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Herbal Extracts in Combination with Nanosilver Inhibit Bacterial Leaf Blight Disease Caused by *Xanthomonas oryzae* pv. *oryzae* in Rice

Nguyen Thanh Hai[1], Dang Hoang Trang[1], and Nguyen Thi Thanh Ha[2]

[1]Faculty of Biotechnology, Vietnam National University of Agriculture, Hanoi 131000, Vietnam
[2] Faculty of Veterinary Medicine, Vietnam National University of Agriculture, Hanoi 131000, Vietnam

Abstract

Bacterial rice leaf blight (BLB), caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), has a massive impact on the quality and productivity of rice. Besides BLB resistant rice cultivars, herbal extracts and nanosilver have increasingly demonstrated their important roles in controlling the disease as alternatives to synthetic chemical pesticides. Therefore, this research aimed to examine the Xoo antibacterial effects of several herbal extracts and nanosilver *in vitro* and *in vivo*. In the study, *Wedelia chinensis Osbeck* Merr., *Clerodendrum fragrans* Vent., *Excoecaria cochinchinensis* Lour., *Polyathia longifolia* var. Pendula, and *Caesalpinia sappan* L. were extracted by maceration with six types of solvents (distilled water, 70% ethanol, chloroform, n-hexane, and 100% acetonitrile), then used in an agar diffusion test to evaluate their *Xoo* antibacterial effects. The results showed that 70% ethanol was the best extracting solvent for the targeted plants. *C. fragrans*, *E. Cochinchinensis*, and *C. sappan* showed significant antibacterial effects with inhibition zone diameters of 28.50 cm, 21.00 cm, and 25.70 cm, respectively. Finally, the individual extract from *C. fragrans*, *E. Cochinchinensis,* and *C. sappan* were combined with nanosilver particles and used to access BLB inhibition capacity *in vivo*, using the rice cultivar IR24 as the target for *Xoo* infection. Application of the *C. fragrans* extract resulted in resistance of IR24 rice to BLB. Similar results were also observed in the infected rice when products combining nanosilver and *E. cochinchinensis* or *C. sappan* were applied to infected rice leaves.

Keywords

Bacterial leaf blight (BLB), *Xanthomonas oryzae* pv. *oryzae*, herbal extract, nanosilver

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Correspondence to
nthaicnsh@vnua.edu.vn

ORCID
Hai Nguyen Thanh
https://orcid.org/0000-0003-0211-1196
Nguyen Thi Thanh Ha
https://orcid.org/0000-0003-2059-7492

Introduction

Rice plays a vital role in agricultural production as well as food security. It was reported that over 40% of the world's population consumes rice as their staple food, and among that 25% consumes rice for half of their daily diet (FAO, 2014). However, rice cultivation is often affected by diseases, among which, bacterial leaf blight (BLB) caused by *Xanthomonas oryzae* pv. *oryzae (Xoo)* is one of the most severe diseases that was first detected in Fukuoka, Kyushu, Japan in 1884 (Tagami and Mizukami, 1962). The BLB disease has widely spread in many countries, especially in all Asian rice producing countries, including Vietnam, leading to what has become a BLB pandemic in rice (Dung and Vien, 2005).

One of the most feasible solutions to control leaf blight disease nowadays is the exploitation and introgression of resistance genes into high-yielding cultivars. However, based on the fact that these cultivars, which are cultured continuously on large scales, have only been introgressed with a single or few resistant QTLs/genes, they are bound to result in significant shifts in *Xoo* pathogens, likely leading to resistance breakdown (Vasudevan *et al*., 2002). Besides, the use of chemicals, such as mercury, copper, and various antibiotics, has been discouraged because of their adverse effects on the agricultural products, surrounding environments and human health. Therefore, recent trends involve the use of herbal products as safely agricultural chemicals to control plant diseases. In nature, plants in general are faced with a plethora of antagonists, and have evolved myriad defense mechanisms by releasing numerous bioactive secondary metabolites (Khanh *et al.*, 2018). Some reports showed that plant-derived compounds produce safe and effective plant protection products (Nascimento *et al.*, 2000; Jabeen, 2011) and may be used as natural pesticides to control plant diseases. Plant-natural products have many advantages in comparison to similar-functioning chemicals, especially regarding environment-friendliness and human health. This could be the key to develop sustainable agriculture production. Some plants, such as *Poncirus trifoliata Rafin* (Rahman *et al.*, 2014) or *Adathoda vasica* (Govindappa *et al.*, 2011), were reported previously to suppress *Xoo* effectively *in vitro* and induce resistance in rice against BLB.

In addition, the technology of silver nanoparticles has achieved significant successes in agriculture in general and in plant protection in particular (Min *et al.*, 2009; Jung *et al.*, 2010; Khot *et al.*, 2012; Mahdizadeh *et al.*, 2015), helping with the production of efficient, safe, and economical products. Several studies have demonstrated the significant antibacterial effects of mixtures of silver nanoparticles and plant extracts (Park *et al.*, 2006; Thanh and Hai, 2014; Gioi and Hai, 2017).

