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4-(4-chlorophenyl) pyridine an antimicrobial compound of plant origin

Rupert Anand Yumlembam and Suresh Govind Borkar

Abstract
The solvent plant extracts of *Terminalia chebula, Simarouba glauca* and *Bixa orellana* showed inhibitory effect when tested against the Xanthomonads by paper disc method. The active fraction of the solvent extracts was separated using thin layer chromatography. Two major peaks on Gas chromatography mass spectroscopy were identified as 4-(4-chlorophenyl) pyridine and phthalic acid respectively. Commercially available compounds of 4-(4-chlorophenyl) pyridine and phthalic acid when tested against the bacteria *Clostridium perfringens* and *Pseudomonas aeruginosa*, and the fungus *Aspergillus niger*, showed that the compound only 4-(4-chlorophenyl) pyridine possess the antimicrobial property against bacteria well as fungi.

Keywords: Xanthomonas, antibacterial compound, 4-(4-chlorophenyl) pyridine, phthalic acid, *Terminalia chebula* and *Bixa orellana*

Introduction
Quick and effective management of plant diseases and microbial contamination in several agricultural commodities is generally achieved by the use of synthetic pesticides. However, in recent years, a large number of synthetic pesticides have been banned in the Western world because of their undesirable attributes such as high and acute toxicity, long degradation periods, accumulation in food chain and extension of their power to destroy both useful and harmful pests (Ortelli *et al.,* 2005) [23]. The widespread application of pesticides to control crop diseases had caused problems to human beings and environment and led to the development of resistance in pathogens to these chemical products (Motoyama *et al.,* 2003) [20]. As an alternative strategy to prevent the spread of diseases, natural compounds of plant origin are being tested for their antimicrobial activities. Naturally occurring biologically active plant products can be a source of new pesticides or serve as templates for the new and more effective compounds (Elkovich, 1988) [9]. According to the research, green plants represent a reservoir of effective chemo-therapeutants and can provide valuable sources of natural pesticides (Balandrin *et al.,* 1985, Hostettmann and Wolfender 1997) [4, 12]. The use of botanicals and antimicrobial agents of plant origin is a time honored practice for control of plant diseases and pests. The necessity to develop a non-toxic, safe and biodegradable alternative to synthetic pesticides has in recent years led to a concerted effort at developing new sources of pesticides from plant parts. The humid tropics, especially the rainforest ecological zones, are endowed with abundant flora of families of plants and herbs with untapped pesticide potentials (Amadioha, 2002, 2003) [2, 3]. Stoll (2000) [31] listed an array of plant families and genera possessing antimicrobial properties, amongst which are *Monodora myristica, Annona Muricata, xylopia aethiopica, Garcinia kola, Gongronema latifolium, Dennitia tripetala, Azadirachta indica, Piper guineenes, Zingiber officinale* and a host of others. Some workers like Amadioha (2000) [2], Kumar and Pamar (1996) [15] and Prakash and Rao (1997) have listed several advantages of plant extract over synthetic chemicals like possession of low mammalian toxicity, minimal health hazards and environmental pollution. Reports on the use of active agents from higher plants in place of chemical fungicides, that are non-phototoxic, more systematic and easily biodegradable are available (Fawcett and Spencer, 1970) [11]. In addition, there is practically no risk of developing pest resistance to these products when used in their natural forms. Similarly, there are fewer hazards to non-target organisms and resurgence has not been reported in the use of botanicals. Further, no adverse effect on plant growth, seed viability or food quality has been reported. Botanicals are less expensive and easily available because of their natural occurrence in abundance.
Plants have been an important source of human medicine for thousands of years. Even today, the World Health Organization estimates that up to 80 percent of people still rely mainly on traditional remedies such as herbs for their medicines (Farnsworth, 1994) [10].

