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RESEARCH ARTICLE

Screening Plant Products for *Ralstonia solanacearum* Inhibition and Characterization of Antibacterial Compounds in *Garcinia indica* and *Tamarindus indica*

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Abstract Bacterial wilt caused by *Ralstonia solanacearum* is a soil borne disease affecting several plant species of different genera. Management of this disease with various strategies provided limited success. Plant product in the management of bacterial wilt is one of the minimum explored areas. In the present study attempts were made to exploit the antibacterial properties of the locally available plant species for the inhibition of *R. solanacearum* and also to characterize the phytochemicals in two of the promising plant species by Gas Chromatography–Mass Spectrometry (GC–MS). Thirty-two plants species were screened for their antimicrobial activity against *R. solanacearum* using eight organic solvents. Out of the 264 tested extracts 123 showed zones of inhibition under in vitro condition. It was observed that alcohol extracts showed better activity against *R. solanacearum* as compared to other solvents. Two plant species viz. *Garcinia indica* and *Tamarindus indica* were highly inhibitory to the pathogen. The phytochemical analysis indicated the presence of saponins, terpenoids, alkaloids, flavonoids and tannins in the extracts. Fractions from solvent–solvent partitioning indicated that alcohol based fraction (Fraction III) was inhibitory to the pathogen. GC–MS analysis of Fraction-III revealed the presence of phytoconstituents viz. 4H-pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl and 2-Furancarboxaldehyde, 5 (hydroxymethyl) in *G. indica*; butanoic acid, and myo-inositol, 4-C-methyl in *T. indica*. The present study is the first report on the use of *G. indica* and *T. indica* in inhibiting *R. solanacearum* and the antimicrobial constituents of the extracts.

Keywords *Ralstonia solanacearum* · Plant products · *Garcinia indica* · *Tamarindus indica*

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Introduction

Ralstonia solanacearum causes bacterial wilt in more than 450 plant species across 54 plant families [1]. It is regarded as one of the most important bacterial phytopathogen. The disease affects mainly the solanaceous vegetables in India [2]. Symptoms of the disease are sudden wilting of plants as the pathogen invades through the sites of root emergence, rapid multiplication and dissemination leading to brownish discoloration and death of the vascular tissue [3]. Control of bacterial wilt is a very difficult task as *R. solanacearum* is known to evolve continuously attaining different genetic variations within the strains [4]. Researchers have tried using various methods such resistant variety [5], selection of healthy planting material [6], crop rotation [7], application of microbial antagonists [8] and organic soil amendments [9] for controlling the disease. Biological control is known to be one of the best methods since it is environmental friendly and therefore an acceptable practice as compared to others. However, bacterial wilt management using a single conventional method is not effective and therefore alternative practices to manage the disease have been reported [10]. Plants represent a reservoir of promising chemotherapeutics [11] and are rich sources of bioactive compounds, such as terpenoids, alkaloids, flavonoids, tannins and polyacetylenes

[12]. In recent times plant extracts are used as compared to synthetic chemicals as an effective way of controlling plant diseases and a lot of interest has been developed in the use of antimicrobial nature of plants for plant disease control [13]. Plant extracts and essential oils have exhibited antimicrobial properties and are used by the researchers as an ecofriendly and economic option in plant disease control [14]. These extracts and secondary metabolites from plants can therefore be considered as a non-toxic and a feasible option over the synthetic bactericides. Thymol, a volatile compound from plants when used as pre-plant soil fumigation was found to reduce bacterial wilt incidence on tomato [15]. *Allium fistulosum* extracts inhibited the growth of *R. solanacearum* phylotype IIB/4NPB in vitro [16]. Sangoyomi et al. [17] reported that aqueous extracts of medicinal plants do not show inhibitory effects on *R. solanacearum*; hence the need to study the effects of different solvent extracts other than water to repress the growth of *R. solanacearum* is required. Extracts of *Myrtus communis*, *Lantana camara*, *Cassia* sp. [18] and *Morinda citrifolia* [19] were active against *R. solanacearum*. Subin and Dilna [20] revealed from their studies that methanol extracts of *Eclipta alba* demonstrated a better antibacterial activity against *X. oryzae*, *X. campestris* and *E. carotovora* whereas the best activity against *R. solanacearum* was exhibited by acetone extracts of *B. diffusa*.

Screening of plant extracts for their antimicrobial activity may provide new antimicrobial substances [21]. Cowan [22] divided the antimicrobial bioactive compounds of plants into terpenoids and essential oils; phenolics and polyphenols; alkaloids; polypeptides and their mixtures (crude extract). Moussa et al. [18] has isolated and identified an active flavonol glycoside, 3-methoxy myricetin 7-O-α-L-rhamnopyranoside which effectively inhibited *R. solanacearum*.

The present study was undertaken with the objective to evaluate the antibacterial activity of extracts from 32 different plants based on the availability but not limited to weedy and invasive alien species against *R. solanacearum*. Further, phytochemical properties of two of the promising plant extracts were analyzed.

Material and Methods

Collection of Plant Material

Thirty-two plant species which were commonly grown and readily available in Goa were collected and their details are given in Table 1. Leaves, root and whole plant were collected separately and washed thoroughly with normal tap water followed by wash with sterile distilled water. The plant materials were completely dried at room temperature under shade for 3–4 days. Dried material was grounded using a grinding machine into fine powder and the powder was stored in polythene bags at 4 °C until further extraction.

Sample Preparation and Extraction

Crude extracts of the plant (leaf, root, whole plant) was obtained following standard procedures described elsewhere. Briefly, 5 g of the dry powder of the plant sample was soaked in 25 mL of organic solvents (80 % ethanol, methanol, chloroform, 80 % ethyl acetate, acetone, dichloromethane, diethyl ether and hexane) to get 20 % (w/v) extract in a 100 mL conical flask. The conical flask was kept on shaker for 48 h at 125 rpm. The infusions were filtered after 48 h using Whatman No. 1 filter paper, then concentrated in vacuum and dried at 40 °C in a hot air oven. After the solvent evaporation, the residue was dissolved in 500 µL of the respective solvent and stored in airtight sterile tubes in a refrigerator at 4 °C until further use. The extract thus obtained was directly used in the assay of antimicrobial activity.

