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Anti-biofilm formation effects of aromatic and medicinal plant extracts on *Xanthomonas axonopodis* pv. *Phaseoli*

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Common bacterial blight (CBB) caused by *Xanthomonas axonopodis* pv. *phaseoli* (Xap) have been recorded as one of the most important seed-borne destructive diseases on beans. Chemical compounds which are incessantly used on beans to manage CBB disease cause serious damage to natural ecosystems and sometimes they show frequent failure for controlling CBB. These consequences are attributed to the use of by-products from aromatic and medicinal plants. The active ingredient of these plants can inhibit the biofilm formation of different bacterial pathogens. In this study, the effect of aromatic and medicinal plant extracts on biofilm formation of *X. axonopodis* pv. *phaseoli* were investigated. The result showed that all plant extracts significantly reduced the biofilm formation of Xap as compared to the control treatment. Twelve of the 14 plant extracts inhibited Xap biofilm formation by >50%. The extract of Clove *(Syzygium aromaticum* (L.), Coriander *(Coriandrum sativum* L.), Thyme *(Thymus vulgaris* L.), Mustard *(Brassica nigra* (L.), Garden Rue *(Ruta chalepensis* L.), Garden cress *(Lepidium sativum* L.), Black cumin *(Nigella sativa* L.) and Black pepper *(Piper nigrum* L.) inhibited Xap biofilm formation by 90.85, 87.67, 84.94, 81.10, 79.93, 77.33, 75.17 and 72.02%, respectively. This clearly indicated that crude extracts of *S. aromaticum, C. sativum, T. vulgaris, B. nigra, R. chalepensis, L. sativum, N. sativa,* and *P. nigrum* had the greatest effect on biofilm formation and to be a potential candidate as anti-biofilm agents in preventing common bacterial disease of beans caused by Xap.

Key words: Anti-biofilm activity, plant extracts, *Xanthomonas axonopodis* pv. *phaseoli.*

INTRODUCTION

Biofilms are a mass of bacterial communities which is formed when bacterial cells are embedded in a matrix of extracellular polymeric compounds attached to a surface. It protects bacteria from internal and external deleterious disorders and an important factor in the disease cycle of both animal and plant bacterial pathogens (Bogino et al., 2013; Dwivedi et al., 2017). Since many bacterial pathogens can form biofilm and its formation can cause serious problems, therefore the development or screening of biofilm inhibiting factors becomes significant.

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The different plant extracts and essential oil substances are playing a great role to inhibit the formation of biofilms in pathogenic plant-bacteria (Sevindik et al., 2017; Pehlivan et al., 2018; Sevindik, 2018; Mohammed et al., 2019). According to Koo and Jeon (2009) and Kim and Park (2013), some biofilm inhibitors have been isolated from medicinal plants, which are advantageous because these inhibitors are generally less toxic and more specific compared to synthetic compounds. Different researchers screened medicinal plants and identified their effect on the biofilm formation of different bacterial pathogens. Kim and Park (2013) evaluated ginger's ability to inhibit *Pseudomonas aeruginosa* PA14 biofilm formation and demonstrated that biofilm development was reduced by 39 to 56% when the ginger extract was added to the culture. Garlic was also found to inhibit biofilm formation in *P. aeruginosa* and prevented nematode death in a limited analysis (Rasmussen et al., 2005). Plant extracts which are belonging to the Meliaceae, Melastomataceae, Lepidobotryaceae, Sapindaceae, and Simaroubaceae families have been reported to significantly reduce the biofilm activities of *Chromobacterium violaceum* ATCC 12472 and *P. aeruginosa* PA1 (Ta et al., 2014).

