



Research Article

Extraction methods and TLC-bioautography for evaluation of antimicrobial activities of endophytic bacteria from medicinal plants

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SUMMARY

Objective: This study was undertaken to evaluate the antimicrobial activities of the bioactive compounds produced by endophytic bacteria isolated from medicinal plants using a rapid thin layer chromatography-bioautography method.

Methods: The metabolites produced by the isolated endophytic bacteria from various medicinal plants were extracted, using various solvents extraction methods. Eight isolates from *Triticum aestivum* were first screened for bioactivity using the disc diffusion method. The bioactive compounds were then fractionated and evaluated in a rapid approach using TLC-bioautography method.

Results: Ethylacetate solvent gave best yield; evaluation of their bioactivities revealed four isolates that produced antimicrobial compounds-*Sphingomonas yabuuchiae* TAR-1, *Enterobacter asburiae* TAR-4, *Pseudomonas putida* TAL-5 and *Delftia tsuruhatensis* TAL-1. They inhibited four to eight pathogens with zone of inhibition ranging from 6.3 to 15 ± 0.5 mm. Another four isolates from *Mentha spicata* and *Aloe vera*- *Pseudomonas entomophila* ALR-12, *Bacillus tequilensis* ALR-2, *Chryseobacterium indologenes* ALR-13 and *Pseudomonas putida* MSS-3, inhibited almost all pathogens tested with zone of inhibition between 7.6 to 16.6 ± 0.6 mm. Fractionation of compounds on TLC produced one to two fractions, which demonstrated bioactivity against *Staphylococcus aureus*, *Bacillus cereus* and *Candida albicans* pathogens.

Conclusion: TLC-bioautography detected antimicrobial activities of the different fractions towards a series of test pathogens. Hence it is a good screening method for isolation of bioactive compounds of pharmacological and agriculture uses.

INTRODUCTION

Endophytes are bacteria or fungi, which reside in the tissues of higher plants without causing any symptoms or apparent harm to the host plant.[1] They are associated with the production of novel secondary natural products which may be antimicrobial, antifungal, anti-inflammatory or antitumor, such as cyclodipeptides, phloroglucinol, graphis lactone A and taxol.[1-3] It has been hypothesized that endophytes residing in medicinal plants have the ability to produce compounds that confer similar benefits as their host plants. Preveena and Bhoire[4] identified several bacterial endophytes with antibacterial activities from the medicinal plant *Tridax procumbens* Linn., which has wound healing properties. Similarly, Strobel *et al*[5] isolated endophyte *Pestalotiopsis microspora* from the inner bark of *Taxus wallachiana*, which produces taxol, an anticancer drug.

The bioprospecting of new isolates and identifying their bioactivity is only a small fraction of the quest to derive and identify the specific compounds that confer the beneficial properties. In lab-scaled experiments, this stage may be accelerated by employing assays that generate results rapidly.

Numerous assays have been adopted to screen and evaluate the bioactivity of the bioactive compounds such as, disc diffusion, microdilution, streak test, bioautography and phytochemical screening methods.[6-9] This can only be achieved with appropriate and efficient separation techniques that allow quick identification of the bioactive compounds. In this study, we demonstrated the use of thin-layer chromatography (TLC)-bioautography to identify fractions to be evaluated for subsequent characterization after successfully developing a solvent system for their separation. This allows for preliminary separation of crude extracts of bioactive compounds into fractions, and the bioactivity of each fraction was rapidly tested. The process reveals the independence or synergy that may exist between compounds which may not be noticed with other methods mentioned above. As such, TLC-bioautography assists in identifying the suitable solvent(s) to be used to achieve separation of the specific bioactive compound(s).

We tested 12 endophytic bacteria isolated from commonly used medicinal plants such as *Triticum aestivum* (wheat grass), *Mentha spicata* (mint) and *Aloe vera*, known

for their antimicrobial nature and ethnobotanical properties.[10, 11] From these, five isolates were selected for further extraction and TLC-bioautography were studied. These selected isolates have been identified in our earlier study as *Sphingomonas yabuuchiae* TAR-1 (KJ780829) *Bacillus tequilensis* ALR-2 (KJ689792), *Chryseobacterium indologenes* ALR-13 (K J 6 3 8 9 8 2), *Pseudomonas entomophila* ALR-12 (KJ689793) and *Pseudomonas putida* MSS-3 (KM280649) using 16S rRNA gene sequences with their sequence deposited in NCBI and accession numbers assigned (Table 1). The isolates were first cultured and crude extracts of their bioactive metabolites were collected from various solvent extraction methods, and their antimicrobial activities determined from TLC-bioautography assay.