Today, Ayurvedic, Hoemoeo and Unani physicians utilize numerous species of medicinal plants that found their way a long time ago into the Hindu Material Media (Narayana and Thammanna, 1987) [21]. Much work has been done on ethno medicinal plants in India (Maheshwari *et al.,* 1986) [17] and interest in a large number of traditional natural products has increased many folds (Taylor *et al.,* 1996) [33]. It has been suggested that aqueous and ethanol extracts from plants used in allopathic medicine are potential sources of antiviral, antitumor and antimicrobial agents (Chung *et al.,* 1995 and Vlietinck *et al.,* 1995) [6, 35]. Similarly, the methanolic leaf extracts of *Acacia nilotica, Sida cordifolia, Tinospora cordifolia, Withania somnifer* and *Ziziphus mauritiana* have also showed significant antibacterial activity against some human as well as plant pathogenic bacteria particularly *Bacillus subtilis, Escherichia coli, Pseudomonas fluorescens, Staphylococcus aureus* and *Xanthomonas axonopodis* pv. m*alvacearum* (Xam) (Mahesh and Satish, 2008) [16]. Use of plant extract having medicinal properties against plant pathogenic bacteria may have significant value due to rapidly degradable antimicrobial compounds in nature and their non harmful effect on plant product and human health. Plant pathogenic bacteria of genus *Xanthomonas* is predominantly found in India and the bacterium is known to cause diseases in cereals, vegetables, fruits and cash crops (Setubal *et al.,* 2005) [27]. Further, this bacterium is reported to have developed resistance to antibiotics like kanamycin, ampicillin, penicillin and streptomycin (Mandavia *et al.,* 1999) [19] and therefore poses a challenge in their control. As plant based bactericide has not yet been developed, the need to work on these lines had an immense importance and therefore atleast 56 medicinal and aromatic plants were evaluated for antibacterial compounds against plant pathogenic bacteria and new antibacterial molecule were reported for the first time.

Material and Method

Assessment of medicinal plants for antibacterial compound

56 medicinal plants were assess for presence of antibacterial compound by using 10 per cent aqueous leaf extract on Xanthomonads.

For preparation of plant aqueous extracts, the leaves of collected plants were thoroughly washed with tap water and macerated separately with sterilized distilled water as 5% (5 gm of leaves in 100 ml distilled water) and 10% (10 gm leaves in 100 ml distilled water) respectively. The extracts were then sieved through double layer muslin cloth and then centrifuged at 4000 rpm for 30 min. The supernatant of the plant extracts were filtered through Whatman No.1 filter paper and sterilized through G4 bacterial filters flask. The aqueous leaf extract was tested on lawn of Xanthomonas bacterium by using paper disc diffusion assay method.

Preparation of plant solvent extract

The medicinal plants whose aqueous extract showed positive results on Xanthomonas were used for preparation of solvent extract. The leaves of selected plants were thoroughly washed with tap water, after soaking it on blotter paper the leaves were macerated in methanol or ethanol solvent to obtain 10% extract respectively (10 gm leaves in 100 ml of solvent). The extracts were then sieved through double layer muslin cloth and then centrifuged at 4000 rpm for 30 min. The supernatant was filtered through Whatman No.1 filter paper and sterilized through G4 bacterial filters flask. The filtered solvent extract was evaporated under vacuum at 45 to 50°C temperature to condense the antibacterial compound and to remove the solvent. The solvent extracted antibacterial compound was dissolved in 0.5ml of distilled sterilize water and further used for confirmation of antibacterial property on lawn of Xanthomonas bacterium by using paper disc diffusion method.

Separation of antibacterial compounds by thin layer chromatography

Thin layer chromatography (TLC) (silica gel) was used for separation of antibacterial compound from the extracts. Silica gel plates were prepared by using 30 gm silica gel with 7.2 gm calcium sulfate binder (i.e. 12%) mixed with 60-65 ml distilled water which was homogenize for 30 sec and the paste was applied on a gel plate at 1 mm thickness by using the spreader on 10 X 20 cm plates within 2 min. The plate was then activated at 130°C for 30 minute before use. The classic solvent mixture BAW: *n*-butanol - acetic acid - water (4:1:5); Forestal: conc. HCl - acetic acid - water (3:30:10) and water were used as solvents for the separation of compounds. The mobile phase i.e. solvent was poured in the TLC tank and the spotted TLC plate (2µl of sample on the TLC plate) was kept in this with approximately 0.5 mm immersed in solvent at the bottom. The tank was closed with a glass lid so as to have the chamber completely filled with the solvent vapors. The plates were kept in the TLC tank till the solvent front reached the top of TLC plate. Then, the plate was removed from the tank and kept in open air at room temperature so as to enable the solvent to get evaporated.

The TLC run plates were observed under UV light and the separated spots were marked. The relative front values were calculated by using the given formula.

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$$\text{Rf value} = \frac{\text{Distance moved by the solute from the origin}}{\text{Distance moved by the solvent from the origin}}$$

Testing antibacterial efficiency of compounds separated on TLC against *Xanthomonas*

For testing efficiency of antibacterial compounds, the separated spots on TLC was carefully scrapped off from the silica plates and the scrapped powder containing antibacterial compound was used to test the efficiency against Xanthomonads by using paper disc diffusion method.