The anti-virulence activity of essential oils from different plant parts against *X. oryzae* pv. *oryzae*, the causal agent of leaf blight disease in rice, was screened by Singh et al. (2017b). In their study, there was a decrement in biofilm formation in 9 plant oils out of 15 without a considerable decrease in total colony-forming units. Similarly, the effect of thyme oil on *X. oryzae* pv. *oryzae* biofilm formation was analyzed by Singh et al. (2017a). The results revealed that thyme oil displayed a significant diminution in swimming, swarming, exopolysaccharide, and Xanthomonadin secretion of the given bacteria. In the same study, disease reduction was observed in an in vitro agar plate assay as lesion length was reduced in thyme oil-treated *X. oryzae* pv. *oryzae* cells when compared with the known treatment (Singh et al., 2017a). The leaf extracts of *Mangifera indica* L. can reduce considerably (36–82%) the biofilm formation by *P. aeruginosa* PAO1 and *Aeromonas hydrophila* WAF38 over the control (Husain et al., 2017). Zhang et al. (2014) also reported that *Rosa rugosa tea* polyphenol extract exhibited inhibition in biofilm formation (67.02 and 72.90%) of *Escherichia coli* K-12 and *P. aeruginosa* PAO1 in a concentration-dependent manner, respectively; however, there was no study about the anti-biofilm formation effects of different plant extracts in the case of *X. axonopodis* pv. *phaseoli* (Xap).

Chemical pesticides applied on beans to control *X. axonopodis* pv. *phaseoli* (Xap) causes serious damage on agricultural and natural ecosystems and showed frequent failure for controlling Xap. These consequences are attributed to the development of eco-friendly alternative disease management strategies (the most effective, economic, and healthy control strategy in the long run). In this connection, the importance of medicinal and aromatic plants in crop protection is being increasingly recognized under the concept of biological control. The active ingredient of these plants can inhibit the biofilm formation of different bacterial pathogens. However, there was no study about the anti-biofilm formation effects of different plant extracts in the case of Xap. Therefore, the objective of this study was to examine the effects of aromatic and medicinal plant extracts for their anti-biofilm formation activities against Xap.

MATERIALS AND METHODS

Crude extract preparation

Based on the procedures described by Ibrahim and Abu-Salem (2014), Houshyar et al. (2014), Yavuz et al. (2017), and Ramena et al. (2018), the stock extract solutions of Cinnamon *(Cinnamon zeylanicum)*, Clove *(S. aromaticum)*, Thyme *(T. vulgaris),* Mustard *(B. nigra),* Black cumin *(N. sativa* L.), Garden cress *(L. sativum),* Ethiopian Mustard *(Brassica carrinata* A. Braun*),* Garlic (*Allium sativum* L.*),* Turmeric *(Curcuma longa* L.*),* Coriander *(C. sativum),* Black pepper *(Piper nigrum* L.), Garden Rue *(Ruta chalepensis* L.*),* Moringa (*Moringa oleifera Lam.)* and Rosemary *(Rosmarinus officinalis* L.*)* were prepared. From each plant extract, 50g fine powder was soaked in 500 ml of 99% (v/v) methanol in a sterile container and incubated at normal temperature for one week. After 7 days, the extract was filtered through Whitman filter paper and processed with Heidolph Rotary Evaporator (distillation machine) for 40°C at 140rpp for the evaporation process. For complete removal of the remaining methanol, the extracts stayed in the water bath for one day at 40°C. Finally, the semi-solid plant extract residues were stored aseptically in a sterilized container at -20°C until use.

Effects of plant extract for anti-biofilm formation activities against Xap

The effects of aromatic and medicinal plant extracts on Xap biofilm formation was evaluated as per the procedures of Coffey and Anderson (2014), Singh et al. (2017a), Singh et al. (2017b), Omwenga et al. (2017), and Choi et al. (2017) with some modifications. Xap isolate was inoculated onto nutrient agar (NA) media and incubated at 28°C for 48 h to prepare a bacterial suspension. A 2mL collection tube/Eppendorf tube was used to quantify the effect of plant extracts on the biofilm formation of Xap isolate. The two-day-old Xap isolate was suspended in sterile distilled water (SDW) and adjusted to 10^8 CFU/mL using a bio-photometer. Once the sterilized collection/Eppendorf tube was prepared, 500 µL (0.5 mL) of nutrient broth (NB) was added to each tube with 50 µL of the prepared Xap suspension. The same amount of plant extracts (50 µL) prepared in 5% Dimethyl Sulfoxide (DMSO) was added to each Eppendorf tube with three replications. A 50 µL of 5% (V/V) DMSO was used as a negative control. After this, each tube was sealed with a parafilm and incubated for 48 h at 28°C without shaking. After 48 h of incubation, the suspensions (planktonic cells) in the Eppendorf tubes were poured out and rinsed with 500 µL of SDW three times to remove the planktonic bacterial cells. The Eppendorf tubes were air-dried for 30 min at room temperature at the laminar cabin. After 30 min air drying, adherent (attached) bacterial cells in the Eppendorf tubes were stained with 500 µL of 0.5% (w/v) of crystal violet (CV) solution and left for 30 min at room temperature. Excess CV (stain) was then removed by washing each tube with 500 µL of SDW three times.

