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ASSESSMENT OF METABOLITES OF *ALTERNARIA BRASSICICOLA* AS A NATURAL BACTERICIDE

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Abstract

Plant pathogenic bacteria cause diseases in a variety of economic plants by using cell wall degrading enzymes. Many fungal species are used as biocontrol agents with promising results against a wide variety of plant pathogens including bacteria. Biocontrol potential of metabolites of *Alternaria brassicicola* was tested against three species of plant pathogenic bacteria viz. *Xanthomonas campestris*, *Pseudomonas syringae* and *Stenotrophomonas maltophilia* under laboratory conditions. Bacterial species were grown on lysogeny broth agar medium by well diffusion and disc diffusion methods and then were exposed to fungal metabolites by using wells and discs. A significant reduction in the bacterial growth was experienced during this study under all the test concentrations of fungal metabolites. Among the tested concentrations, maximum inhibition was shown by 100% followed by 50%. No growth of bacterial colony was seen in the presence of antibacterial agent streptomycin while maximum growth was obtained in pure water. Both the employed methods were effective, however better results were obtained with disc method.

Keywords: *Alternaria brassicicola,* Biocontrol, Fungal metabolites, Natural Bactericide, *Pseudomonas syringae*, *Stenotrophomonas maltophilia*, *Xanthomonas campestris*

INTRODUCTION

Alternaria species are ubiquitous in occurrence, well known for mycotoxin production with different toxicological properties. *Alternaria* metabolites possess phytotoxic, cytotoxic, and antimicrobial properties. Due to these activities such metabolites have attracted chemists, pharmacologists, and plant scientists. Recently more metabolites from *Alternaria* species have been isolated and characterized (Lou *et al.,* 2013). Twenty-six bioactive compounds have been identified from methanolic extract of *Alternaria* and analyzed for their antibacterial and antifungal activities by (Kamal *et al*., 2017).

Plant pathogens are of great concern for crop production throughout the world and demand for a sustainable control mechanism for crop diseases to provide food for ever growing population of the world (Ayaz *et al*., 2021). Plants are attacked by many pathogens, viz, fungi, viruses and bacteria. Almost one hundred bacterial species cause plant diseases. Most bacterial diseases are in tropical or subtropical regions of the world. To cause infection in plants bacteria possess specific pathogenicity factors like cell wall degrading enzyme, phytohormones, exo- polysaccharides, effectors proteins and toxins. A variety of plant diseases are caused by many groups of bacteria viz. *Pseudomonas* (leaf spots, soft rots, cankers, galls,

vascular wilts and blights), *Xanthomonas* (leaf spots, fruit spots, vascular wilts, blights of annual and perennial plants, necrosis, and the canker of grapevines), *Erwinia* (soft rot, wilt of corn, fire blight of apple and pear), *Acidovorax* (leaf spots in corn, watermelon and orchids), *Agrobacterium*, *Pseudomonas syringae* (wilts in tomato), *Ralstonia* (wilts of solanaceous crops), *Arthrobacter* (Douglass-fire bacterial gall), *Clavibacter* (wilts in potato, tomato, and the alfalfa, and *Streptomyces* (potato scab). These bacteria use cell wall degrading enzymes to affect the plants (Sharma *et al.,* 2023).

Xanthomonas spp., a large genus of Gram-negative plant pathogenic bacteria that causes disease to many plants/crops and exploits various virulent factors for pathogenicity (Timilsina *et al.,* 2020) and fitness in host. It has been isolated from fruits and vegetables (Soenens and Imperial, 2020). Black rot disease can reduce more than fifty percent yield is due to *X. campestris* (Rubel *et al*., 2017). *Pseudomonas syringae* is an opportunistic pathogen and attacks woody plants especially when damaged by injury. The main symptoms of the *pseudomonas* spp. are bacterial canker, the dark angular necrotic leaf spots, flower necrosis and blight, twig wilting and dieback, reddening of the lenticels, and fruit collapse (Donati *et al*., 2014). *Stenotrophomonas maltophilia,* infect shoot tips, leaves and fruits that results in plant death (Berg and Martinez, 2015). It is a motile, non-fermentative, gram negative plant pathogenic bacillus bacteria that is readily isolated from wide variety of environments and geographical regions. It has been isolated from soil and a variety of rhizosphere environments. This bacterium is cosmopolitan in its occurrence and is found associated with opportunistic infections (Abbot *et al.,* 2011).