Isolation, Characterization and Pathogenicity of *R. solanacearum* Rs-08-17

Ralstonia solanacearum was isolated from a freshly wilted eggplant from Goa as described by Ramesh and Phadke [23]. It was isolated onto sterile TZC agar plate (Casamino acid, 1 g L^{-1}; peptone, 10 g L^{-1}; glucose, 5 g L^{-1}; agar, 17 g L^{-1} amended with 0.005 % v/v 2,3,5-triphenyl tetrazolium chloride) and the plates were incubated at 28 ± 2 °C on rotary shaker (150 rpm) for 48–72 h. After incubation period, a single fluidal colony was selected, purified and maintained in 30 % glycerol stocks and stored at −80 °C.

Genomic DNA was isolated as suggested by Kumar et al. [24] with some modifications and the final concentration of the DNA was adjusted to 50 ng µL using Nano drop-1000, Thermo fisher scientific, USA. The pathogen was identified as *R. solanacearum* based on the phenotypic characters and confirmed by performing PCR of DNA using Rs759/Rs760 primer pairs [25]. The PCR amplicon was separated on 1.5 % agarose gel and visualized using gel documentation unit (MultiImage Light Cabinet, AlphaImager EC, Alpha Innotech Corporation, San Leandro, CA, USA). Phylotype and biovar of *R. solanacearum* isolate Rs-08-17 were determined by multiplex PCR using a set of phylotype-specific primers as per the method described by Fegan and Prior [26] and by Hayward [3], respectively. Pathogenicity of the isolate was evaluated in a greenhouse maintained at 30 °C. Eggplant seedlings of 25 days were grown in 2 pots (5 seedlings each) and inoculated with the pathogen. Inoculation was done by drenching 20 mL of active bacterial suspension (10^7 CFU mL^{-1}) near the root zone.

Table 1 Antibacterial activity of plant extracts in different organic solvents against *R. solanacearum* Rs-08-17

S. no.	Plant species	Plant part used	Inhibition zone of plant extracts against *R. solanacearum* growth (diameter in mm)[a]							
			80 % ethanol	Chloroform	80 % ethyl acetate	Acetone	Dichloro methane	Diethyl ether	Methanol	Hexane
1	*Murraya koenigii*	Leaves	10.6 ± 0.27	10.3 ± 0.27	–	–	–	–	–	–
		Root	9.0 ± 0.0	1.3 ± 0.27	–	–	–	6.0 ± 0.0	–	–
2	*Eupatorium odorata*	Leaves	8.3 ± 0.54	–	–	–	–	–	10.0 ± 0.0	–
3	*Thuja occidentalis*	Leaves	8.0 ± 0.0	–	15.0 ± 0.47	11.3 ± 0.27	–	–	12.6 ± 0.27	–
4	*Azadirachta indica*	Leaves	12.6 ± 0.54	3.3 ± 0.27	9.0 ± 0.0	–	–	–	–	–
5	*Ocimum tenuiflorum*	Leaves	15.0 ± 0.0	9.0 ± 0.0	13.0 ± 0.0	–	–	–	11.0 ± 0.0	–
6	*Adhatoda vasica*	Leaves	14.6 ± 0.27	4.0 ± 0.47	–	6.0 ± 0.0	7.0 ± 0.0	–	–	–
7	*Garcinia indica*	Leaves	25.3 ± 0.27	3.0 ± 0.0	9.3 ± 0.27	20.6 ± 0.54	–	9.6 ± 0.27	30.0 ± 0.0	–
8	*Moringa oleifera*	Leaves	15.0 ± 0.0	3.6 ± 0.0	14.0 ± 0.47	8.0 ± 0.0	8.3 ± 0.27	12.0 ± 0.0	10.3 ± 0.0	–
9	*Saraca asoca*	Leaves	12.6 ± 0.54	2.0 ± 0.0	9.6 ± 0.27	9.3 ± 0.27	8.0 ± 0.0	–	–	–
10	*Hibiscus* sp.	Leaves	12.0 ± 0.0	–	–	–	7.6 ± 0.27	6.0 ± 0.0	7.6 ± 0.27	–
11	*Jatropha curcas*	Leaves	9.2 ± 0.27	4.5 ± 0.0	–	–	–	–	7.7 ± 0.27	–
12	*Aegle marmelos*	Leaves	12.6 ± 0.27	3.0 ± 0.0	16.3 ± 0.72	12.6 ± 0.27	11.3 ± 0.27	–	10.6 ± 0.27	–
13	*Carica papaya*	Leaves	12.0 ± 0.0	6.3 ± 0.54	8.0 ± 0.47	10.3 ± 0.27	–	–	14.0 ± 0.47	–
14	*Anacardium occidentalis*	Leaves	20.3 ± 0.27	4.0 ± 0.0	9.6 ± 0.27	–	–	10.5 ± 0.47	24.6 ± 0.27	–
15	*Ocimum basilicum*	Leaves	12.0 ± 0.47	13.0 ± 0.47	11.0 ± 0.0	8.6 ± 0.27	9.0 ± 0.0	–	8.0 ± 0.0	–
16	*Cinnamomum zevlancium*	Leaves	21.6 ± 0.27	2.3 ± 0.27	24.6 ± 0.27	12.0 ± 0.0	11.3 ± 0.27	9.0 ± 0.0	12.0 ± 0.0	–
17	*Myristica fragrans*	Leaves	9.0 ± 0.27	–	–	–	7.3 ± 0.27	–	–	–
18	*Gliricidia* sp.	Leaves	12.0 ± 0.0	3.0 ± 0.0	12.6 ± 0.27	–	–	–	9.6 ± 0.27	–
19	*Zingiber officinale*	Rhizome	12.6 ± 0.54	9.6 ± 0.27	12.3 ± 0.27	–	–	–	12.0 ± 0.47	–
20	*Psidium guajava*	Leaves	31.6 ± 0.27	14.0 ± 0.0	12.0 ± 0.0	28.3 ± 0.54	–	–	28.0 ± 0.47	–
21	*Lawsonia inermis*	Leaves	14.0 ± 0.0	6.0 ± 0.47	17.3 ± 0.27	17.6 ± 0.27	8.0 ± 0.0	14.0 ± 0.0	20.6 ± 0.98	–
22	*Averrhoa carambola*	Leaves	15.0 ± 0.0	–	–	9.0 ± 0.0	–	–	11.6 ± 0.0	–
23	*Alstonia* sp.	Leaves	14.0 ± 0.0	1.0 ± 0.0	11.0 ± 0.0	–	6.3 ± 0.27	–	11.3 ± 0.27	–
24	*Canavalia gladiata*	Leaves	14.3 ± 0.27	–	11.6 ± 0.54	16.0 ± 0.0	–	–	10.0 ± 0.0	–
25	*Acacia auriculiformis*	Leaves	25.0 ± 0.0	10.0 ± 0.0	11.3 ± 0.0	23.6 ± 0.0	9.3 ± 0.0	–	9.0 ± 0.0	–
26	*Ricinus* sp.	Leaves	11.0 ± 0.0	24.6 ± 0.27	10.0 ± 0.0	–	–	–	12.6 ± 0.27	–
27	*Physalis weed*	Whole plant	8.3 ± 0.0	11.3 ± 0.0	9.6 ± 0.0	9.0 ± 0.0	–	–	17.6 ± 0.0	–
28	*Mimosa pudica*	Leaves	20.6 ± 0.0	3.0 ± 0.0	14.3 ± 0.0	6.0 ± 0.0	9.0 ± 0.0	–	13.6 ± 0.54	–
29	*Calotropis gigantea*	Leaves	20.0 ± 0.0	2.0 ± 0.0	15.3 ± 0.47	14.0 ± 0.0	7.0 ± 0.0	–	15.0 ± 0.0	–
30	*Tamarindus indica*	Leaves	23.0 ± 0.27	4.0 ± 0.0	13.6 ± 0.54	30.6 ± 0.27	10.0 ± 0.0	–	21.6 ± 0.27	–
31	*Boerhavia diffusa*	Whole plant	15.0 ± 0.0	–	30.0 ± 0.0	12.0 ± 0.0	8.9 ± 0.0	–	16.6 ± 0.0	–
32	*Cymbopogon flexuosus*	Leaves	13.0 ± 0.0	–	31.6 ± 0.54	11.6 ± 0.0	10.0 ± 0.0	–	–	–
33	Control	–	–	8.0	–	–	–	–	–	–

