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Original article

Molecular identification and biological control of *Ralstonia solanacearum* from wilt of papaya by natural compounds and *Bacillus subtilis*: An integrated experimental and computational study

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ABSTRACT

Ralstonia solanacearum is a harmful pathogen that causes severe wilt disease in several vegetables. In the present study, we identified *R. solanacearum* from wilt of papaya by 16S rRNA PCR amplification. Virulence ability of *R. solanacearum* was determined by amplification of approximately 1500 bp clear band of *hrpB* gene. Further, *in-vitro* seed germination assay showed that *R. solanacearum* reduced the germination rate up to 26.21%, 34% and 33.63% of cucumber, bottle guard and pumpkin seeds, respectively whereas shoot and root growth were also significantly decreased. Moreover, growth inhibition of *R. solanacearum* was recorded using antibacterial compound from medicinal plant and antagonistic *B. subtilis.* Petroleum ether root extract of *Rauvolfia serpentina* showed highest 22 ± 0.04 mm diameter of zone of inhibition where methanolic extract of *Cymbopogon citratus* and ethanolic extract of *Lantana camara* exhibited 20 ± 0.06 mm and 20 ± 0.01 mm zone of inhibition against *R. solanacearum*, respectively. In addition, bioactive compounds of *B. subtilis* inhibited *R. solanacearum* growth by generating 17 ± 0.09 m m zone of inhibition. To unveil the inhibition mechanism, we adopted chemical-protein interaction network and molecular docking approaches where we found that, rutin from *C. citratus* interacts with citrate (Si)-synthase and dihydrolipoyl dehydrogenase of *R. solanacearum* with binding affinity of −9.7 kcal/mol and −9.5 kcal/mol while quercetin from *B. subtillis* interacts with the essential protein F0F1 ATP synthase subunit alpha of the *R. solancearum* with binding affinity of −6.9 kcal/mol and inhibit the growth of *R. solanacearum*. Our study will give shed light on the development of eco-friendly biological control of wilt disease of papaya.

1. Introduction

Carica papaya (L.), a commercially well-demanded fruit that is generally popular as papaya or pawpaw and is grown around the world, most commonly in the tropical and subtropical regions (Hossain et al., 2018). In the Indian subcontinent like Bangladesh, among different types of crops and fruits, papaya is widely cultivated due to its nutritious and economical value. *C. papaya* (L.) belongs to the Caricaceae family incorporating all species in six genera like *Carica, Cylicomorpha*, *Jarilla*, *Jacaratia*, *Horovitzia*, and *Vasconcellea* with the genus *Carica*, consisting of only one species. *C. papaya* contains many biologically active compounds two of which are very important namely chymopapain and papain that aid indigestion. Papaya fruit is highly appreciated worldwide for

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its flavor, nutritional qualities, and digestive properties (Fernandes et al., 2006). Papaya has lots of traditional medicinal value including anti-inflammatory activity, wound healing activity (Gurung and Škalko-Basnet, 2009), anti-fertility activity (Pokharkar et al., 2010), anticancer activity (Nguyen et al., 2013), antifungal activity (Chávez-Quintal et al., 2011), antibacterial activity (Nirosha and Mangalanayaki, 2013) etc. A shred of recent evidence reported that pure leaves extract of *C. papaya* increases the amount of RBC and platelet counts in the bloodstream of the murine model without toxicity therefore it boosts up thrombopoiesis and hemopoiesis (Dharmarathna et al., 2013).

Farming is the foremost overwhelming segment of the national economy in Bangladesh contributing 17% of national income (Kaysar et al., 2019). Conversely, according to BBS report almost 45% of the population relies on the agriculture sector for their livelihood. Currently, crop cultivation in Bangladesh has been diversified to fruits and vegetable production to large extent due to the reduction of crop lands as well as for recent demand. Papaya is one of the major fruits in terms of acreage and per hectare production in Bangladesh (Hamim et al., 2014). The cultivation of papaya has gained enormous recognition among the farmers. It creates important sources of income generation leading to economic self-reliance among the farmers in recent years. But, the production of crops and vegetables are hampered each year due to many reasons in the country, one of which is distinctive bacterial diseases (Mondal, 1970). Papaya is very susceptible to diseases caused by many microorganisms especially bacteria and fungi. In Bangladesh, bacterial and fungal diseases of papaya have emerged as significant problems and, in most cases, papaya is susceptible to various pathogens. Climatic factors including temperature, precipitation, humidity and length of the day have a significant negative impact on agriculture (Brammer, 2014). Besides, the tropical climate favours a high prevalence of plant pathogenic microorganisms including bacteria and viruses in Bangladesh (Akhter et al., 2019). A bacterial wilt disease of papaya is caused by *R. solanacearum* (Persley and Ploetz, 2003) having symptoms like epinasty, lower leaves yellowing followed by subsequent defoliation of upper leaves. *R. solanacearum* is a kind of soil-borne and non-spore-forming bacterium which can infect a few hundred host plant species around the globe including potatoes, tomatoes, eggplants, groundnuts, olives, bananas, and ginger (Cai et al., 2018). *R. solanacearum* was previously knowns as *Pseudomonas solanacerum*, causing wilt disease on a wide range of solanaceous plant (Pawaskar et al., 2014). The disease of papaya cripples the economy as their existence can cause bitter economic losses in the production, sale, and exportation of fresh fruit. Thus, bacterial disease identification and regular surveys are necessary to ascertain the prevalence and incidence in other crops.

Accurate identification of microorganism through traditional laboratory tests require a few days whereas that by molecular approaches reduces the identification time to hours (Tsoktouridis et al., 2014). In recent decades, molecular biology techniques have reformed to identify plant pathogenic bacteria. A mainstream strategy is 16S ribosomal gene sequencing (Woo et al., 2008). 16S rRNA investigation has been impressively applied to recognize bacterial species and turned into a valuable genetic tool to study genetic variation among various strains of the pathogen (Islam et al., 2020). Causing disease on host plants and eliciting a hypersensitive response on non-host plants are fundamental features controlled by the *hrp* genes in plant pathogenic bacteria. *hrp* genes command the capability of phytopathogenic bacteria to cause disease in susceptible plants and to trigger the hypersensitive reaction (HR) on resistant plants (Puhar and Sansonetti, 2014). *hrp* genes were extensively studied in nearly all major gram-negative plant pathogenic bacteria including *Xanthomonas* spp., *Pseudomonas spp, Erwinia* spp., *and Ralstonia* spp. (Büttner and He, 2009).

To continue the expanding worldwide interest for food, feed, and fiber, from the eighteenth century forward, farmers have been stepped by step restoring natural agriculture practices with synthetic fertilizers and pesticides. The utilization of chemical synthetic pesticides for controlling different plant diseases is as yet a typical practice particularly in developing countries like Bangladesh. Nevertheless, excessive use of chemical pesticides has been linked to the contamination of the environment as well as food products (Arfaoui et al., 2007). Furthermore, the use of agrochemicals leads to the development of pesticide resistance and eradication of non-target organisms and positive plant–microbe associations from the environment (Bass et al., 2015; Fox et al., 2007; Rivera-Becerril et al., 2017). On the other hand, biological control approaches using bioactive compounds from different medicinal plants and antagonistic microorganisms offer environment-friendly control measures of plant diseases (Wikaningtyas and Sukandar, 2016). Former investigations have reported that some bacteria and plant extracts can serve as excellent biological control agents against soil-borne pathogens (Arfaoui et al., 2007; Mercado-Blanco et al., 2004). Medicinal plants are rich in a wide variety of secondary metabolites such as tannins, alkaloids, and flavonoids, which have been found *in-vitro* to have antimicrobial properties (Lewis and Ausubel, 2006). Biological control of plant pathogens using antagonistic bacteria is an auspicious system for plant protection from soil-borne diseases and has pulled in significant consideration with the mean to diminish the utilization of agricultural synthetic substances (Mizumoto et al., 2007). *B. subtilis* has been extensively studied as an efficient biological agent against various plant diseases due to its capability to produce several antibiotics, lipoproteins, and hydrolytic enzymes (Cavaglieri et al., 2005; Sharma and Sharma, 2008). *B. subtilis* species disclose potential antimicrobial activity against different phyto-pathogenic microbes including *R. solanacerum* (Chung et al., 2008). The importance of an eco-friendly plant protection strategy is greatly emphasized in sustainable agriculture (Rahman et al., 2012). Molecular docking is an *in-silico* approach to determine the binding interactions between ligands and targeted proteins while this promising tool also provides the binding affinity of small compounds (Moraga-Nicolás et al., 2018). This technique can also be used to predict the inhibition mechanism between different chemical compounds and microorganisms. In Bangladesh, appropriate measures to control the wilt disease of papaya biologically are yet to be developed.

The present study is aimed to isolate and identify the causal agent of wilt disease of papaya through biochemical and molecular approaches. Besides, here we propose an effective biological control measure of this disease in an eco-friendly manner. In this study, we also report the underlying *in-vitro* inhibition mechanism of *R. solanacearum* by compound from selected medicinal plants and *B. subtilis* using a molecular docking approach.

2. Materials and methods

2.1. Collection of diseased plant materials

In the current experiment, bacterial wilt infected leaves of papaya were collected from the cultivated papaya land inside the campus area, University of Rajshahi, Rajshahi-6205, Bangladesh, and then the diseases were identified by their symptoms. Then, the samples were transferred to the lab for further experiment.