Thus, this study aimed to evaluate herbal extracts in combination with nanosilver to control rice blight disease caused by *Xanthomonas oryzae* pv. *oryzae* in rice.

Materials and Methods

Materials

The herbs *Excoecaria cochinchinensis* Lour., *Wedelia chinensis Osbeck* Merr., *Polyathia longifolia* var. pendula, *Caesalpinia sappan* L., and *Clerodendron fragrans* Vent. were collected in 2017 and the samples were stored at the Department of Internal Medicine-Diagnose-Pharmacology, Faculty of Veterinary Medicine, Vietnam National University of Agriculture.

The susceptible rice cultivar IR24 was grown at the Botanical Garden of Vietnam National University of Agriculture for the *in vivo* experiment.

Xanthomonas oryzae pv. *oryzae (Xoo)* isolate 07 causing BLB was provided by the Center for Conservation and Development of Crop Genetic Resources, Vietnam National University of Agriculture.

Laboratory chemicals were imported from China including 70% ethanol, chloroform, n-hexane, 100% acetone, 100% acetonitrile, and dimethyl sulfoxide (DMSO).

Nanosilver particles with the stock concentration of 100 ppm, 90% of which had the size of 20-25 nm, were provided by the Department of Biology, Faculty of Biotechnology, Vietnam National University of Agriculture.

A rice blight treatment product, Ankamycin 30SL (Kasugamycin 19 g L^{-1} + Tricyclazole 11 g L^{-1}), was purchased from Hoang An Ltd.

Solid Wakimoto medium was prepared into 10 cm-diameter Petri dishes, about 4 ± 0.2 mm in depth. This medium is for growing bacterial colonies and KB testing. Liquid Wakimoto medium (without Agar) was used to culture and collect *Xoo* bacterial solutions.

Methods

Herbal collection

Mature leaves of *E. cochinchinensis* and *P. longifolia*, which were healthy and intact, were collected and washed under dripping water, then dried until the weight stabilized. The dried leaves were ground into powder (< 0.5 mm) and stored in zipped plastic bags with dehumidifiers.

The bark of *C. sappan*, and the roots and shoots of *C. fragrans* (healthy and intact) were split into small segments. The top three quarters of whole plants of *W. chinensis* were taken (healthy and intact). These samples were dried until the weight stabilized, ground into powder (< 0.5 mm), and stored in zipped plastic bags with dehumidifiers.

Extraction

The five targeted herbs were extracted by maceration with one of six types of solvents (distilled water, 70% ethanol, chloroform, n-hexane, 100% acetone, or 100% acetonitrile). In this technique, plant materials (coarse or powdered) were immersed in a stoppered vessel with a solvent and then allowed to stand at room temperature for at least three days with frequent agitation (Handa *et al.*, 2008). The process aims to soften and break the plant's cell walls to free the soluble phytochemicals. After three days, the mixture was pressed or strained by filtration.

For each type of solvent, the extraction procedures were repeated thrice. The efficiency of the extraction was calculated using the following formula:

$$H(\%) = \frac{\text{weight of crude dried extract (g)}}{\text{weight of dry powered (g)}} \times 100\%$$

The crude extracts were diluted with dimethyl sulfoxide (DMSO) following the ratio of 1 g extract: 10 mL DMSO to obtain the stock extract solutions (100 mg mL^{-1}).

Culturing Xanthomonas oryzae pv. oryzae

Xoo 07 was taken from its -80ºC storage box, activated, and spread on Wakimoto agar medium at 28ºC. After 24 h, a typical single colony (round shaped, smooth, raised, 1-2 mm in size, lemon yellow) was selected and cultured in Wakimoto liquid medium and shaken at 200 rev min^{-1} in a flask for about 16 hours before obtaining the bacteria fluids. Bacterial density was determined by OD measurement at λ= 600 nm to reach 10^8 cells mL^{-1}.

Evaluating the Xoo antibacterial effects of plant extracts

A 100 µL sample of the bacterial solution (10^8 cells mL^{-1}) was cultured on Wakimoto medium. On the medium surface, there were 5 mm-diameter holes containing either DMSO (the control) or one of the different types of extract solutions for the diffusion test. The Petri dishes were then cultured at 28-30ºC for 24 h. The results were analyzed by measuring the diameter of inhibition zones and calculating the average diameters using the following formula:

$$\bar{D} = \frac{\sum_{i=1}^{n} D_i}{n}; s = \sqrt{\frac{\sum_{i=1}^{n} (D_i - D)^2}{n-1}}$$

In which: is the average diameter of the inhibition zones (mm), Di is the diameter of an inhibition zone measured at i time (mm), s is the standard variation, and n is the number of replications (Bauer *et al.*, 1966).

