybee propolis produced from Brazil, Croatian, Argentina, and other countries (Erdogan et al., 2011). CAPE has various biological and pharmacological properties, including antibacterial, antivirus, antioxidant, anti-hyperglycemic, hepatocyte-protective, and antimutagenic properties (Wang et al., 2014). In the previous study, we found that the EC_{50} of CAPE against *R. solanacearum* was 0.17-0.75 mg/mL, suggesting CAPE is a potential *in vitro* inhibitor of plant pathogenic bacteria (Xu et al., 2017). However, the cost of extracting CAPE from natural propolis is very expensive (Jerkovic et al., 2016), and difficult to meet the increasing current market demand (Sforcin, 2016). Considering the residue of propolis extract factory using by supercritical fluid extraction or Pressurized liquid extraction (Erdogan et al., 2011) is an abundant source of CA and CAPE, which may be an ideal alternative to natural propolis. Propolis residue also is one kind of food industry waste in China, total global production every year is greater than 1000 metric tons. So, the aim of this work was to use the propolis residue to replace the natural propolis, in order to develop a novel inhibitor against *R. solanacearum*. It is extremely valuable to develop a cost-effective and efficient method to fully utilize propolis residue as a cheap feedstock for the production of inhibitor against plant pathogenic bacteria.

Antibacterial activities of various extracts from propolis residue (EPRs) against *R. solanacearum* strains including GMI1000, GIM1.76, GIM1.74, GIM1.71 and RS-5 were firstly performed according to an improved protocol (Yang et al., 2016). The EPRs solutions were prepared with 35% ethanol to obtain 0.1 mg/mL concentrations. To avoid cell death, 140 μL of casamino acid-peptone-glucose (CPG) medium, 40 μL of diluted bacterial solution and 20 μL of EPRs with 35% ethanol samples (v/v, T_{Sample}) were added into the wells in succession. In addition, one control was designed as: 40 μL of diluted bacterial solution and 140 μL of liquid CPG medium with 20 μL of 35% ethanol (35%, v/v, T_{Blank}). All plates were homogenized by constantly shaking, and after 10 min, the bacterial growth at T_0 was determined by optical density at 600 nm on a microplate reader (SpectraMax i3, Silicon Valley, CA, USA) with a blank that consisted of liquid CPG medium. Then, the plates were incubated at 30 °C for 24 h. After incubation, the plates were shaken for 15 min to test for TF. The antibacterial activity was reported as an inhibition percentage (%) using Equation (1):

$$\text{Inhibition}\ (\%) = (1-\frac{TF_{\text{sample}} - T0_{\text{sample}}}{TF_{\text{blank}} - T0_{\text{blank}}}) \times 100\% \quad (1)$$

Where $T0_{Sample}$ and TF_{Sample} correspond to the optical density at 600 nm of the strain growth with the existence of the EPRs before (T_0) and after (TF) incubation, respectively. Moreover, $T0_{Blank}$ and TF_{Blank} correspond to the liquid CPG medium with 35% ethanol before and after incubation, respectively.

Figure 1 shows the growth status of five strains of *R. solanacearum* in the presence of various EPRs using different solvents. EPRs of methanol and ethanol both had a strong activity with the antibacterial rate almost over 50% after 24 h treatment at a dosage of 0.1 mg/mL. Among five races treated by EPRs with six various solvents, the RS-5 growth inhibition reached all more than 60%, while it was different from 8.36% to 100% for GIM1.74, and the others were inhibited from 37.71-98.48%. Therefore, the EPRs using different solvents have different impact against five strains. Moreover, Table 1 shows the antibacterial activity comparison of various EPRs and CAPE. For GIM1.74, GIM1.71 and RS-5, 0.1 mg/mL EPRs of chloroform, methanol and ethanol have a significant inhibition effect more than 80%, which is better than that using CAPE solution of 0.2 mg/mL. Especially for GIM1.74, the inhibition effect of EPRs of chloroform and ethanol meet

100%, while CAPE's effect is only around 43%. The EPRs of ethyl acetate has a good effect on GIM1.76 (92.45 ± 5.32%) and GIM1.71 (95.04 ± 1.69%), respectively. The results suggested that propolis residue is a significant antibacterial material, which could be a potential inhibitor of plant pathogenic bacteria *R. solanacearum*.

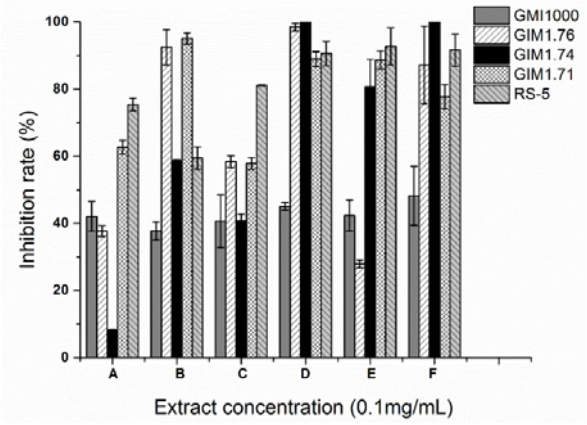

Figure 1. Bacterial growth inhibition (%) of EPRs against *R. solanacearum* strains measured at 600 nm. Solvents: (A) 35% ethanol; (B) ethyl acetate; (C) petroleum ether; (D) chloroform; (E) methanol; (F) ethanol.

Table 1. Antibacterial activity of various EPRs by different solvents against *R. solanacearum* strain (n = 3).

Samples	Inhibition Effect (%)				
	GMI1000	GIM1.76	GIM1.74	GIM1.71	RS-5
0.2mg/mL CAPE solution	55.04 ± 2.56	43.42 ± 3.04	43.47 ± 1.98	43.25 ± 2.18	50.98 ± 4.84
0.1mg/mL EPR of ethyl acetate	37.76 ± 2.70	92.45 ± 5.32	58.78 ± 0.15	95.04 ± 1.69	59.45 ± 3.35
0.1mg/mL EPR of chloroform	45.09 ± 1.17	98.48 ± 1.12	100 ± 0.00	88.92 ± 2.19	90.66 ± 3.62
0.1mg/mL EPR of methanol	42.38 ± 4.62	27.93 ± 1.14	80.69 ± 8.18	88.65 ± 2.69	92.69 ± 5.52
0.1mg/mL EPR of ethanol	48.22 ± 8.78	87.13 ± 11.53	100 ± 0.00	77.68 ± 3.60	91.61 ± 4.75

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