2.2. Isolation and culture of the pathogen

Samples were collected and transported to the laboratory for bacteriological examination in a sterile plastic bag. Then infected

parts were washed through sterile distilled water, then sterilized with 70% ethanol (Ikhsan Zam et al., 2016), and were crushed by mortar pestle and took 1 μl pasted liquid into LB liquid medium containing conical flask and incubated overnight (16 h) at 37 °C and 130 rpm using Richard James Hilfiger orbital shaker RJH-5005. The bacterial isolates were screened on nutrient agar (NA) plates supplemented with peptone 1gm/100 ml, yeast extract 0.5gm/100 ml, and agar 2gm/100 ml from mixed liquid culture of bacteria through streak plate method. Plates were incubated at 37 °C overnight (16 h) and morphologically distinct colonies were chosen and used for further studies. Pure culture of a specific pathogen of wilt disease of papaya was obtained by re-streaking on agar plate based on colony morphology followed by liquid culture.

2.3. Biochemical characteristics of isolated bacteria

In the current study, isolated phyto-pathogenic bacteria were characterized through different biochemical tests. Morphological characterization was done using 100X light microscope (Olympus BX 60). Several biochemical tests such as Gram staining, KOH test, Sulphide-Indole-Motility (SIM) test, Methyl red, Kovac oxidase, Kligler Iron Agar (KIA), Triple Sugar Iron agar (TSI) MacConkey agar, Simmons citrate, Catalase and Urease test were performed as described previously (Brucker, 1986), (Suslow, 1982), (Hasan et al., 2018), (Habiba et al., 2018), (Kovacs, 1956) and (Ali et al., 2017; MacConkey, 1905), (Cox et al., 1977), respectively.

2.4. Molecular identification of isolated bacterial strain

The 16S rRNA gene is common in bacteria, relationships can be measured among all bacteria through this gene (Woese et al., 1985). The comparison of the 16S rRNA gene sequences allows differentiation between organisms at the genus level across all major phyla of bacteria. In this study, the virulence ability of the isolated bacterial strain was also detected by *hrpB* gene PCR amplification.

2.5. Extraction of genomic DNA and optimization of PCR condition

Genomic DNA was extracted from the liquid culture of isolated pure bacterial strains. Genomic DNA was extracted using CTAB (Cetyl Trimethyl Ammonium Bromide) buffer method according to Mas-Ud et al. (2020). Extracted genomic DNA was purified according to the kit instructions (Thermo Fisher Scientific, DNA Purification Kit #K0721). The quality of DNA was measured by 1% agarose gel electrophoresis using a 5 Kb DNA ladder. Quantity of genomic DNA was measured by thermo scientific nanodrop 2000. To identify the isolated bacterial strains, 16S rDNA was amplified through polymerase chain reaction (PCR) using two 16S rDNA universal primers 27F (5′-AGAGTTTGATCCTGGCTC-3′) and 1391R (5′-GACGGCGGTGTGTRCA-3′). A total of 50 μl reaction mixture was prepared as follows; 25 μl 2X concentrated solution (Thermo Scientific, United States), 3 μl for each forward and reverse primer, 15 μl nuclease-free water, and 4 μl template genomic DNA (Frank et al., 2008). The amplification was performed by pre-heating at 95 °C for 4 min followed by 35 cycles at 95 °C, 60 °C, and 72 °C for 1 min denaturation, 30 s annealing, and 2 min extension, respectively. The final extension time was 10 min at 72 °C. A sum of 36 continuous amplification reaction cycles was performed in BIORAD T100 thermal cycler. PCR product was purified by Accu-Prep®, Bioneer purification kits according to manufacturer protocol.

2.6. Sequencing and construction of the phylogenetic tree

PCR product was sent to Invent Biotechnology Ltd, Dhaka, Bangladesh for sequencing. Sequence data were deposited in the GenBank database and the accession number of isolated bacterial strain were obtained as MW150987. For the evolutionary relationship, sequence data were analyzed through Nucleotide blast (BlastN) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) in NCBI GenBank. Then, the evolutionary relationships of taxa were conducted using the Neighbor-Joining method (Saitou and Nei, 1987) and the Maximum Composite Probability method was used to measure the evolutionary distances (Tamura et al., 2004). Here, all the evolutionary analyses have been carried out through the MEGA (Molecular Evolutionary Genetics Analysis) - X software (Kumar et al., 2018).

2.7. PCR amplification of hrpB gene

In response to cell contact with bacteria in plants, the expression of *R.* solanacearum T3SS-encoding and effector genes are stimulated in a six-gene regulatory pathway (Brito et al., 2002). *hrpB* is one of the vital members of this regulatory cascade to induce pathogenicity in the plant (Cunnac et al., 2004a). To amplify *the hrpB* region of the isolated bacterial strain, primers were designed according to Poussier et al. (2000) who used forward primer RShrpBf (5′-TGCCATGCTGGGAAACATCT-3′) and the reverse primer RShrpBr (5′GGGGGCTTCGTTGAACTGC-3′) for PCR amplification of *hrpB* gene in *R. solanacearum.* The PCR was carried out in 0.2 ml PCR tube in total volumes of 25 μl; containing 1.5 μl of genomic DNA, 1 μl of both primers, 1 μl of the dNTPs mix, 2.5 μl of $MgCl_2$, 2.5 μl of Taq buffer B and 0.5 μl of 5U Taq polymerase. PCR reactions were performed in BIORAD T100 thermal cycler programmed for an initial denaturation step of 95 °C for 5 min, followed by 10 cycles of 95 °C for the 30 s, 64 °C for 30 s, 68 °C for 2 min. The final 20 cycles were the same as the first 10 except that an additional 20 s was added to the elongation step for each new cycle, and a final extension step at 68 °C for 7 min. After that, final amplified products were analyzed and visualized in 1.0% agarose gels containing 0.5 μg/ml of Ethidium bromide.

2.8. Seed treatment and germination rate

To determine the pathogenicity of *R. solanacearum* on other plants we performed a seed germination assay using cucumber, bottle guard, and pumpkin seeds. Seeds were collected from Bangladesh Agricultural Development Corporation, Rajshahi. Firstly, *R. solanacearum* was cultured at 37 °C temperature for overnight before the seed treatment. Then culture of the bacterial strain being mixed with three types of seed varieties and thoroughly shake until the bacterial strain was evenly distributed. After treatment, the different types of treated seeds and untreated control seeds were placed into separate petri dishes containing double-layer moistened filter paper and incubated in a dark incubator for 3 days maintaining an inside temperature of 25 °C and relative humidity of 75%. Deionized water (pH = 6.20) was given to the filter paper to maintain sufficient moisture content for germination. The germination data were recorded at 12 h duration. The germination percentage was calculated according to previous studies by Billah et al. (2020) using the following formula

$$\textit{Germination rate}\ (\%) = (\textit{Number of seeds germinated in}\ 3\ d/\ \textit{total number of seeds}) \times 100\ \%.$$

2.9. Analysis of growth parameters

The germinated seeds were cultured in a hydroponic culture media (Hoagland and Arnon, 1938) and seedlings were harvested from the culture media after 5 days of cultivation to determine the shoot and root lengths of the three different types of plants as a growth parameter. Then the shoots and roots were washed with water and their lengths were measured with a digital centimeter slide caliper and registered.

2.10. Antibiotic sensitivity assay

Antibiotic sensitivity of *R. solanacearum* was performed by Bauer et al. (1966) with some modification and disc diffusion method was applied to measure antibiotic susceptibility. In this current study, a total of 19 different antibiotics were selected to observe the sensitivity pattern against the isolated bacterial strain. The isolated bacterial strain was grown overnight in a nutrient broth at 37 °C in a shaker with 150 rpm for the antibiotic sensitivity test. Then, overnight culture of bacterial suspension (1×10^6 CFU/ml) was transferred and gently spread on the nutrient agar plate, and dried. After incubation, clear zones indicated inhibition of growth of the isolated bacteria. Then, the zone of inhibition was measured with the help of a millimeter (mm) scale. The antibiotic sensitivity pattern of the isolate was interpreted using manufactures guidelines.

2.11. Antibacterial assay of plant extracts

Five different medicinal plant parts viz. *L. camara, T. erecta, C. citratus, P. pinnata, R. serpentina*, and five solvents viz. methanol, ethanol, chloroform, acetone, and petroleum ether were used for the preparation of the extract. The plant extract was prepared according to Hossain et al. (2017) and Al-Mamun et al. (2016b). Screening of antibacterial activity of plant extracts against causal pathogen was done by moderate disc diffusion method according to Hasan and Sikdar (2016) and Al-Mamun et al. (2016a). There are four different concentrations were used (10 μg/disc, 20 μg/disc, 30 μg/disc, and 50 μg/disc) for antibacterial assay.

2.12. Evaluation of the antagonistic activity of soil bacteria

To evaluate the antagonistic activity of soil bacteria against *R. solanacearum* of wilt disease of papaya, *B. subtilis* strain was taken from Professor Joarder DNA and Chromosome Research Lab, University of Rajshahi, which was previously confirmed by Hasan et al. (2018). The antagonistic ability of soil bacteria was determined against *R. solanacearum* through the disc diffusion method according to Farah Naqvi et al. (2012). In disc diffusion assay, agar solidified LB medium was inoculated with *R. solanacearum* by plating with a glass rod. The disc of size of 6 mm diameters was made and sterilized in an autoclave at 121 °C for 20 min. Four paper discs were taken to soak the liquid culture of *B. subtilis* with the concentration of 10 μl/disc, 20 μl/disc, 40 μl/disc, and 50 μl/disc, respectively. The four dried discs were placed gently on the four corners on the solidified agar plates that were not closer than 15 mm to the edge of the plate and incubated at 37 °C for overnight. Then inhibition zone by *B. subtilis* was measured by mm scale. Antagonistic properties of *B. subtilis* towards *R. solanacerum* was also measured by the agar well diffusion method. In the agar well diffusion method, the agar plate surface is inoculated by spreading a volume of the *R. solanacearum* inoculum over the whole agar surface. Then, a hole with a diameter of 6 to 8 mm was made aseptically through a sterile cork borer or a tip, and a volume of 10 μl, 20 μl, 40 μl, and 50 μl of liquid culture of *B. subtilis* was poured into the well. After that, agar plates were incubated at 37 °C for overnight. The resulting zone of inhibition was measured by mm scale.