[a] The data represents the mean values of three replicates with standard error; (–) indicates no inhibition; Streptomycin (50 μg/μL) control recorded 40 mm inhibition zone; Zone of inhibition of plant extract was measured after subtracting zone of inhibition if any by the corresponding solvent

In Vitro Antibacterial Activity Assay of Plant Extracts Against *R. solanacearum*

Antibacterial activity of the plant extract against *R. solanacearum* Rs-08-17 isolate was carried out by determining the zone of inhibition using agar diffusion method. 25 μL of overnight grown *R. solanacearum* Rs-08-17 (10^6 CFU mL^{-1}) was added to 100 mL of molten CPG medium and poured into sterile petri plates and allowed to solidify. Four wells of 5 mm diameter each were bored per plate using sterile cork borer and 35 μL of crude extract from each sample was added in the wells. Plain solvent was also added in the

wells which served as negative control. Streptomycin (50 μg μL^{-1}) was used as positive control in the inhibition assay. Plates were incubated at 28 ± 2 °C for 48–72 h. Zone of inhibition of plant extract was measured after subtracting zone of inhibition if any by the corresponding solvent. The experiment was conducted in a completely randomized design and three replicates were maintained per treatment. Standard error of inhibition zone was calculated for each plant species. Activity index (AI) was also estimated using the following formula of Shahidi [27] and expressed as %.

$$\mathrm{AI} = \frac{\text{Diameters of inhibition zone of extract}}{\text{Diameters of inhibition zone of the standard antibiotic}} \times 100$$

Phytochemical Analysis

Ethanol extracts from the two plant species (*Garcinia indica* and *Tamarindus indica*) that showed promising antibacterial activity against the pathogen were selected for preliminary qualitative chemical tests for flavonoids, alkaloids, tannins, terpenoids and saponins. A stock solution of the plant products was prepared by dissolving 1 g of crude extract in 100 mL of respective solvents. This stock was used for further phytochemical screening following the standard methodology described elsewhere.

Tannins

Two mL of each extract was taken and 10 mL of distilled water was added, boiled for 10 min in a test tube. Later a few drops of 0.1 % ferric chloride were added to each test tube and incubated for 10 min. Development of brownish green or a blue black color indicated the presence of tannin [28].

Saponins

Five mL of each extract was taken and to that equal amount of distilled water was added in a test tube and was shaken for 5 min. The mixture was boiled in the water bath for 5–10 min. Frothing during warming indicates the presence of saponins [29].

Flavonoids

Three mL of the extract was taken and 1 mL of 10 % NaOH was added. A yellow coloration showed the presence of flavonoids in each extract [28].

Terpenoids

Each plant extract (500 mg) was taken in a separate test tube and 2 mL of chloroform was added. Later concentrated sulphuric acid was carefully added by the side of the test tube. Formation of a reddish brown color interface layer indicates the presence of terpenoids [30].

Alkaloids

One mL of the extract was taken and to that 2 drops of Mayer's reagent (1.358 g of $HgCl_2$ was dissolved in 60 mL of water and a solution of 5 g of KI in 10 mL of H_2O was added to it and the final volume was made up to 100 mL) was added. Presence of alkaloids was indicated by the formation of a creamy precipitate [31].

Solvent–Solvent Partitioning Method

This method is based on fractionating the components according to polarity. Slight modifications were done in the method described by Terefa et al. [32]. Ethanol extract (15 g, 80 %) of the plant was suspended in about 50 mL of distilled water and thoroughly mixed. This suspension was then shaken with 50 mL of chloroform each time until it became colorless. It was allowed to be partitioned into chloroform-aqueous layer. The chloroform fraction was filtered, evaporated in a rotary evaporator and labeled as Fraction one (F-I). The remaining aqueous fraction was further shaken with n-butanol and allowed to be partitioned into n-butanol-aqueous layer. The n-butanol layer was filtered, concentrated and evaporated to dryness on water bath (40 °C) which was labeled as Fraction two (F-II). The remaining fraction was labeled as fraction three (F-III). All the three fractions were dried and re-dissolved in 500 μL of their respective organic solvents. Antibacterial activity of these fractions was tested against *R. solanacearum* Rs-08-17 according to the method described in the antagonistic section. The fractions showing inhibitory activity against *R. solanacearum* were further analyzed using GC–MS.