At present plant diseases are being controlled by growing resistant varieties and applying pesticides with promising results. However, the use of chemicals as pesticides has posed serious threat to the environment thus limiting their scope in agriculture (Gao *et al*., 2016). Therefore, now a day's scientists are exploiting the beneficial microorganisms as an alternate to the chemical pesticide for the management of crop diseases and a variety of bacteria and fungi have successfully been used for this purpose with promising results against important pathogens of economic crops. Recent researches on the use of fungal strains for controlling plant diseases has attracted the scientists worldwide. The antagonistic potential of such fungi makes them potential bio-pesticides against a wide range of soil and airborne plant pathogens both in greenhouse experiments and during field trials (Zubair et al., 2021). Biocontrol agents interact with the plant pathogens in a variety of ways by exploiting one or a combination of processes directly or indirectly to reduce plant disease (Ayaz *et al.,* 2021). The present study was designed to exploit the secondary metabolites of *A. brassicicola,* as an alternative to chemical control, against devastating bacterial pathogens of economic importance. The aim of present investigations is screening of *A. brassicicola* as a natural bactericide.

MATERIALS AND METHODS

PROCUREMENT OF FUNGAL AND BACTERIAL SPECIES

Fungus species *A. brassicicola* (FCBP-AF-1098) and bacterial species *P. syringae* (FCBP-PB-0009), *S. maltophilia* (FCBP-PB-0095) and *X. campestris* (FCBP-PB-0003) was obtained from Fungal Culture Bank of Pakistan, Faculty of

Agriculture Sciences, University of the Punjab, Lahore, Pakistan.

PREPARATION OF FUNGAL METABOLITES AND BACTERIAL SUSPENSIONS

2% Malt Extract Agar (MEA) and Lysogeny Broth Agar (LBA) media were used for sub culturing of fungal strain and for the evaluation of antibacterial activity of fungal metabolites. The fungal metabolites were obtained using MEA media. Inoculated MEA with full grown culture of *A. brassicicola* and incubated in shaking incubator at 27±2ºC for 27 days, followed by double filtration. 3 concentrations (0%, 50% and 100%) were made for test. Inoculated bacteria in LBA and incubated at 32±2ºC. The bacterial suspensions of test bacterial species were prepared using distilled water.

ASSESSMENT OF ANTIBACTERIAL ACTIVITY OF FUNGAL EXTRACTS

Antibacterial activity of this extract containing fungal metabolites was examined with the help of two methods namely, well diffused method and disc method. Lysogeny broth media was used for this purpose. Bacterial suspensions were spread over LBA media prepared plates. 50µl of fungal extract was loaded to wells. These plates were then incubated at 37°C for 24 hours and examined. Similarly, in disc method, the discs were soaked in different concentrations viz. 0%, 50%, 100% of fungal metabolites for 20-25 minutes approximately. Streptomycin was used as antibacterial agent (Control). Afterwards, 2-3 discs were placed on each petri plate. These petri plates were then incubated at 37°C for 24 hours and examined. After incubation period the zone of inhibition for three target species of bacteria was measured.

FOURIER TRANSFORM INFRARED SPECTROSCOPY

The fungal metabolites were subjected to FTIR for the identification of antibacterial compounds. The spectra were recorded in the region between 4000 to 1000 cm^{-1} (Kosa *et al.,* 2017).

STATISTICAL ANALYSIS

All the collected data was subjected to analysis of variance (ANOVA) to evaluate the significance of the difference (Steel and Torrie, 1980).