2.13. Retrieval of the antibacterial compounds and prediction of the targets

The antibacterial compounds from the plant extracts of *L. camara, R. serpentina*, and *C. citratus* were selected using Dr. Duke's Phytochemical and Ethnobotanical Databases (https://phytochem.nal.usda.gov/) (Duke and Bogenschutz, 2001) while the compounds from *B. subtilis* were selected through the literature studies (Caulier et al., 2019). The structures of the antibacterial compounds were retrieved from the PubChem database of the National Center for Biotechnology Information (https://pubchem.ncbi.nlm.nih.gov/). Further, the interaction of a network of chemicals-proteins was analyzed using the STITCH tool to predict the target proteins of *R. solanacerum* for the antibacterial compounds (Kuhn et al., 2008). Finally, the target proteins were blasted against the essential genes of the bacterium to identify the essential genes using the BlastP program of the DEG database (Luo et al., 2014; Su et al., 2020). The proteins encoded by these essential genes were selected for further analysis.

2.14. Homology modeling and model validation

The homology 3 Dimensional (3D) models of the selected target proteins were generated adopting the SWISS-MODEL which is a widely used web-based integrated service for protein homology modeling (Waterhouse et al., 2018). The best templates were selected based on the query coverage and identity for the homology modeling. After generating the 3D models, the best protein structures were selected by evaluating the several validation assessments scores. PROCHECK, ERRAT, and VERIFY3D were used for the validation of the structures (Colovos and Yeates, 1993; Laskowski et al., 1993; Lüthy et al., 1992).

2.15. Molecular docking

The interactions between the target proteins and their corresponding antibacterial compounds were analyzed employing the molecular docking approaches. Before the molecular docking, the ligands and the target proteins were prepared using the Autodock of MGL tools. Firstly, the structures were optimized adding the required changes such as Kollman and Gasteiger charges. Polar hydrogen atoms were also added to the protein structures. Then, Autodock vina was used for the molecular docking of the ligands against the target proteins (Trott and Olson, 2009). The docking parameters were summarized in Supplementary Table 1. Finally, Discovery studio was used for the visualization of the interactions between the ligands and their target proteins.

2.16. Statistical analysis

For each treatment group, all the investigations were performed with three separate replications. Statistical analysis of the importance of each data treatment category was rendered at $P \leq 0.05$ by one-way ANOVA followed by Duncan's Multiple Range Test (DMRT) by the program IBM SPSS-20. All the graphical diagrams used in the study were prepared by Graph Pad Prism 8.

3. Results

3.1. Isolation and culture of R. solanacearum

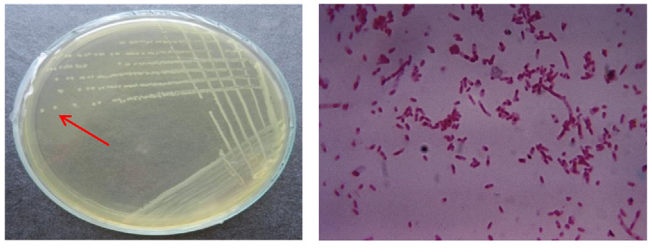
Bacterial liquid culture from wilt disease infected papaya leaves was obtained after overnight incubation at 37 °C. The colony was obtained after subculture on the LB agar medium in 90 mm petri-dishes. The cultural studies of *R. solanacearum* on nutrient agar medium revealed that the colonies were smooth circular, raised, and white in color (Fig. 1a). The optical feature of the colony was opaque and the sizes of colonies were 3 mm on average. These isolates, suspected to be *R. solanacearum,* were subjected to numerous confirmatory biochemical and molecular tests to figure out their identity.

3.2. Biochemical characterizations of R. solanacearum

Morphological observations revealed that cells of *R. solanacearum* were straight rod-shaped, short and motile. The isolated bacteria were pink in color in Gram staining reaction under the light microscope at 100X magnifications (Fig. 1b), authenticating that they were Gram-negative (Table 1). As the bacterial culture of *R. solanacearum* produced a string-like viscous material on a glass slide in the KOH test, therefore it further confirmed its Gram negativity. In the SIM test, indole ring, black precipitation of H_2S and motility was observed. *R. solanacearum* changed the color from yellow to red in the methyl red test. The isolated *R. solanacearum* was capable to produce deep blue color with Kovac reagent indicated that the bacteria were positive for the Kovac oxidase test. Various sorts of carbohydrate (glucose, lactose, dextrose) fermenting ability were observed on KIA, TSI, and MacConkey agar test (Table 1) against isolated *R. solanacearum.* The presence of prussian blue color indicated that *R. solanacearum* were positive to citrate test and used carbon as a source of energy. *R. solanacearum* showed a positive reaction to catalase and urease test through the production of air bubbles and bright pink color upon inoculation to the medium, respectively. Based on our findings regarding morphological and biochemical characterizations, we hypothesized that the isolated bacteria is *R. solanacearum.*

3.3. Molecular identification confirms the isolated bacteria as R. solanacearum

The pathogen of wilt disease of papaya was detected by 16S rRNA gene sequencing. PCR analysis of 16S rRNA using the universal primer 27F and 1391R produced approximately 1400 bp clear band on 1% agarose gel electrophoresis (Fig. 2a). The BlastN search of the GenBank for this 16S rRNA gene sequences unveiled that, it showed 99% similarity (Fig. 3) with the bacteria *Ralstonia solanacearum* strain JN-1 (GenBank accession no. MN508402). These results of 16S rRNA gene sequencing confirm that bacteria isolated from wilt disease of papaya were *R. solanacearum.*



(a) (b)

Fig. 1. Colony morphology and Gram staining of *R. solanacearum*. Here, (a) Colony morphology of *R. solanacearum* isolated from wilt infected leaves of papaya. Red arrow indicates the white round colony of *R. solanacearum* and (b) Gram staining of *R. solanacearum*. Pink color indicates rod shaped cells of *R. solanacearum.*

Table 1
Biochemical characteristics of *R. solanacearum.*

Test name	Reactivity	Appearance	Remarks
Gram test	-ve	Pink color, rod shaped	Gram staining confirmed isolated bacteria Gram negative
SIM test	H_2S+, Indole +, Motility+	Motile, indole ring formation, H_2S production	Isolated bacteria showed mobility, indole ring formation and black precipitation of H_2S
Methyl Red test	+	Color changed from yellow to red in broth medium	Isolated bacteria had ability to utilize glucose to grow in broth culture
Kovac oxidase test	+	Produce deep blue color	Isolated bacteria were Gram negative
Kligler Iron agar test (KIA)	Slant/Butt A/A, H_2S-dextrose (+), lactose(+)	No black precipitation was formed	Isolated bacteria were positive to KIA test
Triple sugar iron test (TSI)	Slant/Butt A/A, H_2S-, gas formation	No black precipitation was formed	Isolated bacteria ferment dextrose, lactose and sucrose
MacConkey agar test	+	No pink color formed around the colony	Isolated bacteria are lactose non-fermenting
Simmon's citrate test	+	Deep blue (prussian blue) color formation	Isolated bacteria metabolized citrate
Catalase test	+	Bubble formation	Breakdown of H_2O_2 to produce water and O_2 gas
Urease test	+	Color changed from orange yellow to bright pink color	Isolated bacteria hydrolyze urea to ammonia

"–ve": Gram-negative; "+": Positive; "–": Negative; "A": Acidic reaction; "K": Alkaline reaction.

3.4. PCR amplification of the hrpB gene demonstrates the virulence ability of R. solanacearum

The hrp gene plays the main function to produce disease in a host plant. In our study, the sequence of the *hrpB* gene was amplified through PCR reaction from genomic DNA of *R. solanacearum* from wilt disease of papaya using *hrpB* gene-specific primer. A successful PCR amplification produced approximately 1500 bp clear band of *hrpB* gene sequence on agarose gel electrophoresis (Fig. 2b). This presence of *the hrpB* gene in *R. solanacearum* indicates its virulence ability to cause disease.

3.5. R. solanacearum inhibits the seed germination of cucumber, bottle guard, and pumpkin

To assess the virulence ability of *R. solanacearum* to other plants, we performed an *in-vitro* seed germination assay using cucumber, bottle guard, and pumpkin seeds. In the present experiment, it was observed that the maximum germination rate of the untreated control group, positive control group seeds treated by *B. subtilis* and cucumber seeds treated by *R. solanacearum* were found to be 80.44 ± 1.11%, 68.88 ± 0.57% and 54.23 ± 1.00%, respectively (Fig. 4a). The germination rate of the *R. solanacearum* treated seeds decreased up to 26.21% which is lower than that of the control seed. In the case of bottle guard seeds result was observed that the highest germination rate of control, positive control group

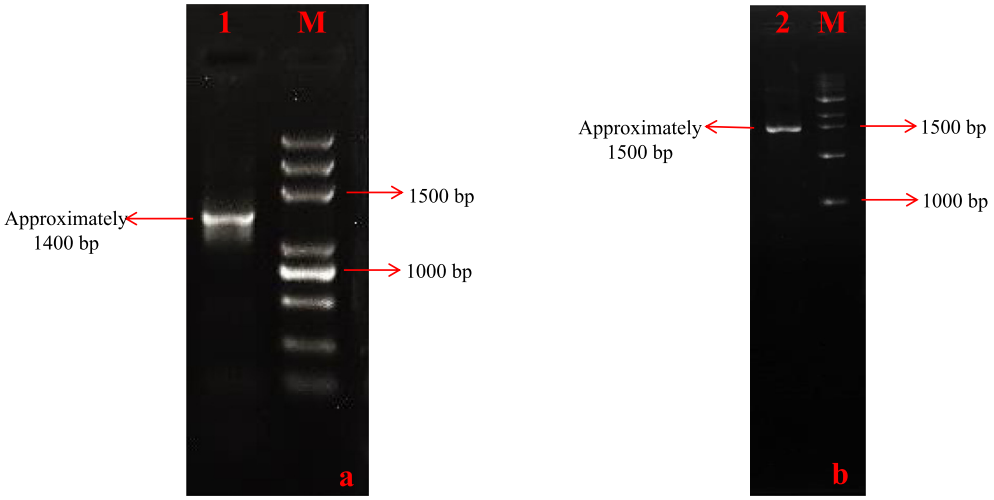


Fig. 2. 1% agarose gel electrophoresis of 16S rRNA and *hrpB* gene. (a) 16S rRNA of *R. solanacearum* (1), (b) *hrpB* gene of *R. solanacearum* (2). M = 1 kb DNA ladder.