Identification of antibacterial compound using GC-MS

Identification of the antibacterial compound was carried out by using Quattro micro Gas chromatography mass spectroscopy (GC-MS) unit. The column type used was DB-5MS with a column length of 30m, fused silica capillary column (0.25mm I.D., 0.25 µm film thickness), and using Carrier gas as Helium. The inlet pressure was held at 5psi for 1 min, then raised at 20 psi/min and held for 4.5 min. The Flow rate maintained was 1 ml/min with an initial column temperature of 50° C and final temperature of 280°C. The rate of temperature change in the column was maintained as 25°C/min for 6 min till the final temperature reaches to 150°C, then it was maintained at 5°C/min for 36 min until it reaches to 280°C with a hold time of 4 min.

Result

Efficacy of an antibacterial compound extractable in the solvent against Xanthomonads

Out of 56 medicinal plants were assessed, only three plants viz., *Bixa orellana, Simarouba glauca* and *Terminalia*

chebula showed to have antibacterial activity. The ethanol and methanol solvents of the plant extracts were vacuum evaporated; the condensed antibacterial compound was dissolved in 0.5 ml distilled water and used for paper disc diffusion assay to determine the antibacterial property on the lawn of Xanthomonads.

The results (Table 1) indicate that the antibacterial compound was extractable in both ethanol and methanol solvents form all the three plants. The zone of inhibition formed by ethanol and methanol extracted antibacterial compound of *Terminalia chebula* was 1.5 cm against *Xanthomonas campestris* pv. *campestris* (Xcc) and *Xanthomonas axonopodis* pv. m*alvacearum* (Xam). The zone of inhibition formed by ethanol extracted antibacterial compound of *Bixa orellana* was 1.20 cm for Xcc and 1.07 cm for Xam, whereas the zone of inhibition formed by methanol extract was slightly more *i.e.,* 1.67 cm for Xcc and 1.50 cm for Xam, respectively. The zone of inhibition formed by ethanol extracted antibacterial compound of *Simarouba glauca* was 1.13 cm for Xam and 1.43 cm for Xcc. However, methanol extracted antibacterial compound of the same plant induced a zone of inhibition of 1.60 cm for Xcc and 1.57 cm for Xam.

Table 1: The zone of inhibition exhibited by antibacterial compound in ethanol and methanol solvent

Paper disc charged with	Solvent extract of medicinal plants					
	Terminalia chebula		***Simarouba glauca***		***Bixa orellana***	
	Zone of inhibition (in cms) against *Xanthomonas sp.*					
	Xcc	*Xam*	*Xcc*	*Xam*	*Xcc*	*Xam*
Ethanol extract	1.50 ±0.26	1.50±0.17	1.43±0.31	1.13±0.32	1.20±0.30	1.07±0.21
Methanol extract	1.50±0.20	1.50±0.20	1.60±0.17	1.57±0.25	1.67±0.15	1.50±0.17

These results indicate that the overall zone of inhibition formed by the methanolic extracts of *Bixa orellana* and *Simarouba glauca,* were higher as compared to zone of inhibition formed by ethanolic extracts of these plants indicating that higher quantity of an antibacterial compound can be extracted with methanolic solvent.

Quantification of antibacterial compound present in the medicinal plants by using equivalent zone of inhibition assay method

The equivalent zone of inhibition assay method was employed for the quantification of the antibacterial compound in the given medicinal plant. For this purpose, known concentration of antibacterial antibiotics like streptomycin sulphate and streptocycline were used at 50, 100, 250 and 500µg concentrations to measure the zone of inhibition formed by these antibiotics against Xcc and Xam by using paper disc diffusion method. A paper disc was dipped in the known concentration of the antibiotics solution and placed on the *Xanthomonas* lawn. The zone of inhibition was measured within 24 hr. The results in Table 2 indicate that the zone of inhibition formed by streptomycin sulphate was higher on Xam than on Xcc whereas, the zone of inhibition formed by streptocycline on Xam was less than that on Xcc. A quantity of 50 µg streptomycin sulphate induced 0.7 to 0.9 cm zone of inhibition, 100 µg induced 0.9 cm, 250 µg induced 1.3 to 1.6 cm and 500 µg induced 1.5 to 1.7 cm zone of inhibition against *Xanthomonas spp*. As regards, streptocycline at 50 µg induces 1cm zone of inhibition, 100 µg and 250 µg induces 1.1 to 1.2 cm and 500 µg induces 1.4 to 1.5 cm zone of inhibition against *Xanthomonas* species.