Evaluating the effect of nanosilver on Xoo bacteria by direct exposure

One hundred µL samples of nanosilver at different concentrations were added to sterilized Eppendorf tubes. In each Eppendorf tube, 100 µL of the bacterial solution (10^8 cells mL^{-1}) was

added, mixed, and allowed to stand for 4 h. Each treated bacterial solution sample was cultured on Wakimoto agar medium for 24 h at 28^0C. The number of colonies was counted to determine the minimum inhibitory concentration of nanosilver (MIC_{nano}).

Evaluating the Xoo antibacterial effect of mixtures of the plant extracts with nanosilver in vitro and in vivo

Five mL of the extract solutions at different concentrations were put into test tubes. Then 5 mL of nanosilver at MIC_{nano} (concluded from the above section) was added into these test tubes and mixed evenly. The *Xoo* antibacterial effect *in vitro* of the mixtures of plant extracts with nanosilver was identified by the agar diffusion test (Bauer *et al.*, 1966).

The IR24 rice cultivar was grown in pots (17 x 19 cm) in greenhouse from March to May in 2017. At the panicle initiation stage, the plants were artificially infected with Xoo 07 using the leaf-clipping method (Kauffman *et al.*, 1973). After 48 h of infection, based on the results obtained in the fourth experiment, eight experimental formulas were chosen for an *in vivo* experiment as follows:

CT1	Distilled water (positive control)
CT2	*E. cochinchinensis* extract solution (3.13 mg mL^{-1})
CT3	*E. cochinchinensis* extract solution (3.13 mg mL^{-1}) + nanosilver (3.13 ppm)
CT4	*C. sappan* extract solution (3.13 mg mL^{-1})
CT5	*C. sappan* extract solution (3.13 mg mL^{-1}) + nanosilver (3.13 ppm)
CT6	*C. fragrans* extract solution (3.13 mg mL^{-1})
CT7	*C. fragrans* extract solution (3.13 mg mL^{-1}) + nanosilver (3.13 ppm)
CT8	Plant protection chemical (antibiotics) - Ankamycin 30SL (negative control).

The BLB resistance effect of each formula was evaluated based on the length of lesions on the rice leaves after 20 days of infection following the standards of the IRRI (1996): Length of lesions < 8 cm: Resistant (R); Length of lesions from 8-12 cm: Moderately resistant (M); Length of lesions > 12 cm: Susceptible (S).

Results and Discussion

Extraction

Maceration is a technique used in wine making and has been widely adopted in medicinal plant research. This technique is one of the easiest and simplest methods; nevertheless, organic wastes become an issue as a large volume of solvents are used and proper management of the waste is needed (Azwanida, 2015).

In the maceration method, the solvents used in the soaking process play a critical role in determining which compounds are extracted from the samples. Solvents are grouped into non-polar, polar aprotic, and polar protic solvents, and ordered by increasing polarity. Water and ethanol are protic solvents, while acetone and acetonitrile are aprotic ones. Chloroform and n-hexane belong to the non-polar group, and are usually used in plant oil extractions. By using solvents with different characteristics, we aimed to evaluate the extracting efficiency of each targeted herb corresponding to each type of solvent, thereby, establishing a reference for further research.

The results showed that following the same extraction protocol but with different solvents, the herbs produced different extracts with distinct colors. The initial conclusion would be that the quality of the herbal extracts (compounds, and physiological and chemical activities) depends largely on the solvent used. This is in line with the results of previously published authors (Thanh and Hai, 2014; Hai and Thanh, 2016; Dat and Tiep, 2016).

The liquid extracts from the plants were speed vacuumed until they reached a stable weight to obtain the crude extract. The final efficiencies of each extraction are shown in Table 1 and Figure 1. It demonstrated that with the same extraction method but with different solvents, each plant resulted in different efficiencies.

Table 1. The weight of crude herbal extracts (g) produced from 20 g of dried powder samples

Solvent plant	Distilled water	Ethanol 70%	Chloroform	n-hexane	Acetone 100%	Acetonitrile 100%
E. cochinchinensis	2.28^{b}	4.06^{a}	1.44^{c}	1.28^{c}	2.44^{b}	3.76^{a}
W. chinensis	2.56^{c}	3.76^{a}	1.38^{e}	1.16^{e}	2.15^{d}	2.93^{b}
C. sappan	3.30^{d}	5.14^{a}	2.16^{e}	1.90^{e}	4.70^{b}	3.78^{c}
P. longifolia	2.18^{c}	3.12^{a}	3.18^{a}	1.65^{d}	2.92^{b}	3.04^{a}
C. fragrans	3.56^{c}	5.36^{a}	1.68^{e}	1.66^{e}	5.02^{b}	3.06^{d}

Note: a, b, c, d, and e in the same row indicate statistically significant differences among means at P<0.05.