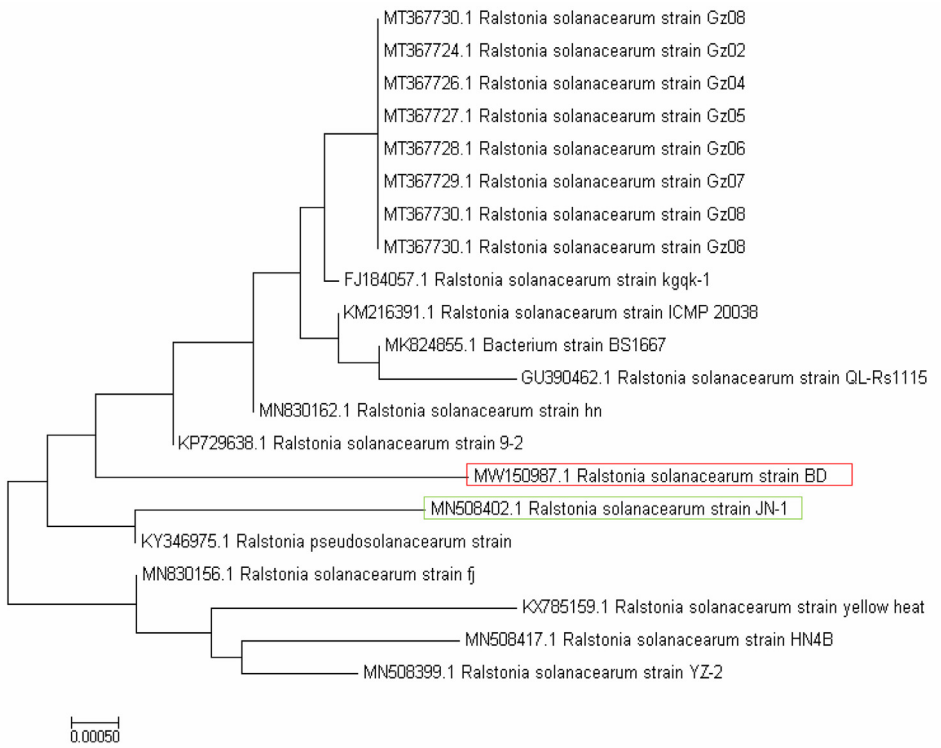


Fig. 3. Phylogenetic tree showing the evolutionary relationship with the *R. solanacearum* (MW150987) isolated from wilt disease of papaya and other related *R. solanacearum* strain. The evolutionary history was inferred using the Neighbor-Joining method. Bootstrap replicate value was kept at 1000. The optimal tree with the sum of branch length 0.02328451 is shown. Red box indicates the isolated *Ralstonia solanacearum* strain BD from wilt of papaya while green box indicates its closest similarities to *Ralstonia solanacearum* strain JN-1.

seeds treated by *B. subtilis* and *R. solanacearum* treated seeds were found to be 87.33 ± 1.02%, 68.44 ± 0.92% and 53.33 ± 1.11%, respectively which is 34% lower than that of control seed (Fig. 4b). And finally, the result was estimated that in the case of pumpkin, the maximum germination rate of control, positive control group seeds treated by *B. subtilis* and *R. solanacearum* treated seeds were found to be 73.75 ± 1.31%, 61.21 ± 0.56% and 40.12 ± 1.05%, respectively which is 33.63% lower than that of untreated control seed (Fig. 4c). From the above results, it can be concluded that the treatment by *R. solanacearum* decreases the seed germination rate in respect to control.

3.6. R. solanacearum delays the root and shoot growth length

After germination, seedlings of cucumber, bottle guard, and the pumpkin, that were grown from the control and *R. solanacearum* treated seeds were showed a significant change in different morphological characters viz. root lengths and shoot lengths. Root and shoot lengths per plant both for control and *R. solanacearum* treated seedlings were measured after 5 DAC (Day after Cultivation). Root lengths of the seedlings were significantly decreased due to *R. solanacearum* treatment in respect to control. We observed that, control and *R. solanacearum* treated cucumber seed-

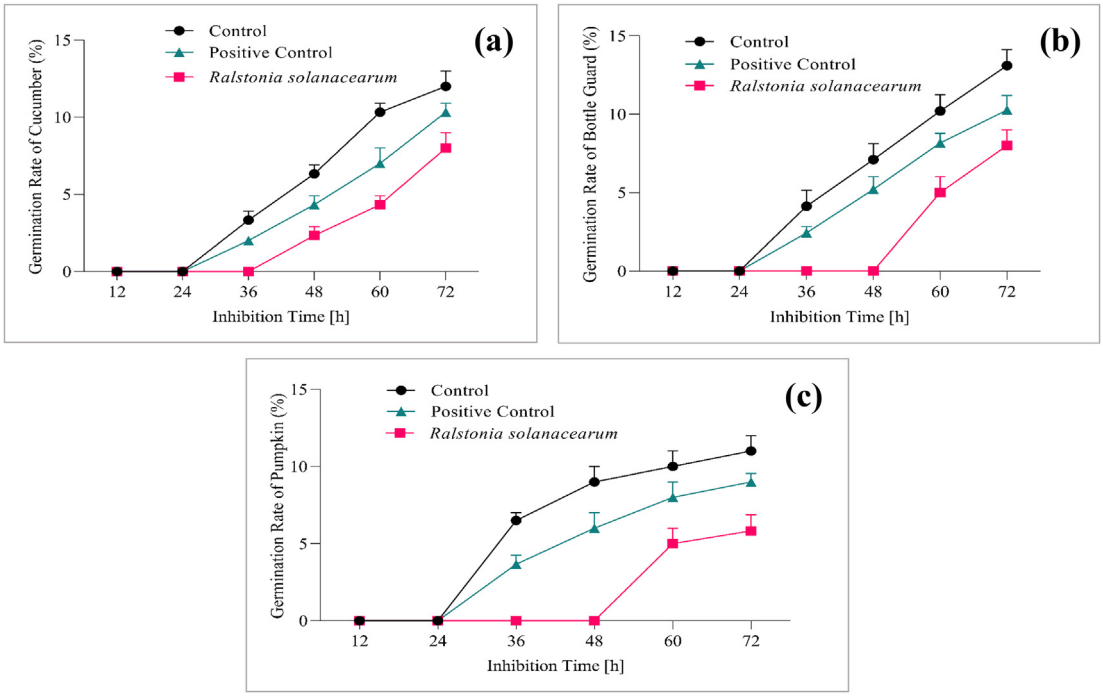


Fig. 4. Effects on germination rate of (a) Cucumber, (b) Bottle Guard and (c) Pumpkin seeds treated by *Ralstonia solanacearum*. Seeds were inoculated by liquid culture of *Ralstonia solanacearum* by shaking using orbital shaker. Seeds treated with *B. subtilis* were considered as positive control. Treated seeds were placed into separate petri dishes and incubated in an incubator maintaining an environment of 25 °C temperature and relative humidity of 75% under dark conditions for 3 days. Seeds germination data were recorded at 12 h interval. The mean of three replications and capped lines represent standard error.

lings were found to be 3.2 ± 1.13% and 1.7 ± 0.92%, respectively which is 46.87% lower than the control (Fig. 5a), where control and *R. solanacearum* treated bottle guard seedlings were found to be 5.6 ± 0.13% and 0.13 ± 0.01%, respectively which is 97.67% lower than the control (Fig. 5b) and finally control and *R. solanacearum* treated pumpkin seedlings were found to be 1.66 ± 1.11% and 0.66 ± 1.09%, respectively which is 60.24% lower than the control (Fig. 5c). Further the shoot lengths of the seedlings were significantly decreased due to *R. solanacearum* treatment in respect to control. It was noticed that shoot lengths of control and *R. solana-*