Table 1. Mean of Biofilm formation of Xap in the presence of some aromatic and medicinal plant extracts at OD 650 nm absorbance and percent biofilm inhibition.

Name of plant extracts	Absorbance	Biofilm inhibition/reduction rate (%)
Cinnamon	0.56 ± 0.03^{e}	68.35 ±1.56^{h}
Clove	0.16 ± 0.01^{L}	90.85 ±0.43^{a}
Thyme	0.26 ± 0.01^{j}	84.94 ±0.59^{c}
Mustard	0.33 ± 0.01^{i}	81.10 ±1.01^{d}
Black cumin	0.44 ± 0.01^{g}	75.17 ±0.90^{f}
Garden cress	0.40 ± 0.00^{h}	77.33 ±0.44^{e}
Ethiopian mustard	0.72 ± 0.02^{c}	59.17 ± 0.43^{j}
Garlic	0.61 ± 0.01^{d}	65.41 ±0.28^{i}
Turmeric	0.91 ± 0.03^{b}	48.04 ±1.94^{k}
Coriander	0.22 ± 0.01^{k}	87.67 ±0.44^{b}
Black pepper	0.49 ± 0.00^{f}	72.02 ±0.51^{g}
Garden Rue	0.35 ± 0.01^{i}	79.93 ± 0.57^{d}
Moringa	0.93 ± 0.03^{b}	47.12 ± 2.02^{k}
Rosemary	0.55 ± 0.01^{e}	68.48 ±0.41^{h}
Control (only Xap)	1.76 ± 0.03^{a}	

Means that do not share a common letter within a column are significantly different from each other at P ≤ 0.05 according to DMRT.

The Eppendorf tubes were air-dried for 30 min again at room temperature. The adherent stained cells were re-solubilized by adding 500 µL of 100% DMSO in each tube and transfer the solution to a sterilized plastic disposable cuvette (Eppendorf UVette®) to measure biofilm. Finally, the intensity of biofilm formation from each plant extract sample was measured and quantified at optical density (OD) 650 nm absorbance using Eppendorf BioPhotometer, and the percentage of biofilm inhibition (biofilm reduction rate) of Xap affected by each plant extract was determined by the following formula.

$$\%\ \text{Biofilm inhibition (biofilm reduction rate)} = \frac{\text{OD at control Sample} - \text{OD at Test Samples}}{\text{OD at Control Sample}} \text{x100}$$

The biofilm assay was done twice having three replicates each and the average OD values were considered.

Statistical analysis

The data were analyzed for statistical significance using analysis of variance (ANOVA) of SAS software version 9.1.3 and mean separation and grouping by letters were carried out using Duncan's Multiple Range Test (DMRT) at 0.99 level of confidence and the average data are presented as means ± standard deviation.

RESULTS

The effects of aromatic and medicinal plant extracts on biofilm formation at OD 650 nm absorbance and their percent inhibition (%) against Xap isolate as compared to the control treatment were presented in Table 1 and Figures 1 and 2. The different aromatic and medicinal plant extracts on bacterial biofilm formation showed a significant (P ≤ 0.01) difference. The effects of all crude plant extracts were found to significantly reduce the biofilm formation of Xap as compared to the control treatment (Table 1). Twelve of the 14 tested plant extracts inhibited Xap biofilm formation by >50%. The extract of *S. aromaticum, C. sativum, T. vulgaris, B. nigra, R. chalepensis, L. sativum, N. sativa,* and *P. nigrum* inhibited Xap biofilm formation at 90.85, 87.67, 84.94, 81.10, 79.93, 77.33, 75.17 and 72.02%, respectively (Table 1). These clearly indicated that crude extracts of *S. aromaticum, C. sativum, T. vulgaris, B. nigra, R. chalepensis, L. sativum, N. sativa* and *P. nigrum* had the greatest effect on biofilm formation of Xap. Reduction by 68.48, 68.35, 65.41, and 59.17% of Xap biofilm formation were obtained by the extract of *R. officinalis, C. zeylanicum, A. sativum,* and *B. carrinata,* respectively. Two out of the 14 extracts reduced Xap biofilm formation by <50% (*M. oleifera* and *C. longa*). Even though they had less effect (<50%) as compared to other plant extracts, they were significantly different by far from the control treatment (Table 1).