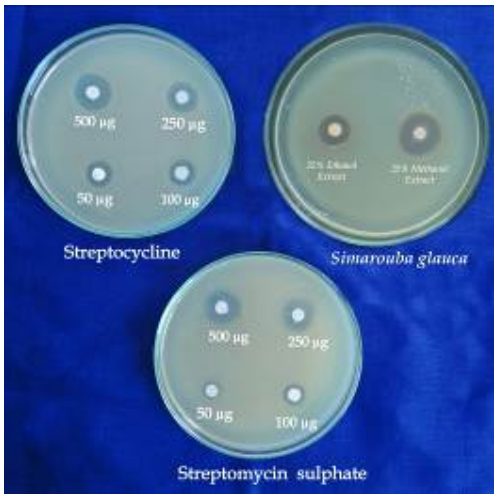
Table 2: Inhibition zones of antibiotics at various concentrations.

Antibiotics concentrations (µg)	Inhibition zone (in cms) of antibiotics.			
	Streptomycin sulphate on *Xanthomonas sp.*		Streptocycline on *Xanthomonas sp.*	
	Xcc	Xam	Xcc	Xam
500	1.5±0.10	1.7±0.17	1.5±0.17	1.4±0.10
250	1.3±0.17	1.6±0.10	1.2±0.20	1.1±0.10
100	0.9±0.17	0.9±0.10	1.2±0.26	1.1±0.20
50	0.7±0.10	0.9±0.20	1.0±0.10	1.0±0.10

These results on inhibition zone formed by antibiotics on *Xanthomonas* were used for quantification of antibiotic compound in the methanolic extracts of all the three plants. On the basis of the zone of inhibition formed by the methanolic extracts (Fig 1), it was concluded that methanolic extracts contained 500 µg concentration of antibacterial compound, whereas the ethanolic extracts contained 100 to 500 µg of antibacterial compound and the content depended on the species of medicinal plant.

Zone of inhibition produced by solvent extracted antibacterial compound of *Bixa orellana* on XCC lawn

Zone of inhibition produced by solvent extracted antibacterial compound of *Terminalia chebula* on XCC lawn



Zone of inhibition produced by solvent extracted antibacterial compound of *Simarouba glauca* on XCC lawn

Fig 1: Quantification of antibacterial compound present in the solvent extract of medicinal plants by using equivalent zone of inhibition assay method

Separation of antibacterial compound by Thin Layer Chromatography (TLC)

The ethanolic and methanolic extracts of the medicinal plants of *Bixa orellana, Simarouba glauca* and *Terminalia chebula,* which possessed the antibacterial property, were used for the separation of antibacterial compound by using TLC with three solvents *i.e.,* water, BAW and Forestal solvent as its mobile phase.

The results indicated that the ethanolic and methanolic extracts of *Bixa orellana* exhibited three bands of Rf values and when run in BAW solvent *whereas* only one band of Rf value was observed for *Terminalia chebula.* In case of water solvent, no separate bands were observed and the test samples spot elongated up to a long distance.

In case of Forestal solvent four distinct bands of different Rf values and were detected for *Terminalia chebula*, three distinct bands of Rf values and were observed for *Bixa orellana* and only one band for *Simarouba glauca.*

Confirmation of antibacterial compound in the separated band on TLC plates

The bands of different Rf values detected in different solvent plates for ethanolic and methanolic extracts of *Simarouba glauca, Bixa orellana* and *Terminalia chebula,* were tested for their possession and efficiency of antibacterial compound by using the direct assay method. The spots of different Rf value illuminated under UV light at 360nm were circled and the illuminating compound was scraped with the help of the blade and was put on the bacterial lawn of *Xanthomonas* as a direct assay method for possession and detection of antibacterial compound in the sample.