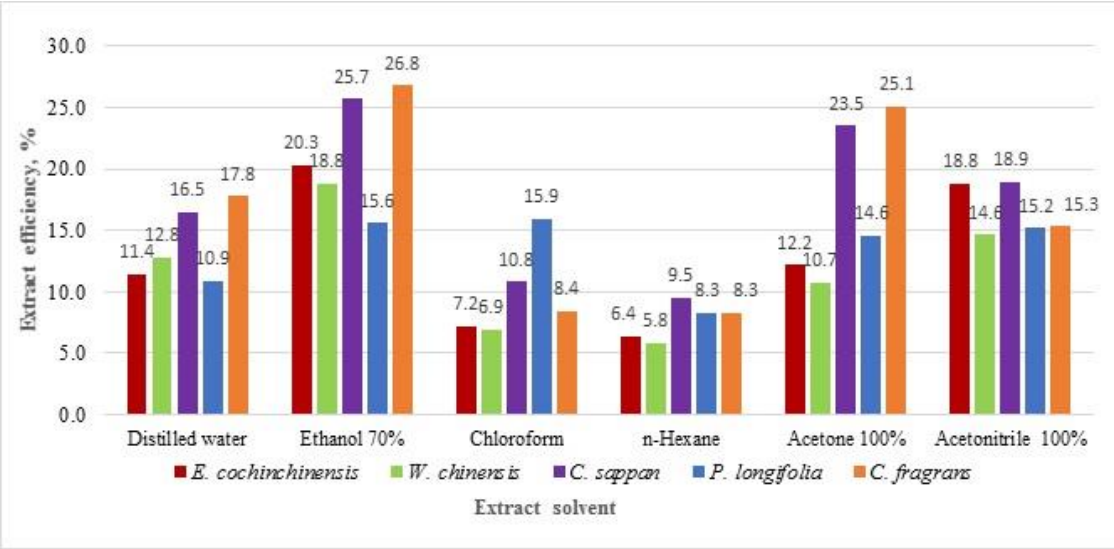


Figure 1. Extraction efficiency by maceration with different solvents (%)

Among the six solvents used, 70% ethanol showed the highest extraction efficiencies for the five plants. On the contrary, the two non-polar solvents, n-hexane and chloroform, showed the lowest efficiencies in almost all the plants (lower than 11%); the exception was *P. longifolia* with a 15.9% efficiency when extracted with chloroform.

When extracting with 70% ethanol, *C. fragrans* had the highest efficiency, with an average of 26.8%. and followed by *C. sappan* with an average of 25.7%. These plants also had the maximum efficiencies when extracted with acetone and distilled water. Although acetonitrile did not produce as high extraction efficiencies as ethanol and acetone when used to extract *C. sappan* and *C. fragrans*, it resulted in the acceptable efficiencies of 18.9% and 15.3%, respectively. From these obtained results, it is possible to conclude that a significant amount of relatively soluble substances were extracted from *C. sappan* and *C. fragrans* via polar solvents.

The antibacterial effects of plant extracts extracted from different solvents

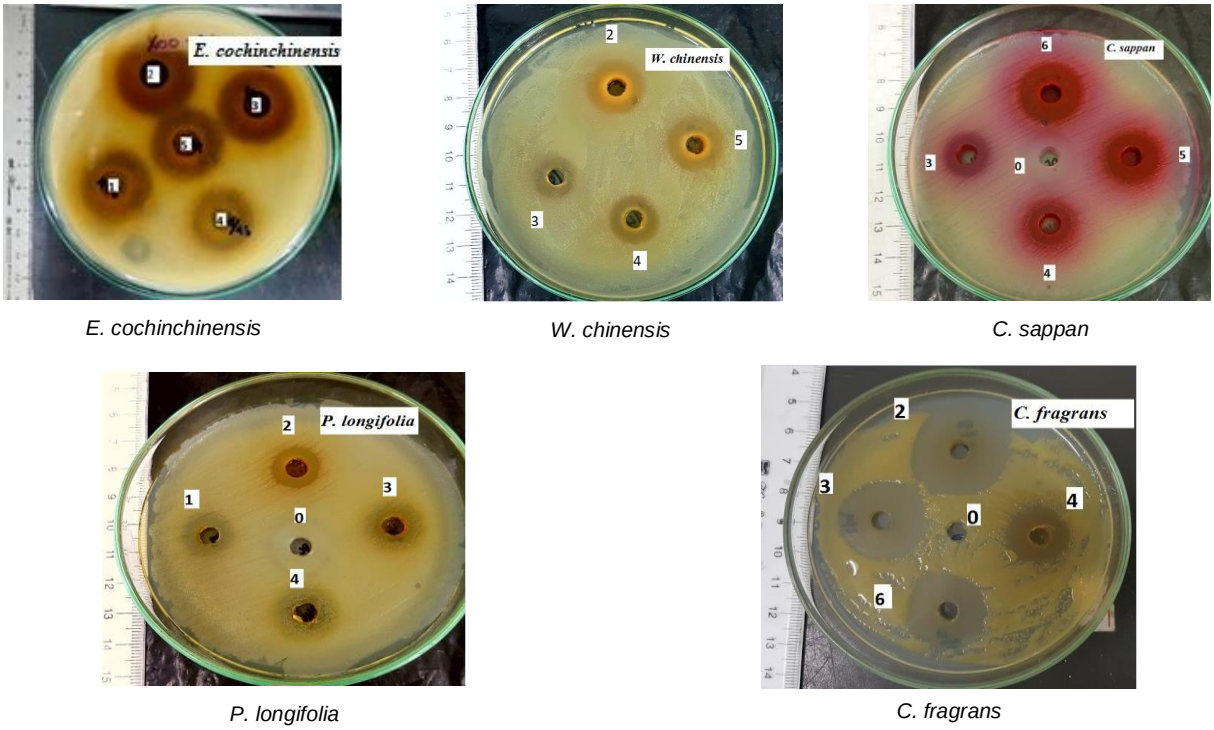
In order to accurately determine the ability of each solvent to dissolve compounds in the target plants that can inhibit *Xoo*, we diluted the crude extracts with 100 mg mL^{-1} DMSO and used the extract solutions in the agar diffusion experiment. The results obtained are shown in Table 2 and Figure 2, it can be seen that all of the five target plants were capable of inhibiting *Xoo* 07. However, the inhibitory capacity of each plant was different. The *W. chinensis* and *P. longifolia* extracts had weak resistance to *Xoo* 07. The inhibition zones obtained owing to