DISCUSSION

Biofilm inhibition is being looked upon as a potential drug target for managing notorious bacterial pathogens because it serves as a pool for antibiotic resistance genes in addition to providing refuge to bacteria from external attack (Sánchez et al., 2016; Singh et al., 2017a). Screening of different aromatic and medicinal plants should be done to detect biofilm inhibitors because the formation of bacterial biofilm can cause serious problems in different settings (Kim and Park, 2013). The anti-biofilm formation activity of aromatic and medicinal plant extracts was tested using the crystal violet method (Figure 3). This method is widely used by different

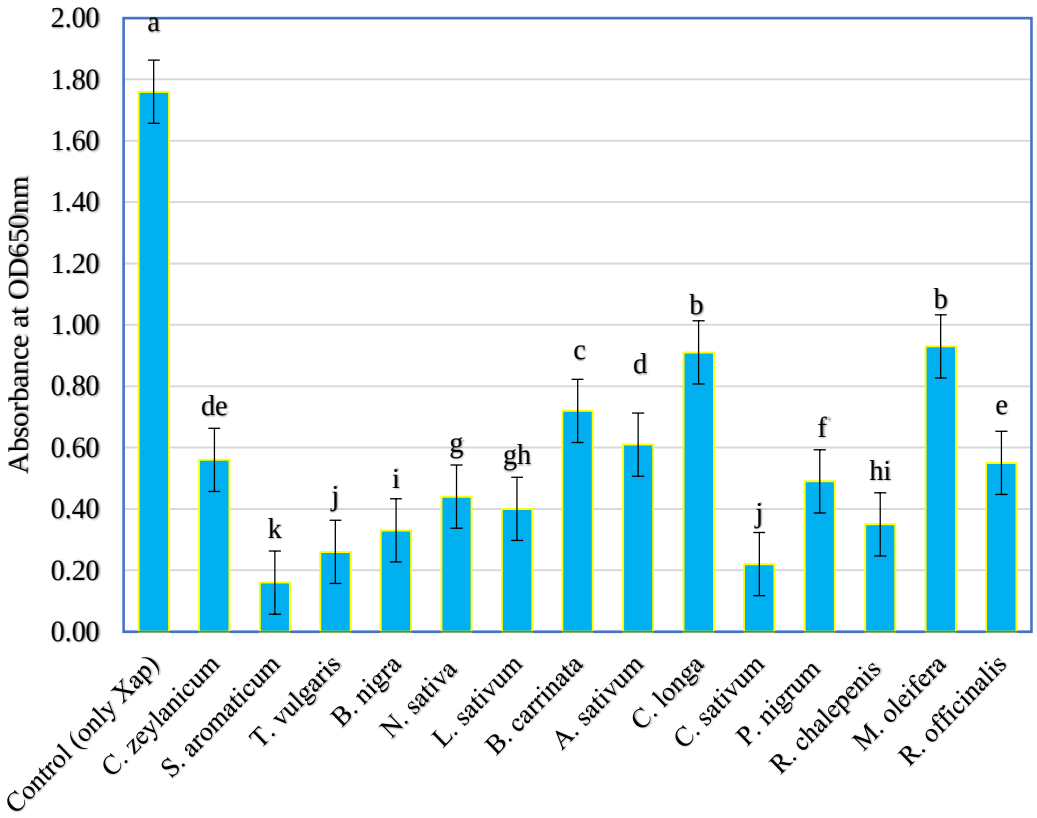

Figure 1. Biofilm formation of Xap in the presence of aromatic and medicinal plant extracts at OD 650 nm absorbance. Means that do not share a common letter in each bar are significantly different according to DMRT (P ≤ 0.05) and vertical bars indicate standard errors of the mean.