RESULTS AND DISCUSSIONS

Anti-bacterial activity of metabolites of *A. brassicicola* was evaluated for their antibacterial activity against three species of bacteria i.e. *X. campestris* and *S. maltophilia* through well and disc methods. For this purpose, different concentrations of fungal metabolites i.e. 0%, 50%, 100% with control were employed. Each treatment was in triplicate. Streptomycin was added as an antibacterial agent. The growth spectrum of *X. campestris* under different test treatments and streptomycin depicted a varying response. Very little growth was observed in the petri plates with streptomycin. Among different concentrations of fungal metabolites maximum inhibition (little growth) of bacterial colony was observed under 100% concentration followed by 50% concentration. However, in 0% concentration of fungal metabolites i.e. in water only, no inhibition (maximum growth) of bacteria was experienced. (Figure 1 and 2, Table 1, Plate 1 and 2). During earlier studies it was shown that the culture filtrates of *Alternaria spp*. significantly reduced the effect of bacteria, similar has been reported earlier (Verma *et al.,* 2007). The growth response of *S. maltophilia* under different test treatments and

streptomycin. The growth response of *S. maltophilia* to streptomycin was found to be similar as that of *X. campestris*. Among different concentrations of fungal metabolites maximum inhibition (Little Growth) of bacterial colony was observed under 100% concentration followed by 50% concentration. However, in 0% concentration of fungal metabolites i.e. in water only, no inhibition (maximum growth) of bacteria was experienced (Figure 1 and 2, Plate 3 and 4, Table 1). During earlier studies it was shown that the culture filtrates of *Alternaria spp*. significantly reduced the effect of bacteria (Gajalakshmi and Abbasi, 2008). *P. syringae* exhibited the similar pattern of growth as depicted in other two target species of bacteria under different test treatments and streptomycin conditions. Under the presence of antibacterial agent streptomycin very little growth was observed. Among different concentrations of fungal metabolites maximum inhibition (little growth) of bacterial colony was observed under 100% concentrations followed by 50% concentrations. However, in 0% concentration of fungal metabolites i.e. in water only, no inhibition (maximum growth) of bacteria was experienced. During earlier studies it was shown that the culture filtrates of *Alternaria spp*. significantly reduced the effect of bacteria. *Alternaria spp*. is considered as potential bio control and growth promoting agents for many crop plants (Savazzini *et al.,* 2009).

FOURIER TRANSFORM INFRARED SPECTROMETER OF FUNGAL METABOLITES

Infra-Red spectral analysis of secondary metabolites of *A. brassicicola* revealed presence of functional group broad stretching at 3281-3300cm^{-1} likewise appearance of peak around 1638-1640cm^{-1} indicate presence of amide 1 group in protein fungal metabolite (figure 3). These active groups in this region could be responsible for antimicrobial activity against phytopathogenic bacteria. Similar peaks correspond to same groups have been reported previously (Kosa *et al.,* 2017).

Table 1: Growth response of *bacterial strains* to metabolites of *Alternaria* by well and disc methods

Growth response of	Using	Concentrations			
		0%	50%	100%	Streptomycin
X. campestris	**Well method**	0.3±0.17 cm	3.0±1.29 cm	3.3±1.17 cm	3.4±0.15 cm
	Disc method	0.3±0.1 cm	2.9±0.15 cm	3.2±0.1 cm	3.4±0.1 cm
S. maltophilia	**Well method**	0.2± 0.1 cm	3.0±0.21 cm	3.3±0.1 cm	3.6±0.1 cm
	Disc method	0.4±0.1 cm	2.7±0.1 cm	3.2±0.1 cm	3.9±0.1 cm
P. syringae	**Well method**	0.3±0.01 cm	2.9±0.01cm	3.2±0.02 cm	3.5±0.01 cm
	Disc method	0.3±0.01 cm	2.8±0.02 cm	3.4±0.01 cm	3.7±0.01 cm