Table 2. Xoo 07 antibacterial effects of 100 mg mL^{-1} herbal extract solutions collected by maceration using different solvents

Solvent for extraction	Concentration of extract solution (mg mL^{-1})	Diameter of inhibition zone (mm)				
		E. cochinchinensis	*W. chinensis*	*C. sappan*	*P. longifolia*	*C. fragrans*
DMSO	Control	0	0	0	0	0
Distilled water	100	14.3 ± 0.6	11.3 ± 0.6	15.8 ± 0.4	12.9 ± 0.5	17.7 ± 0.5
70% ethanol	100	21.0 ± 0.4	14.0 ± 0.4	25.7 ± 0.6	16.2 ± 0.2	28.5 ± 0.4
Chloroform	100	18.3 ± 0.5	12.3 ± 0.2	16.0 ± 0.4	15.3 ± 0.2	27.0 ± 0.4
n-hexane	100	14.8 ± 0.2	11.1 ± 0.5	17.3 ± 0.6	14.2 ± 0.6	19.8 ± 0.6
Acetone 100%	100	11.8 ± 0.2	13.4 ± 0.2	20.2 ± 0.8	13.7 ± 0.5	17.3 ± 0.2
Acetonitrile 100%	100	18.0 ± 0.8	14.4 ± 0.4	21.0 ± 0.8	15.8 ± 0.4	21.3 ± 0.5

the effects of the *W. chinensis* extract solutions fluctuated from 11.1 mm to 14.4 mm, while the inhibition zones from the test of the *P. longifolia* extract solutions ranged from 12.9 to 15.8 mm, depending on the solvent used for the extraction. On the other hand, the *E. cochinchinensis*, *C. sappan*, and *C. fragrans* extract solutions (ethanol solvent) showed noticeable *Xoo* 07 antibacterial effects *in vitro* with average inhibition zones of 21.0 mm, 25.7 mm, and 28.5 mm, respectively.

The results of our study are comparable to the previous reports on *Xanthomonas* spp. Rahman *et al.* (2014) showed that an extract of *Poncirus trifoliata Rafin* resulted in inhibition zones varying from 13.00 mm to 22.10 mm depending on the species of bacteria and the concentration



E. cochinchinensis *W. chinensis* *C. sappan*

P. longifolia *C. fragrans*

Note: DMSO (Control, 0), Extracts from distilled water (1), Extracts from 70% ethanol (2), Extracts from chloroform (3), Extracts from n-hexane (4), Extracts from 100% acetone (5), Extracts from acetonitrile 100% (6).

Figure 2. *Xanthomonas oryzae* pv. *oryzae* antibacterial effects of 100 mg mL^{-1} extract solutions of different plants

of the extract, and for an extract of *Xanthomonas oryzae*, the inhibition zones ranged from 16.02 mm to 21.05 mm. Gioi and Hai (2017) examined the effects of *Piper betle* leaf extracts using a 70% ethanol solvent toward two isolates of *Xoo*, resulting in inhibition zones of 21.33 mm to 23.33 mm. However, all of our target plants in this study could not generate inhibition zones as large as those of an extract of *Adhatoda vasica*, which showed a 41-mm-diameter inhibition zone (Govindappa *et al.*, 2011).

According to Jabeen (2011), only 7 out of 25 plants in his study showed antibacterial effects toward *Xanthomonas oryzae*. Hence, it is important to test and find plants with potential antibacterial abilities. Combining the results of this experiment with the results of the first experiment, we can conclude that *E. cochinchinensis*, *C. sappan*, and *C. fragrans* are potential plants showing antibacterial properties against *Xoo* 07 when extracted by maceration with 70% ethanol. This conclusion was consistent with our predictions when referring to the study of Wang *et al.* (2014), who suggested that ethanol is a universal solvent for extracting and analyzing the chemical composition of herbs. Thus, the extracts of *E. cochinchinensis*, *C. sappan*, and *C. fragrans* by maceration using 70% ethanol will be used in further experiments.