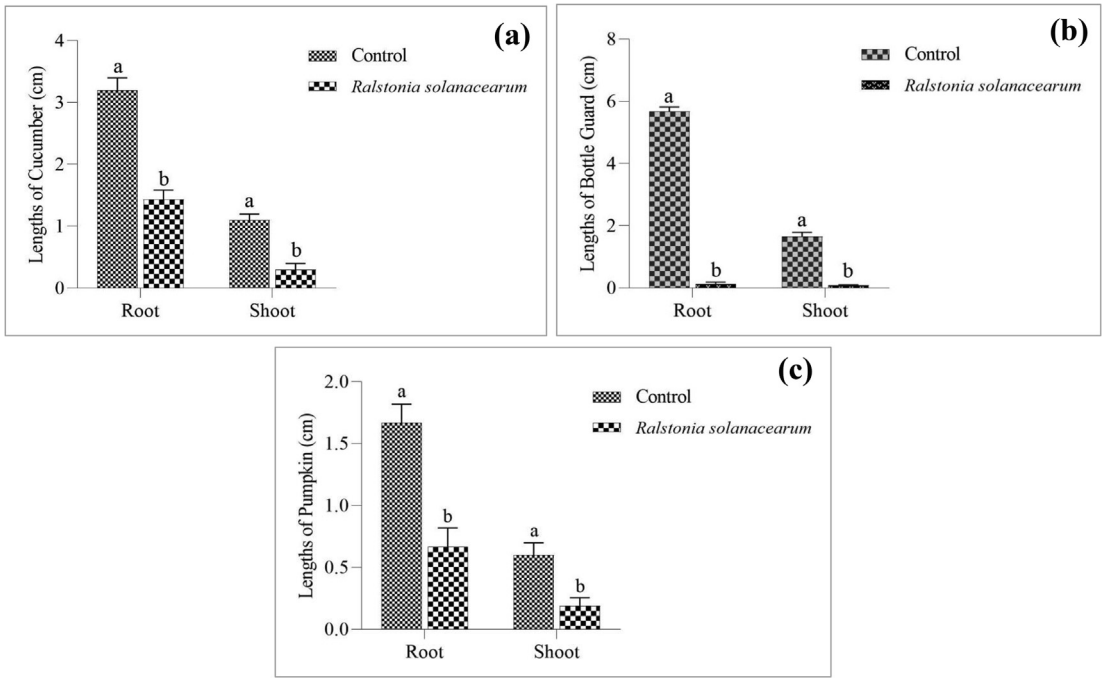


Fig. 5. Changes in root and shoot lengths (a) Cucumber, (b) Bottle Guard and (c) Pumpkin of the *Ralstonia solanacearum* treated seeds. *Ralstonia solanacearum* treated germinated seeds were cultivated in a hydroponic culture medium for 5 days. Shoot and root lengths were measured after 5 days using digital centimeter slide caliper. In each column, similar letters are identical and dissimilar letters are differed significantly as per DMRT. The statistical significance is $P \leq 0.05$.

cearum treated cucumber seedlings were found to be 1.1 ± 0.73% and 0.3 ± 0.62%, respectively which is 72.72% lower than the control (Fig. 5a) where shoot lengths of control and *R. solanacearum* treated bottle guard seedlings were found to be 1.6 ± 0.25% and 0.09 ± 0.01%, respectively which is 94.37% lower than the control (Fig. 5b) and finally shoot lengths of control and *R. solanacearum* treated pumpkin seedlings were found to be 0.6 ± 1.08% and 0.2 ± 0.89%, respectively which is 66.66% lower than the control (Fig. 5c). All the control group increments were found to be statistically more significant than the bacteria treated seeds. Based on the growth parameters we may conclude that *R. solanacearum* treatment inhibits the growth of the proper seedlings of those experimented plants.

3.7. R. solanacearum exhibits diverse antibiotic sensitivity pattern

In the antibiotic susceptibility assay, *R. solanacearum* bacterial pathogen showed a different sensitivity pattern against the tested antibiotics. The highest antibacterial activity was shown by ampicillin (10 μg/disc) with 24.66 ± 0.09 mm diameter zone of inhibition (DZI) followed by streptomycin (10 μg/disc) with 21.66 ± 0. 7 mm DZI, azithromycin (15 μg/disc) with 20 ± 0.0 mm DZI, gentamycin (30 μg/disc) with 19.33 ± 0.9 mm DZI, erythromycin (15 μg/disc) with 19 ± 0.0 mm DZI, clarithromycin (15 μg/disc) with 18.66 ± 0.01 mm DZI, oxytetracycline & neomycin (30 μg/disc) with 18 ± 0.0 mm DZI (Fig. 6). On the other hand, penicillin (10 μg/disc), amoxycillin (10 μg/disc), cefotaxime (30 μg/disc), carbenicillin (100 μg/disc), rifampicin (5 μg/disc), nalidixic acid (30 μg/disc), and cefixime (5 μg/disc) did not show any zone of inhibition against the bacterial strain.

3.8. Bioactive compounds of plant extracts reduce the growth of R. solanacearum

In the current study, bioactive compound of plant extracts from the selected medicinal plants showed a broad spectrum of antagonistic activity against the isolated *R. solanacearum* bacterial strain. In the present study, we prepared extract using leaf of *L. camara*, *T. erecta* and *P. pinnata* while whole plant and root of *C. citratus* and *R. serpentina* were used for the preparation of extract, respectively. The antibacterial sensitivity of different solvents of *L. camara, T. erecta, C. citratus, R. serpentina, P. pinnata* showed a significant reduction in the growth of bacterial strain in terms of zone of inhibition around the disc. In case of *L. camara* ethanolic leaf extract, *C. citratus* methanolic plant extract and *R. serpentina* petroleum ether root extract at concentrations of 10 μg/disc, 20 μg/disc, 30 μg/disc and 50 μg/disc were showed significant responses against the *R. solanacearum*. Among the five medicinal plants, ethanolic leaf extract of *L. camara* showed highest 16.83 ± 0.06 mm, 18.16 ± 0.0 2 mm, 17.63 ± 0.02 mm and 20 ± 0.01 mm zone of inhibition (DZI) at 10 μg/disc, 20 μg/disc, 30 μg/disc and 50 μg/disc concentrations, respectively (Fig. 7a). Methanolic leaf extract of *C. citratus* showed highest 17 ± 0.03 mm, 18 ± 0.0 5 mm, 19 ± 0.07 mm and 20 ± 0.06 mm DZI at 10 μg/disc, 20 μg/disc, 30 μg/disc and 50 μg/disc concentration, respectively (Fig. 7b) while petroleum ether root extract of *R. serpentina* showed highest 16 ± 0.02 mm, 18 ± 0.04 mm, 20 ± 0.03 mm and 22 ± 0.04 mm DZI at 10 μg/disc, 20 μg/disc, 30 μg/disc and 50 μg/disc concentration, respectively (Fig. 7c) against the isolated *R. solanacearum* bacterial strain. On the other hand, the leaf extract of *T. erecta* and *P. pinnata* did not show any sensitivity against the *R. solanacearum* (Fig. 7d, and Fig. 7e). Based on the findings, this results suggest that the natural products from *B. subtilis* and tested medicinal plants might be play as potential source to inhibit the growth of the isolated *R. solanacearum* bacterial strain.

3.9. B. subtilis suppresses the growth of R. solanacearum

In the current study, we used two different methods viz. disc diffusion and well diffusion method to evaluate the antagonistic activity of soil bacterim *B. subtilis* against *R. solanacearum* of wilt disease of papaya. The antagonistic assay of soil bacteria was showed significant reduction in the growth of bacterial strain in terms of zone of inhibition around the disc and well. In case of disc diffusion method, *B. subtilis* showed highest 17 ± 0.9 mm zone of inhibition (DZI) at 50 μl/disc concentration against the *R. solana-*

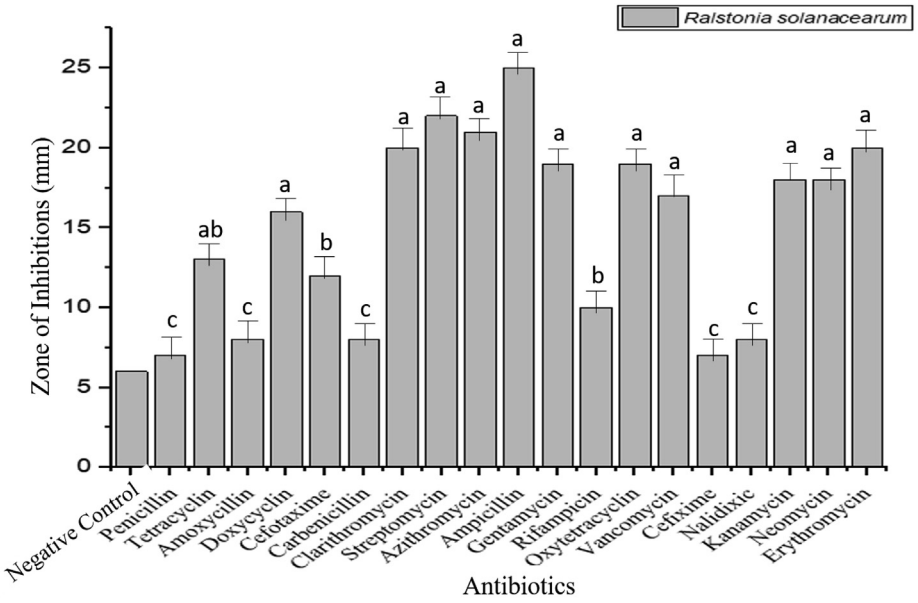


Fig. 6. Antibiotic sensitivity assay and their zone of inhibition against *Ralstonia solanacearum. Ralstonia solanacearum* was grown overnight at 37 °C and spread on LB agar plate, and dried. 6 mm antibiotic discs were placed on the centre of the agar plate and incubated at 37 °C for overnight. Zone of inhibition was measured by mm scale. Disc size (6 mm) was considered as negative control. In each column letters are identical and dissimilar letters are differed significantly as per DMRT. The statistical significance is $P \leq 0.05$.