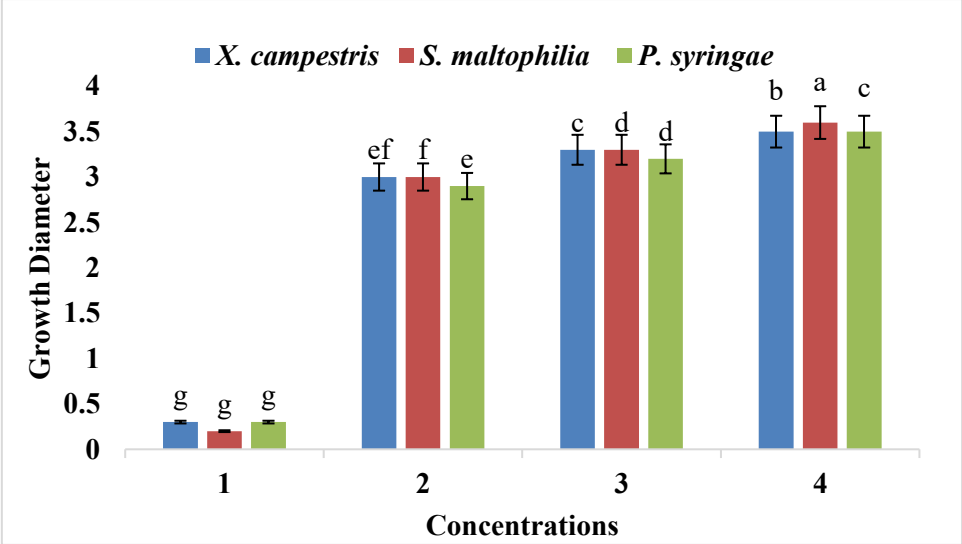

Figure 1: Well method shows inhibition of metabolites *A. brassicicola* against three species of bacteria in statistical analysis. Each treatment mean is sum of three replicates and ± represents standard error (SE). Non-identical letters specify significant difference among the treatments at p≤0.05.

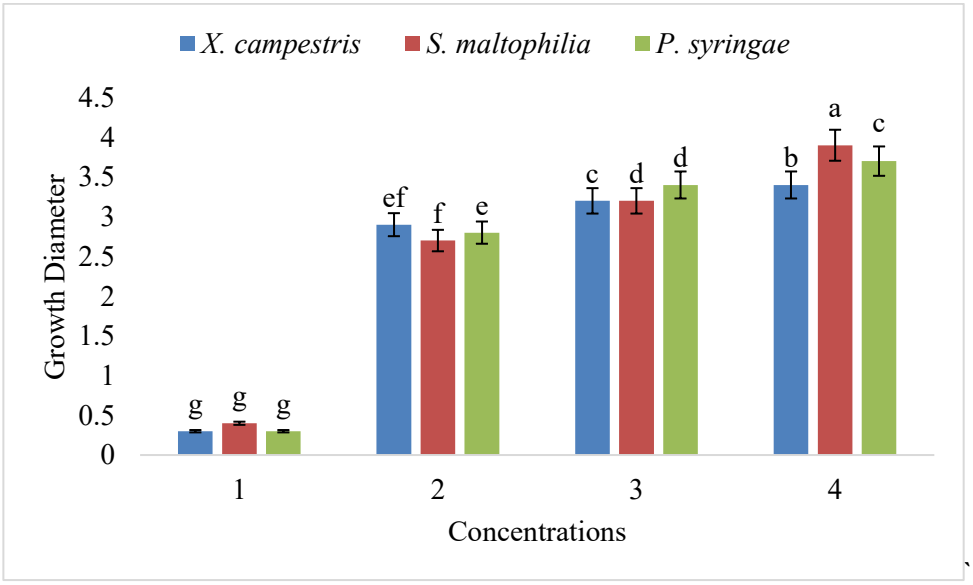

Figure 2: Disc method shows inhibition of metabolites *A. brassicicola* against three species of bacteria in statistical analysis. Each treatment mean is sum of three replicates and ± represents standard error (SE). Non-identical letters specify significant difference among the treatments at p≤0.05.