The Xoo antibacterial effects of silver nanoparticles *in vitro*

To confirm the *in vitro* antibacterial effects and potential application of silver nanoparticles in the prevention and treatment of diseases in plants and animals, we conducted an experiment to test the effects of silver nanoparticles toward *Xanthomonas oryzae* pv. *oryzae* isolate 07.

In a study published in 2017, Gioi and Hai concluded that the minimum inhibitory concentration of nanosilver (MIC_{nano}) *in vitro* toward *Xoo* isolates 04 and 09 was 6.25 ppm. Based on the methods of that study, *Xoo* 07 in our research was directly exposed to silver nanoparticles at six different concentrations (25.00, 12.50, 6.25, 3.13, 1.56, and 0.00 ppm) for 4 h, then cultured onto Wakimoto solid medium, and incubated at 30ºC for 24 h. The results showed that all concentrations of the silver nanoparticles tested, except the 0.00 ppm concentration, were able to inhibit the tested bacteria, of which, the concentrations of 6.25 ppm, 12.50 ppm, and 25.00 ppm showed 100% inhibition (Figure 3). Thus, the results of our study are similar to those of Gioi and Hai (2017) in that we found 6.25 ppm to be the minimum inhibitory concentration of silver nanoparticles.

Other silver nanoparticle studies have found a range of different results. The lowest concentration of silver nanoparticles that inhibited germination of *S. sclerotiorum* was 7 ppm (Min *et al.*, 2009). According to research by Mahdizadeh *et al.* (2015), the MIC of silver nanoparticles varied from 6 ppm to 16 ppm depending on plant pathogenic microorganisms. However, according to a study by Jung *et al.* (2010), a concentration of 100 ppm of silver nanoparticles was required to completely eradicate *Sclerotium cepivorum*, the pathogen of white rot on onions. Similarly, the results of a study by Park *et al.* (2006) showed that 100 ppm was the MIC of silver nanoparticles toward

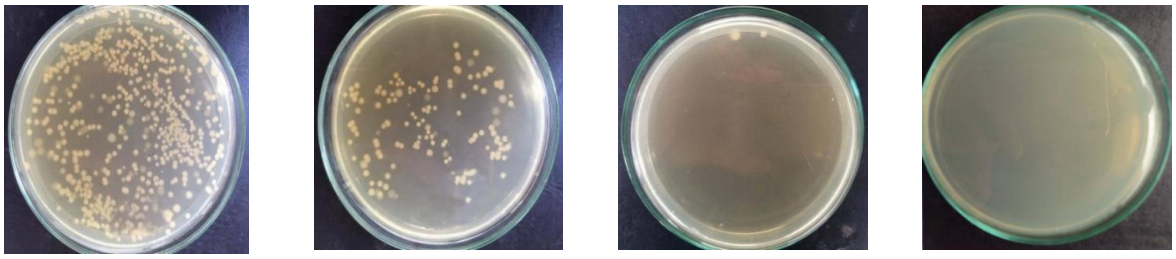


Figure 3. *In vitro* antibacterial effect of silver nanoparticles on *Xoo*

Xanthomonas campestris pv. versicatoria. The difference in MIC values of silver nanoparticles among the above experiments are due to the fact that the inhibiting ability of silver nanoparticles depends very much on their size (Umadevi *et al.*, 2011), and that the antibacterial ability of nanosilver varies depending on the type of bacteria being tested (Gioi and Hai, 2017).

Xoo antibacterial effects *in vitro* of plant extracts at different concentrations, and of mixtures of plant extracts with nanosilver

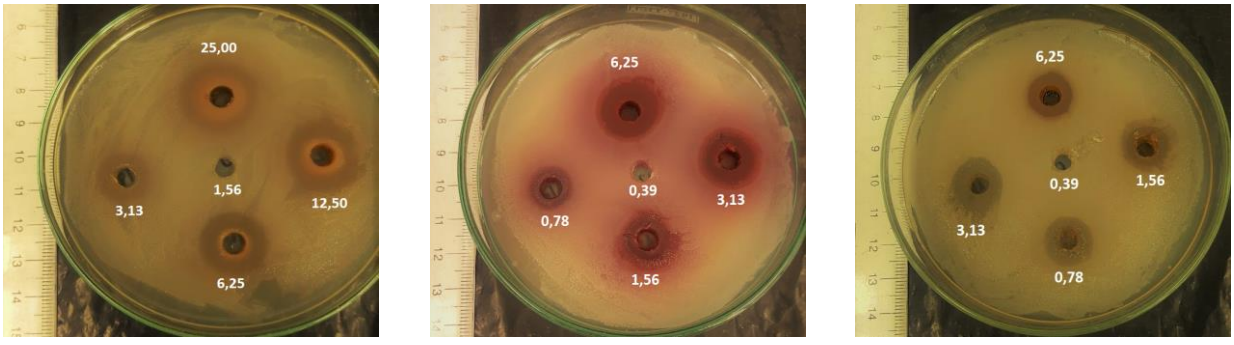
When it comes to producing plant protection products from herbal extracts, it is necessary to determine the minimum concentration of the extracts needed to ensure economic efficiency while saving resources. Therefore, we tested diluted *E. cochinchinensis*, *C. fragrans*, and *C. sappan* extracts (extracted by maceration with 70% ethanol) to determine the minimum concentrations of the extracts (MIC_{DC}) that were still capable of inhibiting bacteria.