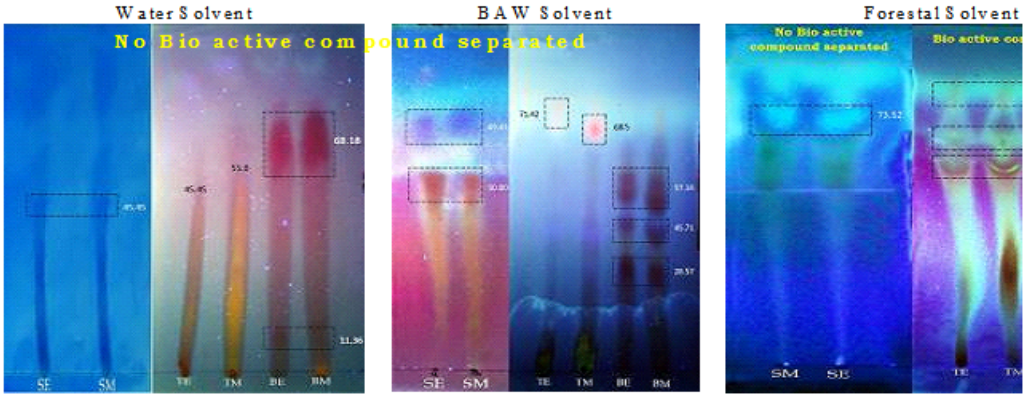
The results (Table 3) indicated that only the Forestal solvent was effective for the separation of antibacterial compound, whereas the water and BAW solvents were ineffective for the separation of antibacterial compound. The material obtained from different Rf values in water solvent and BAW solvent did not show any inhibition zone against the *Xanthomonas* in direct assay method. However, the illuminating spots of different Rf values in Forestal solvents possessed the antibacterial compounds. The antibacterial compounds in methanolic and ethanolic extract of *Terminalia chebula* were present at the Rf values of 58.52, 71.02 and 80.68.

Table 3: Rf (%) value of prominent bands of ethanolic and methanolic extract of medicinal plants possessing antibacterial properties against Xanthomonads after TLC separation

Medicinal plant possessing antibacterial properties against *Xanthomonas*	Antibacterial compound extracted in	Solvent used for separation of antibacterial compound								
		Water solvent		BAW solvent			Forestal solvent			
		Rf value of prominent band and possession of antibacterial property.								
Terminalia chebula	methanol	-	55.00 (-)	17.4 (-)	34.28 (-)	71.42 (-)	25.56 (-)	58.52 (+)	71.02 (+)	80.68 (+)
	ethanol	-	45.45 (-)	17.4 (-)	34.28 (-)	68.57 (-)	28.40 (-)	58.52 (+)	71.02 (+)	80.68 (+)
Simarouba glauca	methanol	-	42.80 (-)	-	50.00 (-)	69.44 (-)	-	-	73.52 (-)	-
	ethanol	-	42.80 (-)	-	50.00 (-)	69.44 (-)	-	-	73.52 (-)	-
Bixa orellana	methanol	11.36 (-)	68.18 (-)	28.57 (-)	45.71 (-)	57.14 (-)	-	57.95 (+)	68.18 (+)	71.02 (+)
	ethanol	11.36 (-)	68.18 (-)	28.57 (-)	45.71 (-)	57.14 (-)	-	57.95 (+)	68.18 (+)	71.02 (+)

(+) indicates the TLC separated prominent band of Rf values with bio active compound
(-) indicates the TLC separated prominent band of Rf values with no bio active compound

In case of methanolic and ethanolic extract of *Bixa orellana,* the antibacterial compounds were present at Rf values of 57.95, 68.18 and 71.02. (Fig 2), whereas in case of *Simarouba glauca,* the illuminating spots of Rf value of 73.52 did not possess any antibacterial compound.



2a 2b 2c

TM- *Terminalia chebula* Methanol
SM- *Simarouba glauca* Methanol
BM- *Bixa orellana* Methanol
TE- *Terminalia chebula* Ethanol
SE- *Simarouba glauca* Ethanol
BE- *Bixa orellana* Ethanol

Fig 2a: Prominent bands of ethanol and methanol extract of medicinal plants separated by TLC plate with water solvent. **Fig 2 b:** Prominent bands of ethanol and methanol extract of medicinal plants separated by TLC plate with BAW. **Fig 2c:** Prominent bands of ethanol and methanol extract of medicinal plants separated by TLC plate with Forestal solvent

Fig 2: Prominent bands of ethanol and methanol extract of medicinal plants separated by TLC plate with different solvents

These results clearly indicate that the antibacterial compounds against *Xanthomonas* were present in *Terminalia chebula* and *Bixa orellana,* and can be extracted in ethanol and methanol extract, and the bioactive material can be separated on TLC using Forestal solvent at Rf values of 58.52, 71.02 and 80.68.

Quantification of the antibacterial compound separated at different Rf values on TLC

The test material obtained from the different Rf values was examined for its antibacterial activity against the *Xanthomonas* by assessing zone of inhibition in direct assay method. The results (Table 4, Fig 3) indicated that the bioactive material obtained at 58.52 Rf value for *Terminalia chebula* produced the inhibition zone of 1 to 1.4 cm whereas the bioactive material at Rf value of 71.02 produced an inhibition zone of 0.5 cm. In case of *Bixa orellana,* the bioactive material of Rf values of 57.95 and 68.18 produced an inhibition zone of 1.0 cm, whereas the bioactive material at Rf value at 71.02 produced an inhibition zone of 0.5 cm.