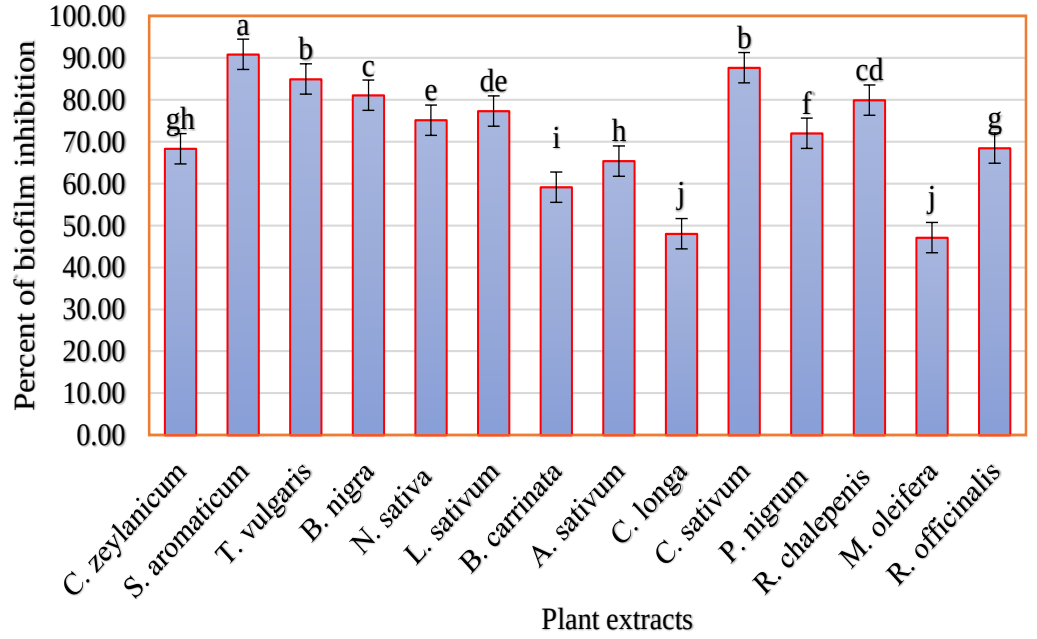

Figure 2. Biofilm formation reduction rate of Xap affected by aromatic and medicinal plant extracts. Means that do not share a common letter in each bar are significantly different according to DMRT (P ≤ 0.05) and vertical bars indicate standard errors of the mean.