Gas Chromatography–Mass Spectrometry (GC–MS) Analysis

GC–MS technique was used to identify the components present in the extract (F-III from solvent–solvent partition) showing inhibition of the pathogen. GC–MS analysis of ethanol fraction (F-III) of two extracts (*G. indica* and *T. indica*) was carried out at Sophisticated Analytical Instrument Facility, Indian Institute of Technology—Bombay, Powai (Mumbai) using an Agilent Technologies 7890A GC systems interfaced to a Mass Spectrometer Jeol The AccuTOF GCV JMS-T100GCV with Hewlett–Packard HP-5MS (30 m × 0.25 mm × 0.25 μm) column. For GC–MS detection, an electron ionization energy system with ionization energy of 70 eV was used. Helium gas (99.999 %) was used as the carrier gas at a constant flow rate of 1 mL min^{-1}. An injection volume of 2 μL of the F-III ethanol extract was

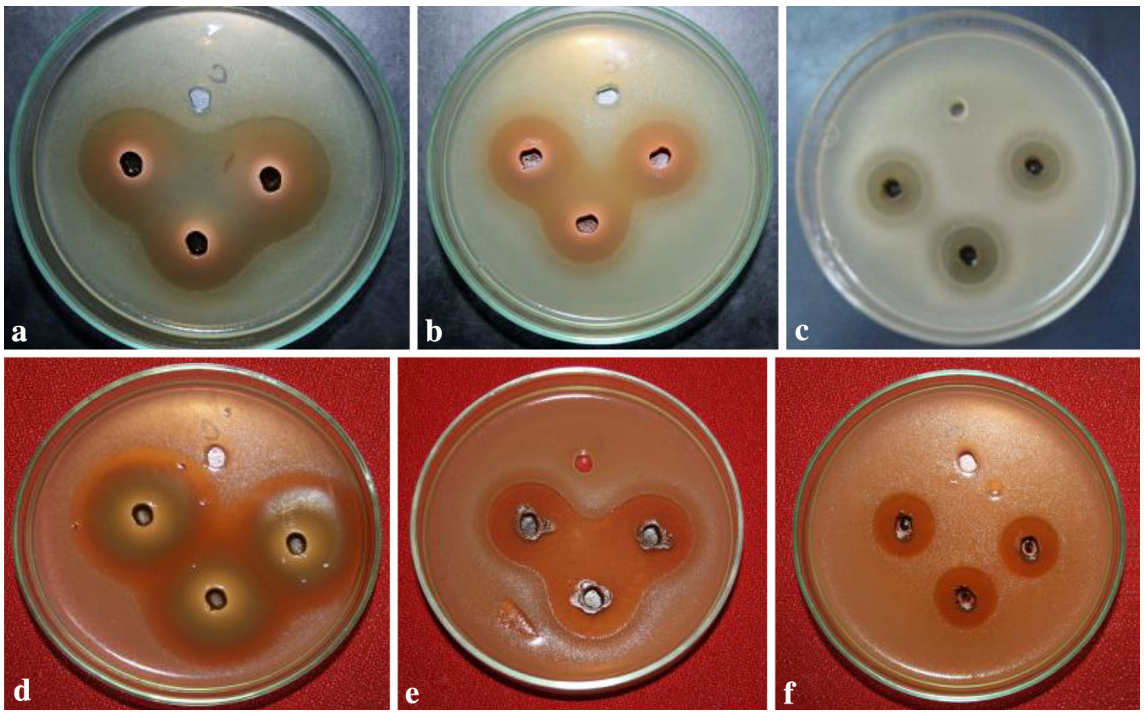

Fig. 1 Inhibition of *R. solanacearum* by various plant extracts; **a** ethanol extract of *T. indica*; **b** methanol extract of *T. indica*; **c** acetone extract of *T. indica*; **d** ethanol extract of *G. indica*; **e** methanol extract of *G. indica*; **f** acetone extract of *G. indica*

used at a split ratio of 20:1. Injector temperature of 250 °C and Ion-source temperature of 200 °C were set. The oven temperature was set as follows: 80 °C (isothermal for 1 min) with an increase of 8 °C/min to 200 °C, followed by 8 °C/min to 250 °C with a 5 min isothermal at 280 °C. GC was run for 36 min. Interpretation on mass spectrum GC–MS was done using the SAIF-IIT database. The spectrum of the unknown component was compared with the spectrum of the known component available in the respective library. From the database, name, molecular weight and structure of the components of the F-III were ascertained.

Results and Discussion

Isolation, Characterization and Pathogenicity of *R. solanacearum* Rs-08-17

Bacterial wilt caused by *R. solanacearum* is regarded at one of the economically important diseases of plants, since the pathogen is soil borne and has a broad host range in addition to high genetic variation within the strains. In the present study, *R. solanacearum* was isolated on TZC medium from the wilted eggplant and the isolate was assigned the number Rs-08-17, based on phenotypic characters [33]. *Ralstonia solanacearum* colonies are white with pink centre producing abundant extracellular polysaccharide. Further its identity was confirmed using Rs759-760 PCR primer set [25] as 282 bp amplicon was produced in PCR. *Ralstonia solanacearum* Rs-08-17 belonged to Phylotype I and biovar 3 based on species specific multiplex PCR and biovar determination test. Its virulent nature was confirmed on eggplant as it could successfully cause 100 % wilting within 7 days of inoculation by soil drenching method.