TM- *Terminalia chebula* Methanol
BM- *Bixa orellana* Methanol
TE- *Terminalia chebula* Ethanol
BE- *Bixa orellana* Ethanol

Fig 3: Zone of inhibition exhibited by antibacterial compounds at different Rf (%) value against Xanthomonas after TLC separation with Forestal solvent.

Table 4: Zone of inhibition exhibited by antibacterial compounds at different Rf (%) values against *Xanthomonas* after TLC separation

Medicinal plant with antibacterial properties against *Xanthomonas*	Antibacterial compound extracted in	Rf value in Forestal solvent	Zone of inhibition (in cms) against *Xanthomonas sp.*
Terminalia chebula	Methanol	25.56	0.0
		58.52	1.4
		80.68	1.0
		71.02	0.5
	Ethanol	28.40	0.0
		58.52	1.0
		80.68	1.0
		71.02	0.5
Bixa orellana	Methanol	57.95	1.0
		68.18	1.0

		71.02	0.5
	Ethanol	57.95	1.0
		68.18	1.0
		71.02	0.5

When these results were equated with the results of zone of inhibition of antibiotics, it was evident that the antibacterial bioactive material separated at different Rf values varied from 50 to 500 µg of Streptocycline. The bioactive material at the Rf values of 57.95 and 68.18 producing 1cm of inhibition zone were equated to 50 µg of Streptocycline, whereas the bioactive material which produced 1.4 cm of inhibition zone was equivalent to 500 µg of Streptocycline. The bioactive material at Rf value of 71.2 producing 0.5 cm of inhibition zone must have less than 50 µg of antibacterial bioactive material.

Identification of extracted antibacterial compound using GC-MS

Identification of antibacterial compound was carried out using Quattro micro GC-MS unit. Full scan of the sample of ethanolic and methanolic extracts of *Terminalia chebula* and *Bixa orellana* was run with a temperature programming of 280°C *i.e.,* 36 min and the detection of the compound was based on the relative retention time and the size of the peak. The readings of these two parameters of the tested compound were searched in the library search (NIST MS Search 2.0) to identify the test compound.

The results for the retention time of major peak of antibacterial compound of the plant extract indicated that the ethanolic extract of *Terminalia chebula* had two major peaks at retention time of 12.44 and 17.03 min, whereas the methanolic extract of the same plant had three major peaks at retention time of 12.56, 15.34 and 17.03 min. The ethanolic extract of *Bixa orellana* had two major peaks at retention time of 12.47 and 17.03 min, whereas the methanolic extract of the same plant had two major peaks at retention time of 12.42 and 17.02 min. It was apparent from the data that the test compound in methanolic and ethanolic extract of both these plants produced two major peaks, one at 12.42 to 12.56 min and another at 17.02 to 17.03 min.

The library search of retention time of 12.42 to 12.56 min matched the presence of 4-(4-chlorophenyl) pyridine with a molecular weight of 189 and the probability of this compound was from 94.3 to 96.7%.

The library search of retention time of 17.02 to 17.03 min matched the presence of phthalic acid, 6-ethyl-3-octyl isobutyl ester with a molecular weight of 362 and the probability of this compound was from 7.04 to 7.20%.

These results (Table 5) indicated that the medicinal plants of *Terminalia chebula* and *Bixa orellana* contain two antibacterial compounds *viz.,* 4-(4-chlorophenyl) pyridine and phthalic acid.

Table 5: Retention time of major peak of antibacterial compound using Quattro micro GC-MS unit.

Medicinal plant having antibacterial properties against *Xanthomonas*	Test compound in extracted in Solvent	Major peak	Probable compound	Probability (%)	Molecular formula.	Molecular weight.
Terminalia chebula	Methanol	12.44	4-(4-Chlorophenyl) pyridine	96.7%	$C_{11}H_8CIN$	189
		17.03	Phthalic acid, 6-ethyl-3-octyl isobutyl ester	7.20%	$C_{22}H_{34}O_4$	362
	Ethanol	12.56	4-(4-Chlorophenyl) pyridine	96.7%	$C_{11}H_8CIN$	189
		17.03	Dibutyl phthalate	7.04%	$C_{16}H_{22}O_4$	278
Bixa orellana	Methanol	12.42	4-(4-Chlorophenyl) pyridine	96.3%	$C_{11}H_8CIN$	189
		17.02	Phthalic acid, isobutyl octadecyl ester	8.57%	$C_{20}H_{30}O_4$	334
	Ethanol	12.47	4-(4-Chlorophenyl) pyridine	96.7%	$C_{11}H_8CIN$	189
		17.03	Phthalic acid, isobutyl octyl ester	6.89%	$C_{20}H_{30}O_4$	334