Based on the antimicrobial activity of the Ag^{+2} ion, which has bacterial cell wall breaking capabilities, a combination of silver nanoparticles with the plant extracts would likely increase their antibacterial effects. Because the MIC of silver nanoparticles was previously determined as 6.25 ppm, a 2-times lower concentration of 3.13 ppm nanosilver was applied in the test and combined with the plant extracts to investigate their synergistic effects on bacteria. The results are shown in Table 3 and Figure 4.

Table 3. *In vitro* antibacterial effects of plant extracts with and without the supplement of silver nanoparticles

	Dilution factor of extract solution (corresponding concentration – mg mL^{-1})									
	2^{-1} (50)	2^{-2} (25)	2^{-3} (12.5)	2^{-4} (6.25)	2^{-5} (3.13)	2^{-6} (1.56)	2^{-7} (0.78)	2^{-8} (0.39)	2^{-9} (0.2)	2^{-10} (0.1)
Excoecaria cochinchinensis										
Extract solution	+	+	+	+	+	-	-	-	-	-
Extract solution + nanosilver	+	+	+	+	+	+	+	-	-	-
Caesalpinia sappan										
Extract solution	+	+	+	+	+	+	-	-	-	-
Extract solution + nanosilver	+	+	+	+	+	+	+	+	-	-
Clerodendron fragrans										
Extract solution	+	+	+	+	+	+	-	-	-	-
Extract solution + nanosilver	+	+	+	+	+	+	+	+	-	-

Note: (+): Inhibition zone observed; (-): No inhibition zone



Excoecaria cochinchinensis *Caesalpinia sappan* *Clerodendron fragrans*

Figure 4. *In vitro* antibacterial effects of plant extracts on *Xoo*

The lowest concentration of the *C. sappan* and *C. fragrans* extract solutions still able to inhibit the tested bacteria strain was 1.56 mg mL^{-1}. For *E. cochinchinensis*, the minimum inhibitory concentration increased to 3.13 mg mL^{-1}. The results of our experiment differed with the results of Hai and Thanh (2016) who used an *E. cochinchinensis* extract to suppress *Staphylococcus* spp. and *Streptococcus* spp. They found that the minimum inhibitory concentration of the extract of *E. cochinchinensis* from a chloroform solvent was 0.195 mg mL^{-1}. This variance could be explained by the different levels of resistance of each bacterium toward the extracts of *E. cochinchinensis*. The results also showed that the plant extracts, when mixed with silver nanoparticles, had better *Xoo* inhibitory effects. Namely, the *C. sappan* and *C. fragrans* extracts, each at the concentration of 0.39 mg mL^{-1}, when mixed with 3.13 ppm of silver nanoparticles were able to inhibit the bacteria. The *E. cochinchinensis* extract solution, at the concentration of 0.78 mg mL^{-1}, when mixed with a 3.13 ppm of nanosilver solution also showed antibacterial activity. These results are in line with the study by Gioi and Hai (2017) who showed increasing bactericidal effects of plant extracts by mixing them with silver nanoparticles, namely, the mixing of a *Piper betle* extract with nanosilver to inhibit Xoo *in vitro*.

Effects of mixtures of the plant extracts with nanosilver on inhibiting rice blight caused by Xoo *in vivo*

The results of the previous experiments showed that the extracts of *E. cochinchinensis*, *C. sappan*, and *C. fragrans* were able to inhibit *Xoo in vitro*, and had better performances when mixed with silver nanoparticle solutions. The results presented in Table 4 and Figure 5 showed that *Xoo* 07 was highly pathogenic in the IR24 rice variety. In the positive control formula (CT1), in which distilled water was sprayed on the leaves, after 20 days of infection, the lesion lengths averaged 15.0 cm, and the leaves were no longer green and lost their ability to conduct photosynthesis (Figure 5). According to the evaluation criteria, the IR24 was highly susceptible to *Xoo* 07 causing BLB.

Kasugamycin, the main active ingredient in the Ankamycin product, an antibiotic extracted from the fermentation of Streptomyces kusagaensis, and one of the available antibiotics still effective with *Xoo*, was used as the negative control in the experiments. As shown in Figure 5, when Ankamycin was applied to the infected leaves, the lengths of the lesions in CT8 were significantly shorter in comparison to CT1 (from 15.00 cm to 1.50 cm).