In Vitro Antibacterial Activity Assay of Plant Extracts Against *R. solanacearum*

Plants are a rich source of bioactive compounds and can therefore be exploited for infectious disease management [34]. Very little work has been done to determine the potential use of natural plant products in bacterial wilt management. Antibacterial activity towards *R. solanacearum* by various plant extracts has already been reported [35, 36]. A total of 264 crude extracts were prepared from 32 plant species and were screened against *R. solanacearum* Rs-08-17 for its antibacterial activity. Results of in vitro studies on antibacterial activities of ethanol, methanol, chloroform, ethyl acetate, acetone, dichloromethane, diethyl ether and hexane extracts against the growth of *R. solanacearum* are mentioned in Table 1 and Fig. 1. One hundred twenty-three extracts showed variable inhibitory activity against the pathogen and the inhibition zones were in the range of 6.0–31.6 mm.

Chanda and Kaneria [37] reported the accumulation of bioactive compounds as secondary metabolites in all plant cells, however their concentration was high in leaf tissues [38]. Antibacterial activity was observed in the organic solvent as compared to water extract, which indicated that the active compounds responsible for the bactericidal activity were more soluble in the organic solvents [39]. The in vitro inhibitory activity (inhibition zone) of ethanol extracts of plants against *R. solanacearum* ranged from of 8.0–31.6 mm; the highest in *P. guajava* (31.6 mm) followed by *G. indica* (25.3 mm), *A. auriculiformis* (25.0 mm) and *T. indica* (23.0 mm) extracts. With chloroform extract, the highest diameter of inhibition zone was displayed by *Ricinus* sp. (24.67 mm) followed by *P. guajava* (14.0 mm). Ethyl acetate extract of *Cymbopogon flexuosus* and *B. diffusa* produced the maximum inhibition zones of 31.6 and 30.0 cm respectively. Diameters of inhibition zone of 30.6, 28.3 and 20.6 mm were recorded for acetone extract of *T. indica*, *P. guajava* and *G. indica* respectively. Though inhibition zones were also produced by dichloromethane and diethyl ether extracts of some of the plant species, it was less than 14.0 mm. Inhibition zone of >21.0 mm was recorded by methanol extracts of *T. indica* (21.6 mm), *A. occidentalis* (24.6 mm), *P. guajava* (28.0 mm) and *G. indica* (30.0 mm). Hexane extract of the plant species were not inhibitory to *R. solanacearum* as shown in Table 1. Overall observations proved that 33 ethanol extracts, 26 methanol extracts, 25 ethyl acetate extracts, 19 acetone extracts, 15 dichloromethane extracts and 5 diethyl ether extracts showed better zones of inhibition. A total of 20 plant extracts viz. 8 ethanol, 5 methanol, 4 acetone and 3 ethyl acetate extracts showed >20 mm size zones of inhibition (Table 1).

Different solvent extracts showed different phytoconstituents depending on their solubility or polarity in the solvent [22] that later resulted in variable antibacterial activity of the extract. Though many mechanisms have been suggested for the inhibition, it is likely that these extracts inhibit the multiplication of bacterial cells as evidenced by the lack of growth.

Activity index (AI) of the plant extracts in various solvents was determined. AI of 50 % or more was considered as active product for further study. Ethanol extracts of *P. guajava* (79 %), *G. indica* (63.3 %), *T. indica* (57.5 %), *A. auriculiformis* (62.5 %), *C. zevlancium* (54.1 %), *M. pudica* (51.6 %), *A. occidentalis* (50.8 %) and *C. gigantea* (50 %) showed AI of 50 % and more. Ethyl acetate extracts of *C. flexuosus*, *B. diffusa* and *C. zevlancium* showed AI of 79, 75 and 61.5 % respectively. Acetone extracts of *T. indica* (76.6 %), *P. guajava* (70.8 %), *A. auriculiformis* (59.15 %) and *G. indica* (51.67 %) showed AI of 50 % and more. Methanol extracts of *A. occidentalis* (61.6 %), *T. indica* (54.1 %) and *L. inermis* (51.6 %) were effective. Dichloromethane and diethyl ether extracts showed <30 % activity index (Table 2).

Methanol is the most potent solvent for extracting phytochemicals from plant materials [40]. However, it was observed that ethanol extracts of various plant species were highly inhibitory to *R. solanacearum* followed by methanol extracts as compared to the other organic solvents. Organic solvents alone except chloroform as control did not have any inhibitory effect against the pathogen. Bioactive compounds may interfere with bacterial cell wall synthesis [41], protein synthesis, and nucleic acid synthesis, breaking the peptide bonds, acting as chelating agents, inhibiting the metabolic pathway and preventing the utilization of available nutrients by the microorganisms thus hampering their survival [42].

After careful consideration on the efficiency of bacterial inhibition and the availability of plant species, *G. indica* and *T. indica* were selected for further characterization. Both the plant species grow abundantly in the western India and their usefulness to mankind is known for centuries. Varalakshmi et al. [43] reported the antimicrobial properties of *G. indica* on certain microbes. *T. indica* has broad spectrum antibacterial activity [44] due to the presence of flavonoid and polyphenols in their leaves [45]. However, their antimicrobial activity against *R. solanacearum* was not known.

Phytochemical Analysis

Based on the above results, ethanol extracts of promising plant species were re-tested for their activity after 1 year of storage and it was found that the inhibitory activity of *G. indica* and *T. indica* remained high and unchanged. The compounds present in these species which are responsible for antibacterial activity were not studied. Hence it was decided to explore the antibacterial compounds in these plant species. Therefore, these two species were selected for qualitative phytochemical screening by conventional standard protocols. Several studies have also shown that alkaloids, saponins, tannins, flavonoids and phenolic compounds possess antimicrobial activities [46]. When phytochemical analysis of the ethanol extracts *G. indica* and *T. indica*, were conducted, the presence of saponins, terpenoids and alkaloids were recorded. Phytochemical analysis revealed the presence of flavonoids in *G. indica* and tannins in *T. indica* (Table 3). Saponins are widely distributed amongst plants and are believed to protect the plant against pathogens due to its antimicrobial activity [47]. Tannins are known for their astringent property and antimicrobial activity [22]. Zhao et al. [48] reported the antibacterial activity of flavonoids derived from *D. odorifera* against *R. solanacearum*.