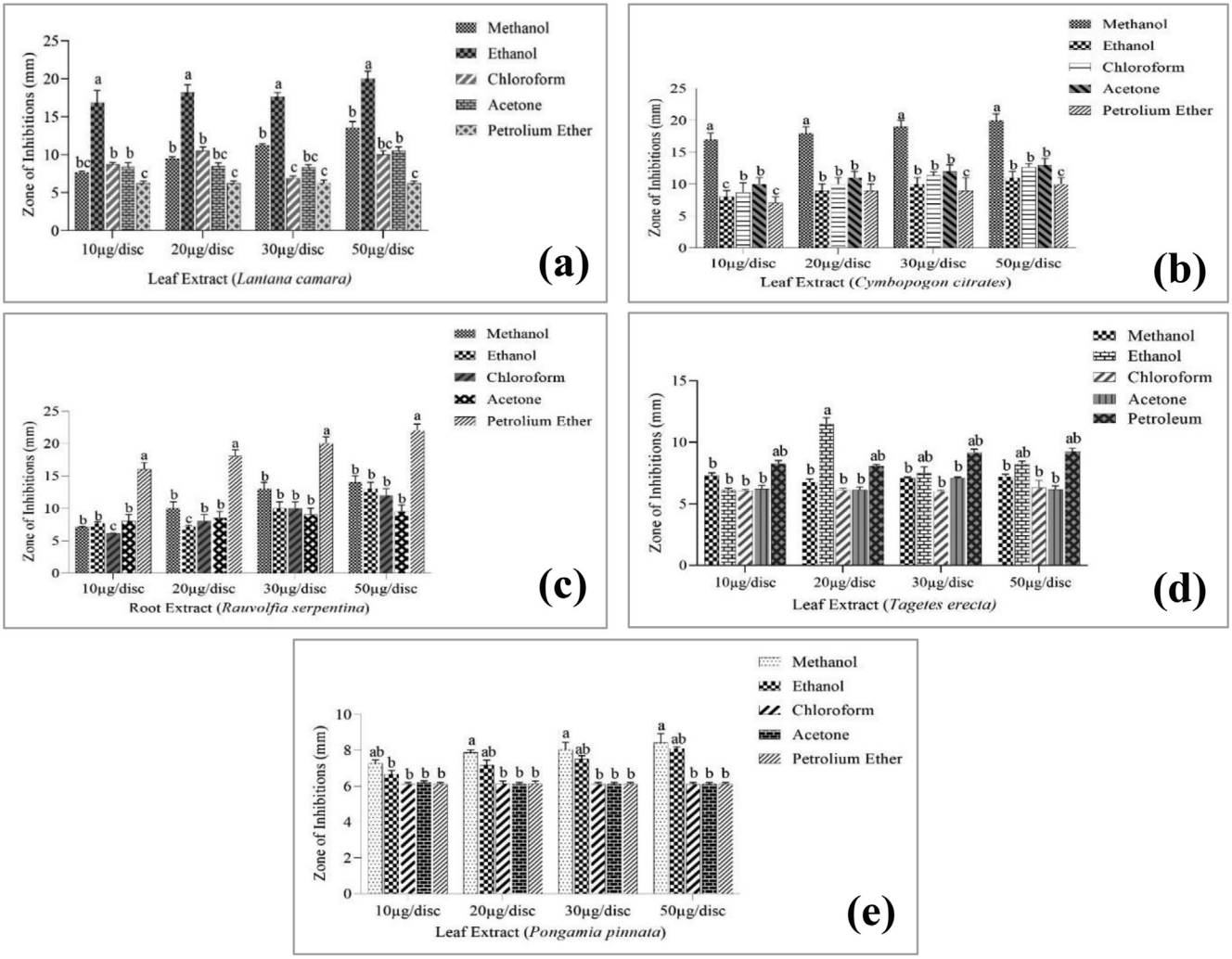


Fig. 7. Antagonistic activity of plant extracts against *Ralstonia solanacearum.* Plant extracts with five solvents (methanol, ethanol, chloroform, acetone, petroleum ether) were applied at four different concentrations (10 µg/disc, 20 µg/disc, 30 µg/disc, and 50 µg/disc). Agar plate containing *Ralstonia solanacerum* and discs of plant extracts incubated at 37 °C for overnight. Zone of inhibition was measured by mm scale where (a) ehtanolic extract of *Lantana camara* showed highest 20 ± 0.01 mm zone of inhibition at concentrations of 50 µg/disc, (b) methanolic extract of *C. citratus* showed highest 20 ± 0.06 mm zone of inhibitions at concentrations of 50 µg/disc, (c) petroleum ether extract of *Rauvolfia serpentina* showed highest 22 ± 0.04 mm zone of inhibitions at concentrations of 50 µg/disc, (d) ethanolic extract of *Tagetes erecta* showed highest 12 ± 0.04 mm zone of inhibitions at concentrations of 20 µg/disc and (e) methanolic extract of *Pongamia pinnata* showed 9 ± 0.01 mm zone of inhibitions at concentrations of 50 µg/disc. In each column letters are identical and dissimilar letters are differed significantly as per DMRT. The statistical significance is $P \leq 0.05$.

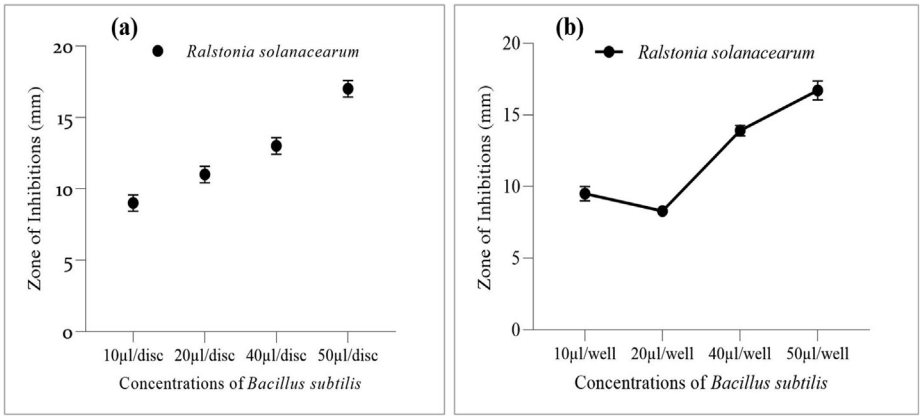


Fig. 8. Antagonistic assay of soil bacteria (*Bacillus subtilis*) against *Ralstonia solanacearum. Bacillus subtilis* at a concentrations of 10 µl/disc, 20 µl/disc, 40 µl/disc, and 50 µl/disc were placed on *Ralstonia solanacearum* inoculated agar plate and incubated it at 37 °C for overnight. Zone of inhibition was measured by mm scale. (a) *Bacillus subtilis* showed highest 17 ± 0.9 mm zone of inhibition at concentrations of 50 µl/disc against the *Ralstonia solanacearum* by disc diffusion method. (b) *Bacillus subtilis* showed highest 16.66 ± 0.5 mm zone of inhibition at concentrations of 50 µl/well against the *Ralstonia solanacearum* by well diffusion method.

cearum bacterial strain (Fig. 8a). On the other hand, in the well diffusion method, *B. subtilis* showed highest 16.66 ± 0.5 mm zone of inhibition (DZI) at 50 μl/well concentration against the *R. solanacearum* (Fig. 8b). Depend on the above-mentioned results we can conclude that, the soil bacteria play a vital role as a dynamic source to inhibit the growth of the isolated *R. solanacearum* bacterial strain.

3.10. Retrieval of the antibacterial compounds and prediction of the targets

A total number of 30 antibacterial compounds were found for the three plant extracts (Supplementary Table 2) whereas 29 antibacterial compounds were found for *B. subtilis* (Supplementary Table 3). Total 53 protein hits were found for all the compounds of the plant extracts (Supplementary Table 2) and 23 protein hits were found for the compounds of *B. subtilis* (Supplementary Table 3). Among all of the hits, 5 proteins were found essential for the survival of the bacterium *R. solanacearum* (Table 3). This study also revealed that quercetin (*C. citratus and B. subtilis*) interacts with F0F1 ATP synthase subunit alpha, rutin (*C. citratus*) interacts with citrate (Si)-synthase and dihydrolipoyl dehydrogenase, eugenol (*L. camara*) with Cysteine synthase CysM and dipicolinic acid (B. subtilis) interacts with 4-hydroxy tetrahydrodipicolinate reductase.

3.11. Homology modeling and model validation

In total, five models were generated for the selected five proteins where the identities of all templates were more than 60% (Table 2). Model validation assessment scores revealed that all models were highly reliable. In Ramachandran plot analysis by PROCHECK, more than 90% residues were found in the most favored regions for all models. Overall quality factors of all models by ERRAT were also more than 90%. Additionally, all models passed in the Varify3D model test.

3.12. Molecular docking

A molecular docking approach was adopted for the study of the interaction between the antibacterial compounds and their corresponding targets. Rutin found in *C. citratus* was showed the highest binding affinity of −9.7 kcal/mol and −9.5 kcal/mol while interacting with citrate (Si)-synthase and dihydrolipoyl dehydrogenase of *R. solanacearum*, respectively (Table 3, Fig. 9d and Fig. 9e) and (Fig. 10c and Fig. 10d). Another compound named quercetin, which was present in both *C. citratus* and *B. subtilis* interacts with F0F1 ATP synthase subunit alpha with a binding affinity of −6.9 kcal/mol (Table 3, Fig. 9b and Fig. 10b). Besides, two more compounds, dipicolonic acid from *B subtilis*, and eugenol from *L. camara*, were also showed interaction with 4-hydroxy tetrahydrodipicolinate reductase with a binding affinity of −6 kcal/mol (Table 3, Fig. 9c and Fig. 10e) and cysteine synthase CysM (−5.4 kcal/mol), respectively (Table 3, Fig. 9a and Fig. 10a). These binding interactions between antibacterial compounds and essential target proteins of *R. solanacearum* indicate that antibacterial compounds may block the synthesis of these essential genes and reduce the growth of *R. solanacearum*.