The six formulas (CT2-7) drawn from this research all reduced the pathogenicity of *Xoo* 07 in the IR24 rice variety. In the absence of silver nanoparticles, the rice plants sprayed with the *C. fragrans* extract solution achieved disease resistance according to the criteria of the IRRI (7.17 cm lesion length average). The *E. cochinchinensis* and *C. sappan* extract solutions also showed significant disease resistance with lesion lengths of 9.10 cm and 8.23 cm, respectively (moderate resistance). The mixture of the *C. sappan* extract with silver nanoparticles (CT5) and the mixture of the *C. fragrans* extract with silver nanoparticles (CT7) resulted in

Table 4. IR24 rice varieties after being artificially infected and treated with different formulas

	CT1	CT2	CT3	CT4	CT5	CT6	CT7	CT8
Length of lesions (cm)	15.00 ± 0.82	9.10 ± 0.85	7.03 ± 0.21	8.23 ± 0.54	4.50 ± 0.85	7.17 ± 1.03	4.40 ± 0.43	1.50 ± 0.41
Proportion of infected plants (%)	100	66.12	40.66	49.75	36.12	41.33	31.25	29.75
Evaluation of disease resistance	S	M	R	M	R	R	R	R

Note: S - Susceptible, M - Moderate resistance, R - Resistance.

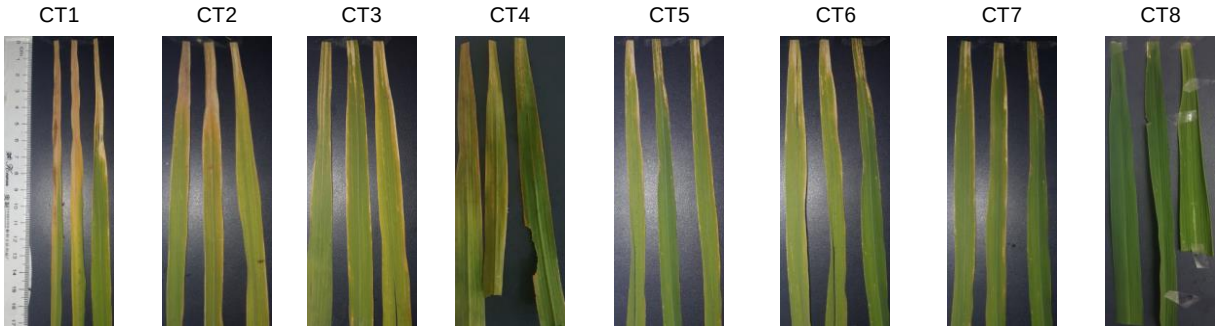


Figure 5. Length of lesions on IR24 rice variety after artificial inoculation

excellent disease resistance, as the lengths of the lesions were significantly shorter compared to the control (15.00 cm verses 4.40-4.50 cm). Silver nanoparticles also increased the effects of the *E. cochinchinensis* extract, and the rice plants treated with CT3 were resistant to BLB.

According to Jabeen (2011), *Terminalia chebula*, *Amomum subulatum*, and *Thuja orientalis* extracts inhibited BLB *in vivo*; the lesion lengths were much shorter than those of the control. The *Terminalia chebula* leaf extract gave the best results as BLB management increased by 83.25% compared to the control. Also, according to the study, when applied in the field, *Terminalia chebula* extracts showed noticeable results in managing BLB caused by *Xoo*. *Piper betle* leaf extracts, according to Gioi and Hai (2017), have also shown positive BLB control abilities *in vivo*.

The results of our study and the cited authors have confirmed the ability of herbal extracts to inhibit *Xoo in vitro* and control rice bacterial leaf blight *in vivo*. These studies are providing increasing support of the feasibility of replacing toxic chemicals with non-toxic plant-derived compounds for pest and disease management, thereby, reducing environmental pollution, ensuring food safety and public health, and developing clean and sustainable agriculture.

Conclusions

Among the experimental solvents, 70% ethanol resulted in the highest extraction efficiencies for all five target plants. At concentrations of 100 mg mL^{-1}, the *C. fragrans* extract resulted in the largest inhibition zones averaging 28.5 ± 0.4 mm in diameter, followed by *C. sappan*, and then *E. cochinchinensis*, *W. chinensis* and *P. longifolia* showed poor resistance to *Xoo*.

Extracts of *E. cochinchinensis*, *C. sappan*, and *C. fragrans* (extracted with 70% ethanol solvent) still had *Xoo* antibacterial activity after being diluted. The minimum inhibitory concentration (MIC_{DC}) of *C. fragrans* and *C. sappan* was both 1.56 mg mL^{-1}, but the MIC_{DC} for *E. cochinchinensis* was 3.13 mg mL^{-1}. When mixed with 3.13 ppm of nanosilver, the MIC_{DC} of *C. sappan* and *C. fragrans* reduced to 0.39 mg mL^{-1}, and the MIC_{DC} of *E. cochinchinensis* reduced to 0.78 mg mL^{-1}. Using the *E. cochinchinensis*, *C. sappan* and *C. fragrans* extracts independently or mixing them with 3.13 ppm of nanosilver all resulted in *Xoo* inhibitory effects *in vivo*.

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