Testing of commercial preparation of identified antibacterial compound

These two compounds available commercially were tested for their antibacterial property against the bacterium Xanthomonas. The results indicate that the compound phthalic acid does not show any antibacterial activity against Xanthomonas in the inhibition zone assay while the compound 4-(4- chlorophenyl) pyridine formed the inhibition zone against the test bacterium Xanthomonas (Fig. 4) indicating the presence of antibacterial property in this compound. Also, the results in Table 6 (Fig. 5) shows that the 4-(4- chlorophenyl) pyridine formed the inhibition zone against the test bacterium *viz.,* (gram positive bacteria) *Clostridium perfringens*; (gram negative bacteria) *Pseudomonas aeruginosa* and *Escherichia coli*; and the fungus *Aspergillus niger* and the inhibition zone increases with the increase in dose of the chemical.

Inhibation zone produced by 4-(4-chlorophenyl)pyridine against Xanthomonas bacterium

Fig 4: Inhibition zone formed by commercial chemical 4-(4-chlorophenyl) pyridine against *Xanthomonas* bacterium

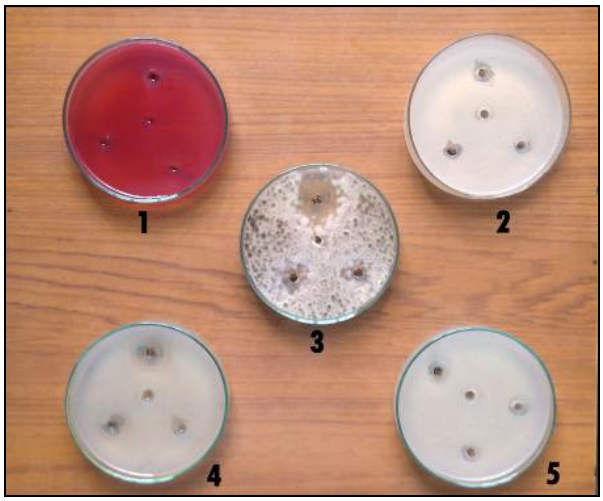


Fig 5: Inhibition zone formed by commercial chemical 4-(4-chlorophenyl) pyridine against *Pseudomonas aeruginosa* (1), *Clostridium perfringens* (2), *Aspergillus niger* (3), *Escherichia coli* (4) and *Bacillus* spp (5)

Discussion

The antibacterial compound present in *Terminalia chebula* and *Bixa orellana* was extractable in ethanol and methanol solvent. The vacuum-dried compound of solvent extraction was also tested for its antibacterial property against *Xanthomonas*. It was observed that this solvent extracted compound also produced the inhibition zone of 1.5 cm for *Terminalia chebula* and 1 to 1.67cm for *Bixa orellana*, indicating that the antibacterial compound present in the extract was equivalent to 500 µg of streptomycin sulphate (500 µg of streptomycin sulphate produce 1.5 cm of inhibition zone against Xcc and 1.7 cm of inhibition zone against Xam)

Sukanya *et al.* (2009) [32] scored the maximum *in vitro* inhibition in methanol extracts of *Chromolaena odorata* which formed the inhibition zone of 10, 9, 12 and 12 mm against *Escherichia coli, Staphylococcus aureus, Xanthomonas vesicatoria* and *Ralstonia solanacearum*, respectively, followed by chloroform extract of the same plant leaf with inhibition zone of 8, 4, 4 and 4 mm, respectively. Shirsat (2008) [29] also showed the anti-phytopathogenic activity of crude and methanol extract of leaves, stem bark, seed and dry fruit of *Terminalia thorelli* against four phytopathogens by disc diffusion method. Crude aqueous extract of plant parts taken at 5 mg concentration showed zone of inhibition ranging from 11 to 22 mm. Xam was found to be highly susceptible with the highest zone of inhibition suggesting the strong inhibitory activity of these extracts against the selected bacterial pathogens. These pathogens were more sensitive to the methanol extracts forming 13 to 28 mm zone of inhibition which suggested that the methanol extract was little more effective than crude extract. Methanolic extracts of plants generally posses terpines and phenolics, which were reported by different workers as antimicrobial compounds (Dwivedi, 2007; Sanches *et al.,* 2005; Begum *et al.,* 2004; Manach *et al.,* 2001) [8, 26, 5, 18].