Table 2 Activity index of plant extracts in inhibiting *R. solanacearum*

S. no.	Plant species	Activity index[a] (%)						
		80 % ethanol	Chloroform	80 % ethyl acetate	Acetone	Dichloro methane	Diethyl ether	Methanol
1	*Murraya koenigii* (*leaf*)	26.6	25.8	0.0	0.0	0.0	0.0	0.0
	Murraya koenigii (*Root*)	22.5	3.3	0.0	0.0	0.0	15.0	0.0
2	*Eupatorium odorata*	20.8	0.0	0.0	0.0	0.0	0.0	25.0
3	*Thuja occidentalis*	20.0	0.0	37.5	28.3	0.0	0.0	31.6
4	*Azadirachta indica*	31.5	8.3	22.5	0.0	0.0	0.0	0.0
5	*Ocimum tenuiflorum*	37.5	22.5	32.5	0.0	0.0	0.0	27.5
6	*Adhatoda vasica*	36.6	10.0	0.0	15.0	17.5	0.0	0.0
7	*Garcinia indica*	63.3	7.5	23.3	51.6	0.0	24.1	75.0
8	*Moringa oleifera*	37.5	9.1	35.0	20.0	20.8	30.0	25.8
9	*Saraca asoca*	31.5	5.0	24.1	23.3	20.0	0.0	0.0
10	*Hibiscus* sp.	30.0	0.0	0.0	0.0	19.1	15.0	19.1
11	*Jatropha curcas*	23.1	11.2	0.0	0.0	0.0	0.0	19.3
12	*Aegle marmelos*	31.6	7.5	9.1	31.6	28.3	0.0	26.6
13	*Carica papaya*	30.0	15.8	7.5	25.8	0.0	0.0	35.0
14	*Anacardium occidentalis*	50.8	10.0	24.1	0.0	0.0	26.2	61.6
15	*Ocimum basilicum*	30.0	32.5	27.5	21.6	22.5	0.0	20.0
16	*Cinnamomum zevlancium*	54.1	28.3	61.6	30.0	28.3	22.5	30.0
17	*Myristica fragrans*	22.5	0.0	0.0	0.0	18.3	0.0	0.0
18	*Gliricidia* sp.	30.0	27.5	31.6	0.0	0.0	0.0	24.1
19	*Zingiber officinale*	31.6	24.1	30.8	0.0	0.0	0.0	30.0
20	*Psidium guajava*	79.0	35.0	30.0	70.8	0.0	0.0	70.0
21	*Lawsonia inermis*	35.0	35.0	43.3	44.1	27.5	35.0	51.6
22	*Averrhoa carambola*	37.5	0.0	0.0	22.5	0.0	0.0	29.1
23	*Alstonia* sp.	35.0	22.5	27.5	0.0	20.8	0.0	28.3
24	*Canavalia gladiata*	35.8	0.0	29.1	40.0	0.0	0.0	25.0
25	*Acacia auriculiformis*	62.5	25.0	28.3	59.1	23.3	0.0	22.5
26	*Ricinus* sp.	27.5	61.6	25.0	0.0	0.0	0.0	31.6
27	*Physalis weed*	20.8	28.3	24.1	22.5	0.0	0.0	44.1
28	*Mimosa pudica*	51.6	7.5	35.8	12.5	22.5	0.0	34.1
29	*Calotropis gigantea*	50.0	5.0	38.3	35.0	5.0	0.0	37.5
30	*Tamarindus indica*	57.5	10.0	34.1	76.6	25.0	0.0	54.1
31	*Boerhavia diffusa*	37.5	0.0	75.0	30.0	22.2	0.0	41.5
32	*Cymbopogon flexuosus*	32.5	0.0	79.0	29.0	25.0	0.0	0.0

[a] AI (%) = 100 × diameters of inhibition zone of extract ÷ diameters of inhibition zone of the standard antibiotic

The ethanol extracts were further subjected to solvent fractionation and all the three fractions were tested for *R. solanacearum* inhibition. Ethanol fraction (F-III) of *G. indica* and *T. indica* showed inhibition of 21.5 and 20.5 mm respectively. Lesser degree of inhibition (*G. indica*: 2 mm and *T. indica*: 4 mm) was shown by chloroform fraction (F-I). N-butanol fractions (F-II) did not inhibit the pathogen (Table 3). Therefore, it was decided to identify the compounds present in the ethanol fractions (F-III) of *G. indica* and *T. indica* by GC–MS analysis.

GC–MS Analysis

The active principles of fraction III of *G. indica* and *T. indica* are presented in Tables 4 and 5 with their retention time (RT), molecular formula (MF), molecular weight (MW) and peak area (%). Fraction III extract of *G. indica* produced 9 peaks in GC–MS analysis as illustrated in Fig. 2. Out of these peaks, compounds were identified for 4 peaks corresponding to their respective retention time of 5.9, 7.2, 30.5 and 32.2. Two antimicrobial compounds viz. 4H-Pyran-4-one, 2, 3-dihydro-3,5-

Table 3 Phytochemical analysis of ethanol extracts of *G. indica*, *T. indica* and antibacterial activity of solvent–solvent partitioning fractions (F-I, F-II, F-III) against *R. solanacearum*

Characters	*G. indica*	*T. indica*
Phyto-chemicals[a]		
Flavonoids	+	–
Tannins	–	+
Saponins	+	+
Terpenoids	+	+
Alkaloids	+	+
Antibacterial activity of fractions[b]		
Chloroform (F-I)	2.0	4
n-Butanol (F-II)	0	0
Ethanol (F-III)	21.5 ± 0.70	20.5 ± 0.70

[a] The experiment was repeated two times with two replications; (+) indicates presence (–) indicates absence of the chemical

[b] Zone of *R. solanacearum* inhibition (diameter in mm) by the ethanol fractions obtained by solvent–solvent partitioning; mean of three replications

dihydroxy-6-methyl, a flavonoid and 2-Furancarboxaldehyde,5 (hydroxymethyl), an aldehyde compound were identified based on mass spectrum (Fig. 3).