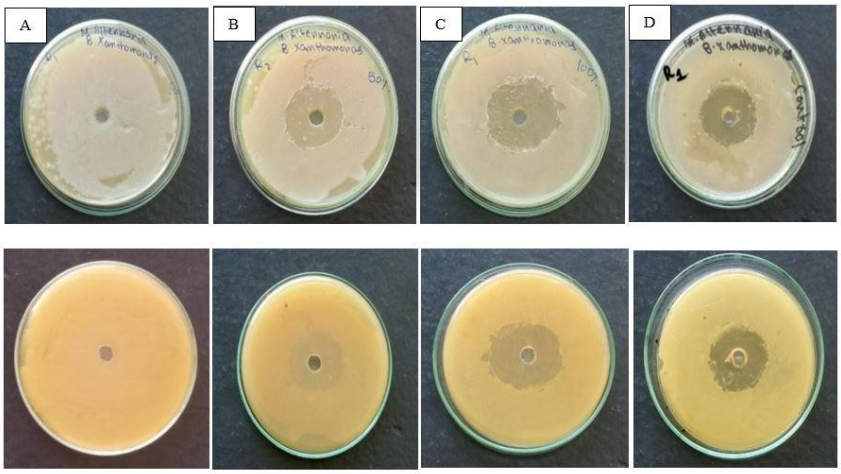

Plate 1: Growth response of *Xanthomonas campestris* to metabolites *of Alternaria brassicicola* by well method.

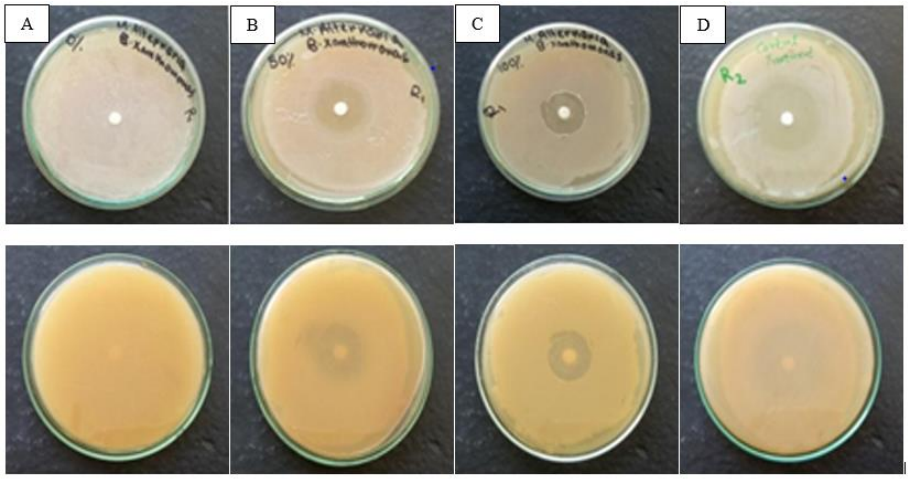

Plate2: Growth response of *Xanthomonas campestris* to metabolites *of Alternaria brassicicola* by disc method.

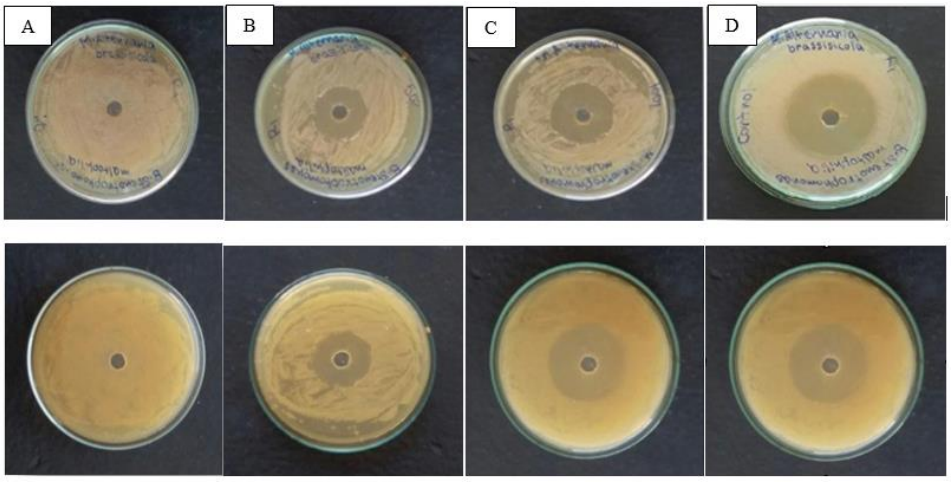

Plate 3: Growth response of *Stenotrophomonas maltophilia* to metabolites *of Alternaria brassicicola* by well method.