Table 3
Binding energy of the protein–ligand interactions.

Source	Compounds	Target protein	Binding energy (Kcal/mol)
C. citratus	Rutin	citrate (Si)-synthase	−9.7
		dihydrolipoyl dehydrogenase	−9.5
C. citratus and *B. subtilis*	Quercetin	F0F1 ATP synthase subunit alpha	−6.9
B. subtilis	Dipicolonic acid	4-hydroxy-tetrahydrodipicolinate reductase	−6
L. camara	Eugenol	cysteine synthase CysM	−5.4

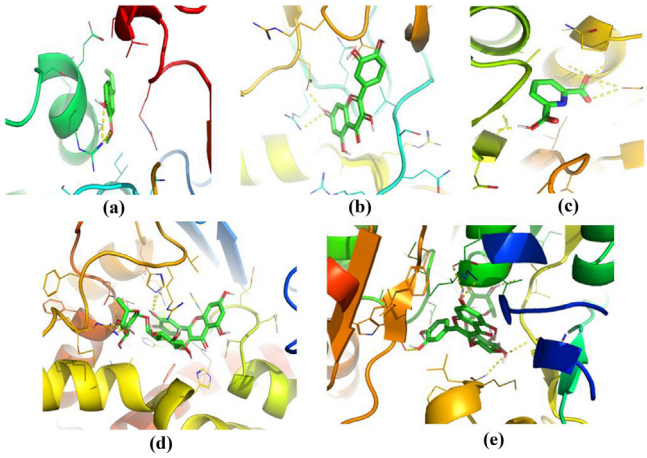


Fig. 9. Interactions of antibacterial compounds with their target proteins. Here, (a) eugenol with cysteine synthase CysM, (b) quercetin with F0F1 ATP synthase subunit alpha, (c) dipicolonic acid with 4-hydroxy-tetrahydrodipicolinate reductase, (d) rutin with citrate (Si)-synthase, and (e) rutin with dihydrolipoyl dehydrogenase.

4. Discussion

R. solanacearum, a causal agent of bacterial wilt disease, is a terrible hindrance to the production of papaya plants in both tropical and temperate regions. The wilt disease of papaya is becoming the main impediment in the successful cultivation of papaya; therefore, it is necessary to conduct studies on various physiological and biochemical characteristics of the causal agents. Isolation and proper biochemical characterizations are the key for identifying pathogenic bacteria. In the present study, *R. solanacearum* was isolated on LB agar medium from wilt infected leaves of papaya. We obtained a smooth, raised, and white colony of *R. solanacearum* as the pure culture which was round in shape on an LB agar plate. Our result supports the findings of Stanford and Wolf (1917) who

Table 2
Generation of homology models and model validation assessment scores.

Name of proteins	Templates PDB ID	Identity (%)	Validation assessment score		
			Ramachandran plot (Most favored regions)	ERRAT value (%)	Varify3D (%)
cysteine synthase CysM	2jc3.1.A	64.73	93.5%	97.56	98.31
F0F1 ATP synthase subunit alpha	6oqv.1.B	69.59	90.8	93.30	90.25
citrate (Si)-synthase	4e6y.1.A	65.34	93	94.10	94.41
dihydrolipoyl dehydrogenase	5u25.1.A	70.22	93.4	95.95	86.95
4-hydroxy-tetrahydrodipicolinate reductase	4f3y.1.A	64.64	92.8	98.76	96.96

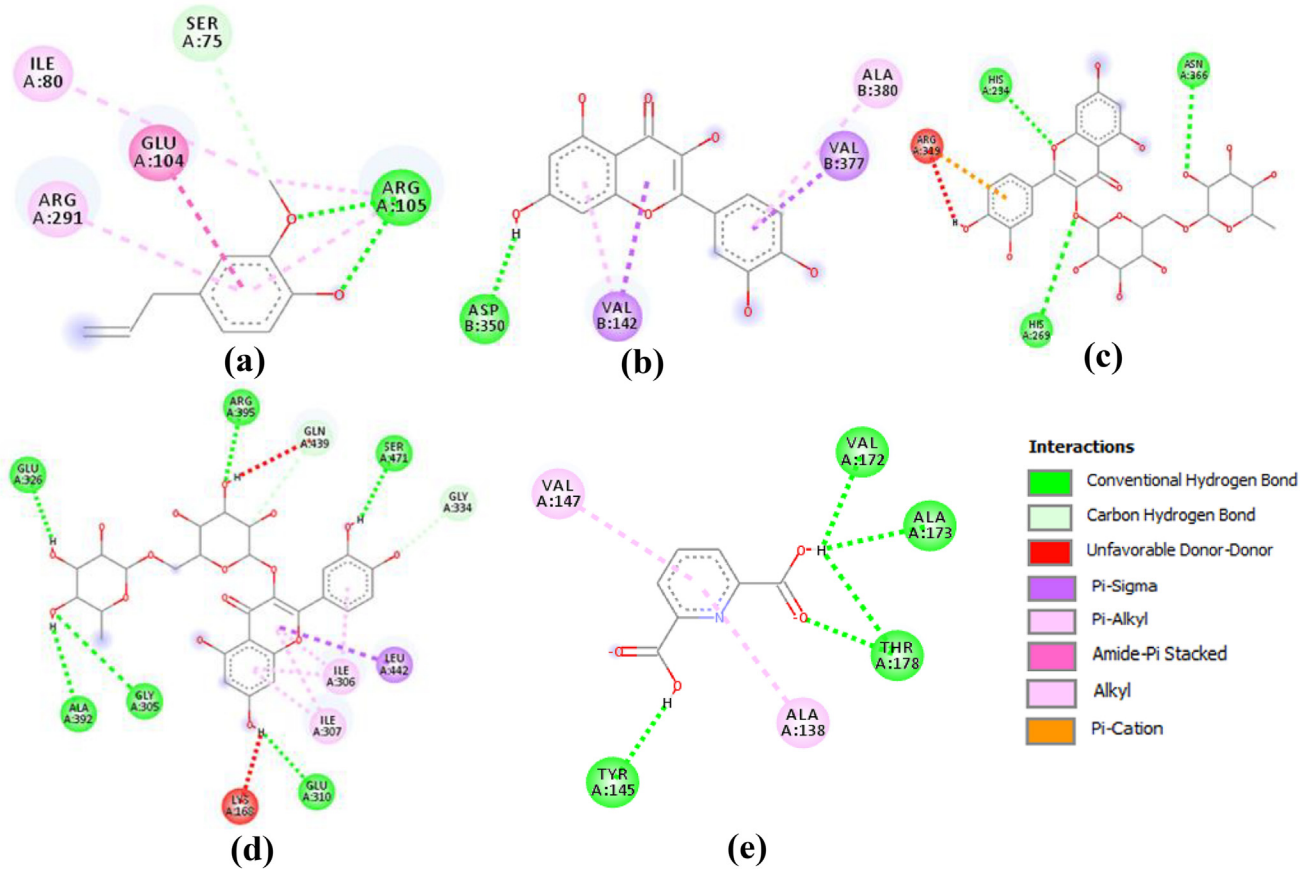


Fig. 10. 2D diagram of protein–ligand interaction sites. Here, (a) eugenol with cysteine synthase CysM, (b) quercetin with F0F1 ATP synthase subunit alpha, (c) rutin with citrate (Si)-synthase, (d) rutin with dihydrolipoyl dehydrogenase, and (e) dipicolonic acid with 4-hydroxy-tetrahydrodipicolinate reductase.

mentioned a similar colony morphology of *R. solanacearum* as white, raised, circular and smooth. In Gram staining, cells of *R. solanacearum* were found to be straight rods and they retained pink color. These results are following the findings of Antony et al. (2014). The thread-like viscous appearance in the KOH test was noticed which is parallel with the result of Popoola et al. (2015) who reported that strains of *R. solanacearum* produced slime thread which is an indication of Gram-negativity. The motility of *R. solanacearum* strain BD was confirmed as the bacteria were spreading away from the line of inoculation. H_2S production was noticed by the appearance of black precipitation. Seleim et al. (2014) also found positive motility for all *R. solanacearum* isolates from wilt infected tomato plants. In the biochemical test, *R. solanacearum* strain BD isolated from wilt disease of papaya showed positivity to methyl red and Kovac oxidase which is confirmatory with the findings of Zhang et al. (2011). Different sugar fermenting ability of *R. solanacearum* was determined by KIA, TSI, MacConkey and Urease test. We observed that *R. solanacearum* produced gas in the TSI medium. Previously Rath and Addy (1977) reported that *P. solanacearum* produced acid when grown in a medium containing either dextrose, sucrose, lactose or glycerol. *R. solanacearum* was able to utilize carbon as its sole source of energy in citrate medium and showed a positive response in catalase activity. Our results correlate with the findings of Kataky (2017), Maji and Chakrabartty (2014), and Murthy and Murthy (2012).