Fraction III extract of *T. indica* produced 5 peaks in GC–MS analysis (Fig. 4). Out of these peaks, compounds were identified for 3 peaks corresponding to their respective retention time of 3.6, 14.7 and 36.5. The fraction revealed the presence of a carboxylic acid ester (Butanoic acid, butyl ester) and fatty acid ester compound (Myo-inositol, 4-C-methyl) based on mass spectrum (Fig. 5) with antimicrobial properties.

This is the first report on the antibacterial effect of *G. indica* and *T. indica* against *R. solanacearum* and the presence of antimicrobial compounds in the leaf extracts. As the future line of work the key compound responsible for bacterial inhibition among the compounds reported by the analysis is under consideration. This future narrowing down of the antimicrobial compound could lead to the development of plant based antimicrobial agent for plant disease management.

Table 4 Phytochemicals identified in the ethanol extracts of the leaves of *G. indica* by GC–MS

Peak no.	RT (min)	Peak width	Area (%)	Compound name	MF	MW	Compound nature	Activity[a]
1	3.1	0.124	8.3	–	–	–	–	–
2	3.3	0.045	3.8	–	–	–	–	–
3	4.8	0.027	3.5	–	–	–	–	–
4	5.9	0.026	3.7	4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl	$C_6H_8O_4$	144.127	Flavonoid fraction	Antimicrobial, Anti-inflammatory, Antiproliferative
5	6.2	0.044	4.5	–				
6	7.2	0.034	23.8	2-Furancarboxaldehyde, 5(hydroxymethyl)	$C_6H_6O_3$	679.8	Aldehyde compound	Antimicrobial, preservative
7	29.1	0.10	7.9	–				
8	30.5	0.12	10.1	Neo tigogenin	$C_{27}H_{44}O_3$	416.64	Steroid sapogenin	
9	32.2	0.17	33.8	Spirostan-12-one, 3 hydroxy-(3B, 5A, 25R)	$C_{27}H_{42}O_4$	430.62	Steroidal sapogenin	Induction of apoptosis

[a] *Source* Dr. Duke's: Phytochemical and Ethnobotanical Databases (online database)

Table 5 Phytochemicals identified in the ethanol extracts of the leaves of *T. indica* by GC–MS

Peak no.	RT (min)	Peak width	Area (%)	Compound name	MF	MW	Compound nature	Activity[a]
1	3.1	0.082	27.6	–	–	–	–	–
2	3.4	0.033	4.1	–	–	–	–	–
3	3.6	0.025	10.4	Butanoic acid, butyl ester	$C_8H_{16}O_2$	144.21	Carboxylic acid ester	Antibacterial
4	14.7	0.099	26.9	Myo-inositol,4-C-methyl	$C_7H_{14}O_6$	194.18	Fatty acid ester	Antimicrobial, Antioxidant
5	36.5	0.18	30.8	Spirostan-12-one, 3 hydroxy-(3^{β}, 5α, 25R)	$C_{27}H_{42}O_4$	430.62	Steroid saponin	Induction of apoptosis

[a] *Source* Dr. Duke's: Phytochemical and Ethnobotanical Databases (online database)

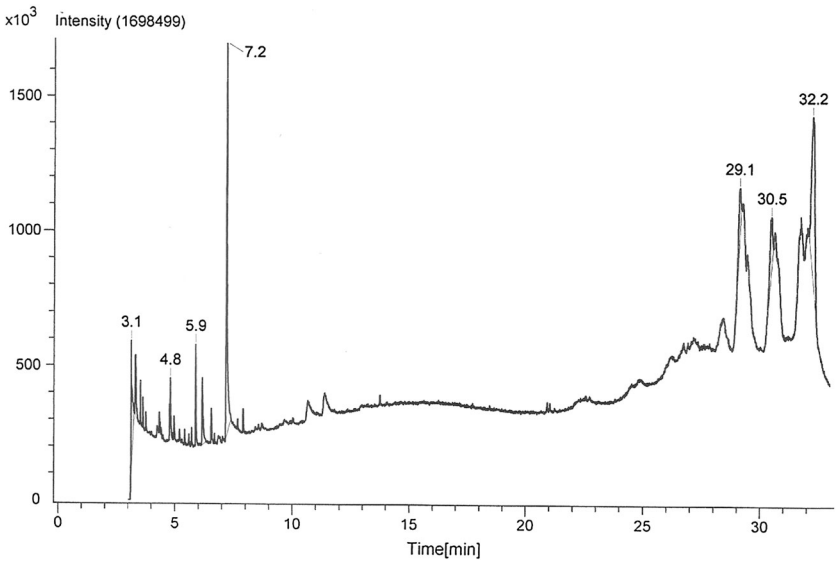

Fig. 2 GC–MS chromatogram showing chemical constituents of ethanol extract (F-III) of the leaves of *G. indica*