In our studies, the other medicinal plants, *Madhuca indica* and *Azadirachta indica,* did not show any antibacterial property in aqueous extracts against *Xanthomonas* although Patil and Ghoderao (1997) [24], Opara and Wokocha (2008) [22], Akhtar *et al.,* (1997) [1], Shenge and Akpa (2003) [28] and Hulloli *et al.,* (1998) [13] reported *Azadirachta indica* to be the effective one against *Xanthomonas.* Further, the antibacterial compound was separated by using Forestal solvent on TLC. The other solvents particularly water and BAW were not efficient in the separation of antibacterial compound from the methanolic and ethanolic extract of *Terminalia chebula* and *Bixa orellana.* Only Forestal solvent was the effective one in separation of antibacterial compound. The separation of antibacterial compound on TLC plate by Forestal solvent produced four prominent bands of different Rf values of 25.56, 58.52, 71.02 and 80.68 for the methanol extract of *Terminalia chebula,* while the corresponding Rf values for ethanolic extracts of *Terminalia chebula* were 28.40, 58.52, 71.02 and 80.68. The Rf values for ethanol and methanol extracts of *Bixa orellana* were similar *i.e.,* 57.95, 68.18 and 71.02.

When the bioactive material obtained at different Rf values where tested for the antibacterial activity, it was found that Rf values of 58.52, 71.02 and 80.68 contained antibacterial compound in the methanolic and ethanolic extracts of *Terminalia chebula,* whereas the Rf values of 57.75, 68.18 and 71.02 contained the antibacterial compound in the methanolic and ethanolic extract of *Bixa orellana*. The inhibition zone produced by these antibacterial compounds present at these Rf values was 1 to 1.4 cm *dia.*, which was equivalent to 50 to 500 µg (50 µg streptocycline produce 1 cm inhibition zone, whereas 500 µg produce 1.5 cm inhibition zone against *Xanthomonas axonopodis*). The inhibition zone produced by ethanol extract of *Terminalia chebula* and methanolic and ethanolic extracts of *Bixa orellana* was 1 cm *dia.* whereas the inhibition zone produced by methanol extract of *Terminalia chebula* was 1 to 1.4 cm *dia*. Khuntong and Sudprasert (2008) reported that purified fraction of *Suregada multiforum* exhibited stronger inhibition than the crude extract against *Xanthomonas campestris.*

Identification of antibacterial compound

The identification of antibacterial compounds was carried out using GC-MS. The antibacterial compounds showed two major peaks in ethanolic extract of *Terminalia chebula* at retention time of 12.44 and 17.03 min, whereas three major peaks were shown in methanolic extracts, with a peak retention time of 12.56, 15.34 and 17.03 min. In case of *Bixa orellana,* two major peaks at retention time of 12.44 and 17.03 min were obtained for ethanol extract and methanol extracts. When the retention time was compared with the library search of GC-MS unit (NIST MS Search 2.0), the library search of retention time of 12.42 to 12.56 min matched the presence of 4-(4-chlorophenyl) pyridine with a molecular weight of 189 and the probability of this compound was from 94.3% to 96.7%. The library search of retention time of 17.02 to 17.03 min matched for the presence of phthalic acid, 6-ethyl-3-octyl isobutyl ester with a molecular weight of 362 and the probability of this compound was from 7.04% to 7.20%. The commercially available 4-(4-chlorophenyl) pyridine when tested against the three bacteria and one fungus and was found to have antimicrobial property (Fig 4). Thomas (1985) reported that the compounds 4-(3-chlorophenyl) pyridine and 4-(4-chlorophenyl) pyridine to be useful intermediates in the synthesis of antibacterial product. Cross *et al.* (1963) [7] reported that phthalic acid produced by *Gibberella fujikuroi* had antimicrobial and other pharmacological activity. Srinivasan *et al.* (2009) [30] reported that ester of phthalic acid was the major compound present in the essential oil of *Leea indica* (Burm.f) Merr flower which have moderate antimicrobial activity. However, in our findings the compound phthalic acid does not show any antibacterial activity.

In conclusion, the results of the present investigation clearly indicated that 4-(4-chlorophenyl) pyridine possess the antimicrobial properties and that this compound is present in the medicinal plant *Terminalia chebula*. Thus *Terminalia chebula* is a source of new antimicrobial compound 4-(4-chlorophenyl) pyridine.

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