(A) 0% Concentration (B) 50% Concentration (C) 100% Concentration (D) Streptomycin

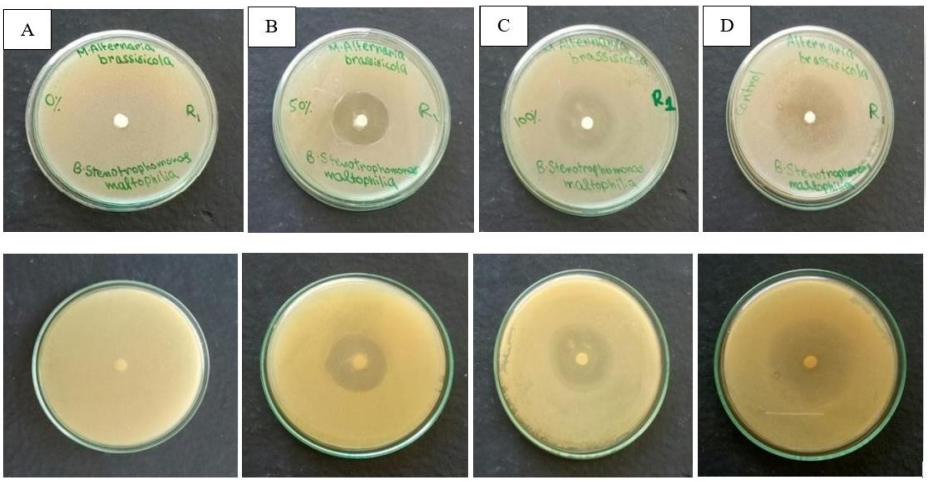

Plate 4: Growth response of *Stenotrophomonas maltophilia* to metabolites *of Alternaria brassicicola* by disc method.

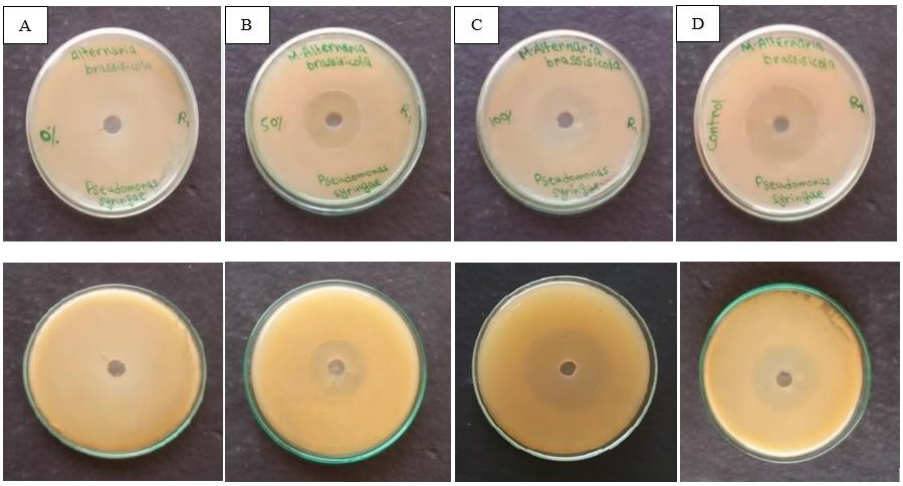

Plate 5: Growth response of *Pseudomonas syringae* to metabolites *of Alternaria brassicicola* by well method.