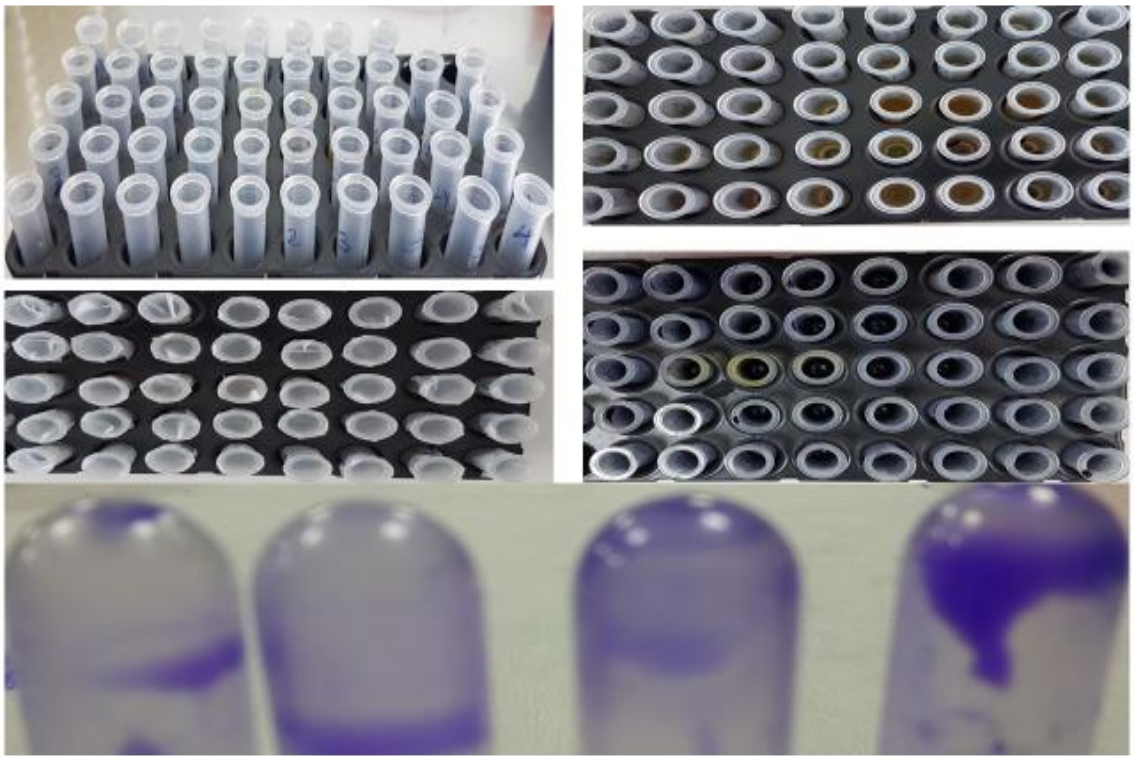

Figure 3. Anti-biofilm formation activity of aromatic and medicinal plant extracts against Xap using the crystal violet staining method.

scientists (Coffey and Anderson, 2014; Namasivayam and Vivek, 2016; Singh et al., 2017a, b; Omwenga et al., 2017; Choi et al., 2018) because it is not expensive and could be repeated many times in order to ensure accurate results. In our study, the aromatic and medicinal plant extracts (*S. aromaticum, C. sativum, T. vulgaris, B. nigra, R. chalepensis, L. sativum, N. Sativa, P. nigrum, R. officinalis, C. zeylanicum, A. sativum,* and *B. carrinata*) used in crystal violet assay indicated anti-biofilm formation activity against Xap. Different results were obtained for the various tested aromatic and medicinal plant extracts. Namasivayam and Vivek (2016) screened the anti-biofilm formation effect of six medicinal plant extracts and all the tested extracts were reduced significantly the biofilm formation of *P. aeruginosa*. Singh et al. (2017a) investigated the biofilm effect of *T. vulgaris* plant oil against *Xanthomonas oryzae* and reported that the biofilm formation of *X. oryzae* was significantly decreased in a concentration-dependent manner. Likewise, the anti-biofilm activity of medicinal plant essential oils against *X. oryzae* indicated a reduction in biofilm formation of *X. oryzae* (Singh et al., 2017b). In another study reported by Kim and Park (2013) the different concentrations of ginger extract tested prevented the amount of biofilm formation by 39-56% less than the amount in the control treatment (without plant extract). The anti-biofilm effect of several medicinal plant extracts against *Streptococcus* mutants and *Candida albicans* were screened in Korea by Choi et al. (2017), eight out of 37 plant extracts were presented biofilm inhibitory activities against both bacterial pathogens. The polyphenolic extract of *R. rugosa* exhibited inhibition in biofilm formation by 67 and 73% against *E. coli* and *P. aeruginosa,* respectively (Zhang et al., 214).

CONCLUSION AND RECOMMENDATION

The present study investigated about 14 aromatic and medicinal plant extracts that exhibited various biological activities. Some of these plants have never been studied anywhere against the selected bacterial pathogen. To the best of our knowledge, no reports are available regarding the anti-biofilm activity of Xap by these plant extracts. Therefore, these aromatic and medicinal plants could be used to manage *Xanthomonas* pathogenesis and hinder its dissemination. Generally, *S. aromaticum, C. sativum, T. vulgaris, B. nigra, R. chalepensis, L. sativum, N. sativa,* and *P. nigrum* are the most potentially useful extracts in preventing and inhibiting the biofilm formation of Xap.

As a recommendation, further studies should be carried out to determine other methods toward increasing yield of

potent plant extracts. Also, studies on purification of crude extracts to ascertain pure compounds and their concentrations.

CONFLICT OF INTERESTS

The author has not declared any conflict of interest.

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