Rapid and accurate identification of causal agents of plant diseases can minimize economic losses. Nucleic acid-based amplification methods like polymerase chain reaction (PCR) are promising strategies for the rapid identification of plant pathogenic bacteria (Srinivasa et al., 2012). The 16S rRNA gene contains variable regions that have been extensively used for the differentiation between the species and genera. In our study, we obtained a clear band of approximately 1400 bp of 16 s rRNA from *R. solanacearum* of wilt disease of papaya after PCR amplification. A similar result was obtained by She et al. (2017) who found 1423 bp fragment 16S rRNA gene for 24 isolates from wilt disease infected *Cucurbita maxima*. A BlastN search followed by phylogenetic tree analysis confirmed the isolated causal agent of wilt disease of papaya as *R. solanacearum*. *Hrp* genes act as the main contributing genes of the bacterium to cause pathogenicity mainly by encoding proteins for type three secretion system (T3SS) (Cunnac et al., 2004b). According to Genin et al. (1992) and Kanda et al. (2003) most of the *hrp* gene-coded proteins form part of T3SS and their expression is regulated by *hrpB*. In our observations, successful PCR amplification and clear band in the 1% agarose gel electrophoresis indicate the presence of *hrpB* gene in *R. solanacearum* isolated from wilt disease of papaya. Therefore, the presence of the *hrpB* gene in *R. solanacearum* confirms its virulent ability. Coll and Valls (2013) reported that the main pathogenicity determinant in *R. solanacearum* is T3SS, and using this system the bacteria injects type III effector proteins into host cell during infection (Erhardt et al., 2010; Tampakaki et al., 2010). Aldon et al. (2000) also reported that the *hrpB* gene is the main component of T3SS and it is unique to *R. solanacearum* that activates the virulence mechanisms when the bacterium detects the plant cell wall component. Jacobs et al. (2012) found that one-half of the *hrpB* regulated genes are induced in bacteria isolated from the xylem of bacterial wilt-infected plants. Based on our findings, the presence of the *hrpB* gene also confirms *R. solanacearum* was successfully identified and it can cause wilt disease in papaya.

In this present study, evaluation of the ability to inhibit the germination rate of three different types of seeds viz. cucumber, bottle guard, and pumpkin seeds were conducted by treating *R. solanacearum*. Previously studies were conducted on evaluating bacterial pathogenicity of *R. solanacearum* towards 6–7 day-old tomato seedlings inoculated by leaf clipping method (Kumar et al., 2017) but there was no works were reported regarding seedlings of cucurbits, bottle guard and pumpkin inoculated by *R. solanacearum*. *R. solanacearum* is a phytopathogenic bacterium that colonizes the xylem vessels of host plants leading to lethal wilt disease. It has been observed that *R. solanacearum* triggered disease symptoms in the seedlings at the inoculation site and eventually spread down to the root region (Kumar et al., 2017). In our experiment, it was observed that, in each case, the germination rate over time had been delayed and inhibited through bacteria inoculation with the control. In the experiment, a germination test was carried out where the seeds of cucumber were used as control and after 72 h of incubation, the germination rate was 80.44 ± 1. 11% while seeds inoculated with *R. solanacearum* showed 64.32 ± 1.45%, resulting in a significant delay in seed vigor over control. A similar delayed result was observed in the germination test of bottle gourd and pumpkin seeds inoculated with *R. solanacearum* in where, the germination rate was 53.33 ± 1.11% and 40.12 ± 1.0 5%, respectively after 72 h of incubation. These observation indicate that when seeds were inoculated with *R. solanacearum*, bacterial colonization impairs seed germination resulting in a significant delay in seed vigor over control. These observations are consistent with the result from an earlier study as the effect of the bacterium *Azotobacter chroococcum* inoculation on barley seeds showed a delay in germination (Harper and Lynch, 1981). A similar effects were observed with wheat as they were attributed to damage by seed-borne species of *aspergillus* and *penicillium* (Griffin, 1966). Thomas et al. (2007) found similar findings by the inoculation of papaya seeds with *Microbacterium* sp., *Pantoea* sp., *Cedecea* sp., *Brevundimonas* sp., *Bacillus* sp., Sphingomonas sp., *Mrthylobacterium* sp. *and Agrobacterium sp*. inoculate, which led to delayed germination or slow seedling growth initially, during the first 2–3 weeks after sowing. While the above-mentioned studies on the effect of microbial inoculation on germination of various vegetable species were conducted under different conditions, our study was employed under optimum laboratory conditions and unveiled new data on the delay in seed germination of the three different seeds when they were inoculated at the seedling stage of development with the *R. solanacearum*. This is an apparent disadvantage of crop production, as our experiment confirms that the bacterial isolate has the potential to attack vegetable species at all stages of development.

The spread, distribution, and environmental reservoirs of antibiotic-resistant bacteria are vital issues. In recent years various potential sources were examined intensively to explain the spread of antibiotic-resistant bacteria. The development and distribution of liquid manure are known to lead to the expansion of multi-resistant bacteria (Roschanski et al., 2017; Sengupta et al., 2013). In the antibiotic susceptibility assay, isolated *R. solanacearum* bacterial pathogen showed different sensitivity patterns against the antibiotics. The highest antibacterial activity was shown by ampicillin, streptomycin, azithromycin, gentamycin, erythromycin, clarithromycin, oxytetracycline and neomycin. On the other hand, penicillin, amoxycillin, cefotaxime, carbenicillin, rifampicin, nalidixic acid, and cefixime did not show any significant zone of inhibition against the *R. solanacearum*. Verma et al. (2014) found the inhibitory action of several antibiotics *against R. solanacearum* to control wilt in tomato, brinjal, and capsicum. The remarkable genetic plasticity of the microorganisms, the high selective pressure, and the mobility of the world population have created resistant bacterial strains that have spread worldwide. Some naturally available medicinal, aromatic, and spice-producing plants are very much effective against microbial flora (Sarter et al., 2011; Tripathi and Dubey, 2004). The discovery of natural materials as alternatives to the synthetic antibacterial agents has been quite interesting in the past 20 years (Chehregani et al., 2007). In our study, the ethanoic leaf extract of *L. camara*, petroleum ether root extract of *R. serpentina*, and methanol extract of *C. citratus* were showed significant response against the *R. solanacearum*. On the other hand, the leaf extract of *T. erecta* and *P. pinnata* did not show any sensitivity against the bacterium. Previous studies using the extracts from *L. camara* species showed that they were able to inhibit the growth of Gram-positive and negative bacteria (Breijyeh et al., 2020). Alemu et al. (2013) found an inhibitory effect of methanol extract of *L. camara* against *R. solanacearum* which supports our findings. A study conducted by Paret et al. (2010) demonstrated the suppression of *R. solanacearum* using *C. citratus* which is consistent with our present findings. The antagonistic soil microorganisms inhibit the growth of other microorganisms including plant pathogens by antibiosis, competition, and exploitation. The present study was conducted to identify the antagonistic activity of *B. subtilis* soil bacteria against the causal agent of bacterial wilt of papaya. In the case of the disc diffusion method, *B. subtilis* showed the highest 17 ± 0.9 mm zone of inhibition (DZI) against the *R. solanacearum* bacterial strain. On the other hand, the well diffusion method of *B. subtilis* showed the highest 16.66 ± 0.5 mm zone of inhibition (DZI) against the causal pathogen (*R. solanacearum*) of papaya. Farahat et al. (2017) found promising findings to control *R. solanacearum* from wilt of tomato using *B. subtilis* whereas Ji et al. (2008) found the growth inhibition of *R. solanacearum* from wilt of mulberry using *B. subtilis*. Our result clearly demonstrated that the *B. subtilis* bacteria possess the ability to inhibit the growth of *R. solanacearum*. Therefore, by using soil microbes, we can control the severity of the disease without chemical control that is more environmentally friendly.

Network pharmacology and molecular docking analysis demonstrated the mechanism of inhibition of bacterial growth by the compounds from plant extracts and *B. subtilis*. Antibacterial compounds found in these plant extracts interact with the essential genes of *R. solanacearum* and eventually inhibit the growth of the bacterium. In our study, we found that *C. citratus* contain the antibacterial compounds rutin which was identified to interact with citrate (Si)-synthase and dihydrolipoyl dehydrogenase. These two genes are essential for the survival of *R. solanacearum*. Inhibition of these genes eventually affects the growth of the bacteria. A study conducted in China by Yang et al. (2013) also reported that rutin strongly inhibits the growth of *R. solanacearum*. Another compound, quercetin from this plant extract inhibits the bacterium targeting F0F1 ATP synthase subunit alpha which is also essential for *R. solanacearum*. However, *B. subtilis* secreted another compound dipicolonic acid which also inhibits this bacterium by inhibiting 4-hydroxy-tetrahydrodipico linate reductase. By molecular docking study, we found that *L. camara* plant extracts inhibit *R. solanacearum* by eugenol. Eugenol affects the growth of the bacteria by interacting with cysteine synthase CysM. Bai et al. (2016) reported that eugenol from lilac extract had the strongest inhibitory effect on *R. solanacearum* while studying tobacco bacterial wilt. Former studies reported that the inhibitory effect of the antibacterial compound is linked to the disruption of the cell membrane and membrane proteins, breakdown of cell wall biosynthesis, and obtrusion with the enzymatic activity (Kim et al., 2002; Nguyen et al., 2013). In our study, we identified five essential genes of *R. solanacearum* which are vital for its existence. The compound from plant extracts and *B. subtilis* interact with these essential genes and finally inhibit the growth by blocking the biosynthesis of the essential genes.

5. Conclusion

R. solanacearum causes great economic loss by producing wilt disease in wide range of plants all over the world. Therefore, control of wilt disease caused by *R. solanacearum* has become a major concern for the scientific community. A number of strategies are available for minimizing the severity of this disease. Most of them are based on chemical pesticides which are harmful for biotic community. For this reason, an alternative environmentally friendly control measurement is needed. In our study, we found that antibacterial compound from plant extracts and *B. subtilis* can suppress the growth of *R. solanacearum*. Finally, *in-silico* molecular docking study unveiled that, quercetin, eugenol, rutin and dipicolonic acid from selected medicinal plants and *B. subtilis* can inhibit the growth of *R. solanacearum* by interacting with the essential protein of *R. solanacearum*. Our study will be helpful for developing environmental friendly control measure of *R. solanacearum* of wilt disease of papaya.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2021.07.069.

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