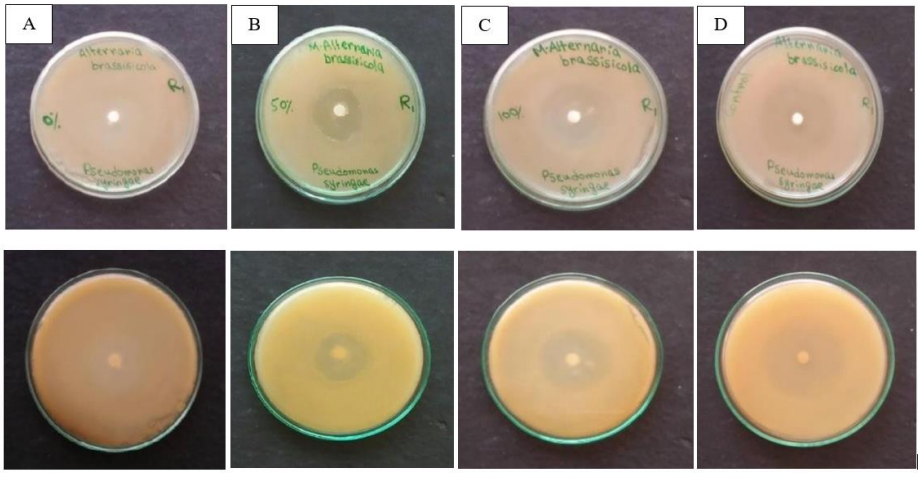

Plate 6: Growth response of *Pseudomonas syringae* to metabolites *of Alternaria brassicicola* by disc method.

(A) 0% Concentration (B) 50% Concentration (C) 100% Concentration (D) Streptomycin

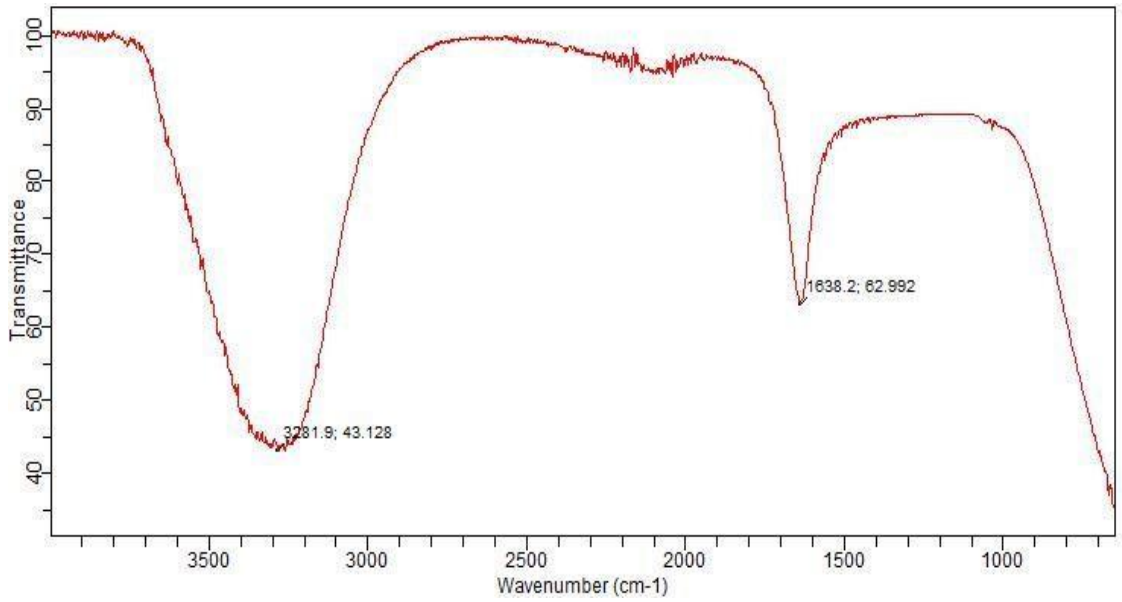

Figure 3: Fourier Transform Infrared Spectrometer of Fungal Metabolites of *Alternaria brassicicola.*

CONCLUSION

Biological control is the most important method to reduce plant diseases caused by different plant pathogenic bacteria. The present study concludes that the secondary metabolites of *Alternaria brassicicola* have the potential to be used as antibacterial agents against selected plant pathogenic bacteria.

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This study was supported by all authors.

AUTHOR'S CONTRIBUTION

All authors contributed equally.

DATA AVAILABILITY STATEMENT

All the data is primary.

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CONFLICT OF INTEREST

There is no conflict of interest.

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