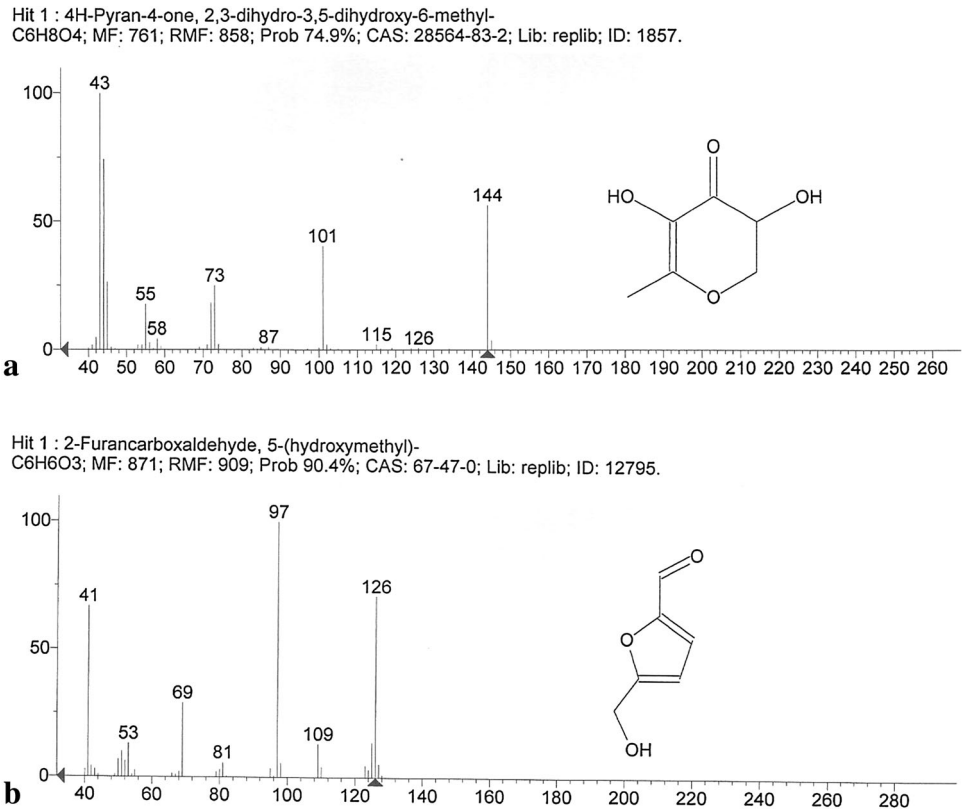

Fig. 3 Mass spectrum of compounds from ethanol extract (F-III) of the leaves of *G. indica*. **a** 4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl (RT: 5.9); **b** 2-Furancarboxaldehyde, 5(hydroxymethyl) (RT: 7.2)

Conclusion

Plant species analyzed in the present study for antibacterial activity have opened up a new perspective in the management of bacterial wilt caused by *R. solanacearum*. In this direction, in vitro results of the study confirmed the potential of *G. indica* and *T. indica* extracts as a promising antibacterial product for controlling the bacterial wilt. However, isolation of individual phytochemical constituents and subjecting them

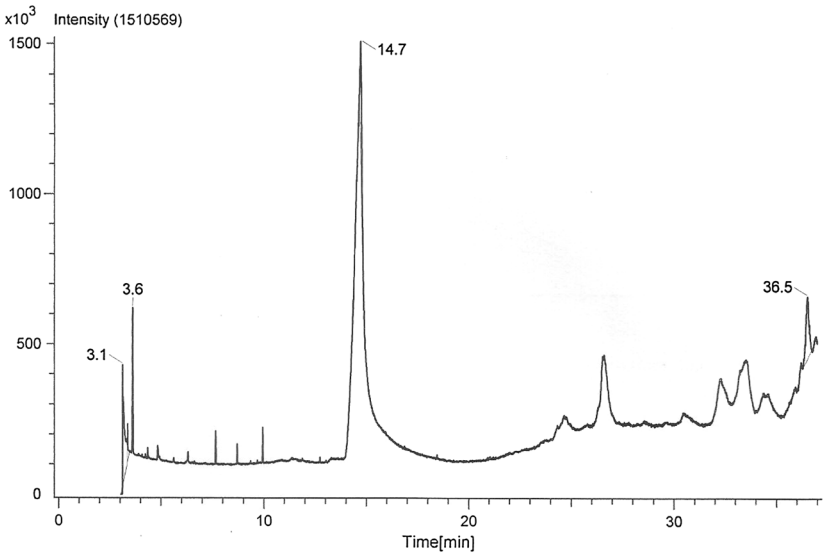

Fig. 4 GC–MS chromatogram showing chemical constituents of ethanolic extract (F-III) of the leaves of *T. indica*

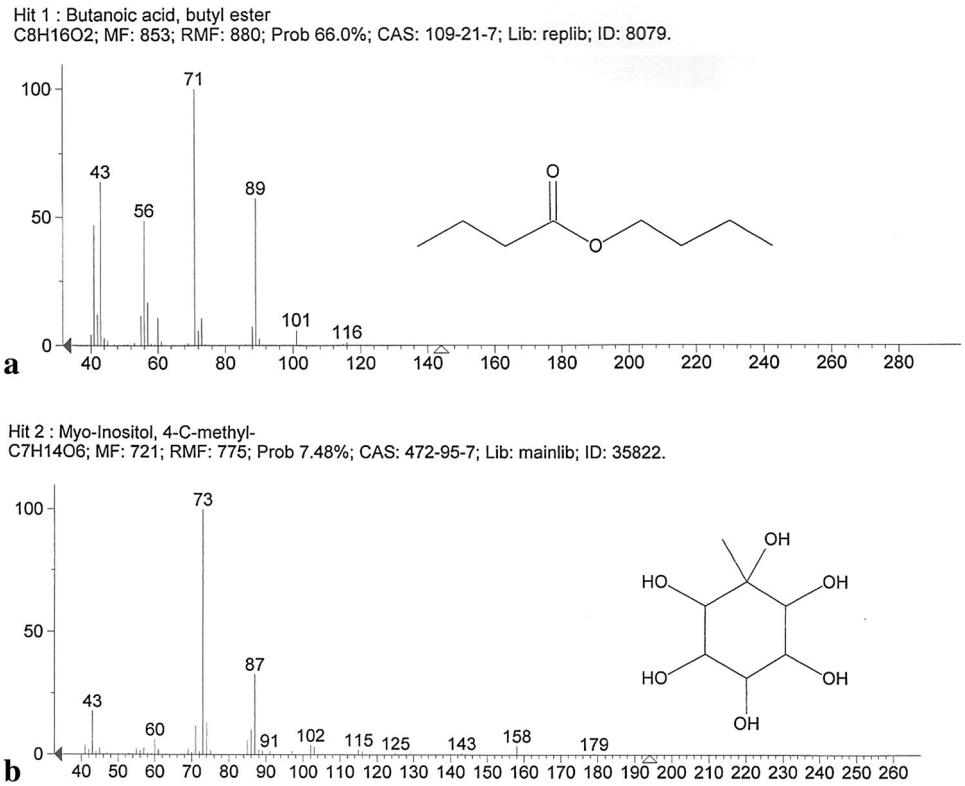

Fig. 5 Mass spectrum of compounds from ethanol extract (F-III) of the leaves of *T. indica*. **a** Butanoic acid, butyl ester (RT: 3.6); **b** Myo-inositol,4-C-methyl (RT: 14.7)

to biological testing may have even more potency against the pathogen.

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Compliance with Ethical Standards

Conflict of interest We declare that we have no conflict of interest.

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