MATERIALS AND METHODS

Isolation of endophytic bacteria and molecular characterization

The herbal plants *T. aestivum*, *M. spicata* and *A. vera* were collected from Sungai Buloh (3.235283 N, 101.568342 E), Selangor, Malaysia. The endophytic bacteria were isolated and identified based on the analysis of their 16S rDNA gene sequences as described in our earlier work.[12] The sequences were deposited in Genbank of National Centre for Biotechnology Information (NCBI) and accession number obtained (Table 1).

Preliminary screening of antimicrobial activity by disc diffusion assay

The antimicrobial activities of bacterial endophytes were tested against bacteria and yeast pathogenic strains- *Pseudomonas aeruginosa* ATCC 10145, *Staphylococcus aureus* ATCC 33591, *Bacillus cereus* ATCC 14579, *Salmonella typhimurium* ATCC 14028, *Proteus vulgaris* ATCC 8427, *Klebsiella pneumoniae* ATCC 10031, *Escherichia coli* ATCC 25922, *Streptococcus pyogenes* ATCC 12384 and *Candida albicans* ATCC 90028 using the Kirby-Bauer disc diffusion method.[13] All pathogens were obtained from Microbiology Laboratory of Monash University Malaysia. The bacteria pathogens were pre-cultured overnight in Mueller Hinton broth at $35 \pm 2^\circ\text{C}$, and 5 mL of the suspension culture was then pipetted and centrifuged at $6,000 \times g$ for 5 min. The pellets were suspended in sterile distilled water and density adjusted to 0.5 McFarland standard. The suspension was seeded onto Mueller Hinton agar plates for antimicrobial testing. The endophytic bacteria were cultured in nutrient broth, incubated for 18-24h at $35 \pm 2^\circ\text{C}$ and centrifuged at $6,000 \times g$ for 5 min. The supernatant was filtered with a sterile 0.2 μm filtering unit and 20 μL of the supernatant obtained was impregnated onto sterile discs and placed on seeded agar plates. The plates were incubated at $35 \pm 2^\circ\text{C}$ for 48h and zone of inhibition measured. The experiment was performed in triplicates. Gentamicin (10 μg) and chloramphenicol (30 μg) standard antimicrobial discs were used as positive control.

Solvent extraction methods for high extract yield

The endophytic bacteria strains (TAR-1, ALR-2, ALR-13, MSS-3 and ALR-12) from storage, were first cultured in nutrient broth at $35 \pm 2^\circ\text{C}$ (150 rpm, 18 h), in which 25mL of the culture was then transferred into sterile Erlenmeyer flask (1L) of 475 mL nutrient broth and incubated

at $35 \pm 2^\circ\text{C}$ (150 rpm, 36 h). The culture was centrifuged at $8,000 \times g$ for 5 min to obtain the cell-free supernatant. The metabolites in the cell-free supernatant were exhaustively extracted separately with ethylacetate, diethylether and n-hexane in the ratio of 1:1 (supernatant:solvent). The organic phase was concentrated in rotatory evaporator (Rotavap) at 37°C , freeze-dried (LABCONCO freeZone 4.5 - 105°C) and weighed. The percentage yields of the two extracts were calculated from the following equation:

% extractive value (yield % w/v) = weight of dry extract (mg)/volume taken for extraction (1L) $\times$ 100

Phytochemical screening of the bioactive compounds

The nature of compounds present in the extracts were identified by testing for the presence of alkaloids, flavonoids, anthraquinone, cardiac glycosides and tannins using methods described by Parekh and Chanda.[14] To test for alkaloids, 2 mL of 1% HCl was added to 1mL (5 mg mL⁻¹) of crude metabolites and 6 drops of Wagner's reagent. A brownish/red precipitate indicated the presence of alkaloids. For detection of flavonoids 5 mg of crude metabolite was dissolved in 1 mL distilled water and made up to 2mL with diluted NaOH followed by addition of 2mL of diluted HCl. A yellow solution that turns colourless indicated the presence of flavonoids. For detection of presence of anthraquinone (Borntrger's test), equal volume of crude metabolite was mixed with 10% ammonia solution. A pink violet or red colour in the ammoniacal layer indicated presence of anthraquinone. For detection of presence of cardiac glycosides (Keller-kiliani test), 1mL of glacial acetic acid with 1mL of FeCl₃ and 1mL of concentrated H₂SO₄ were added to 2mL of extract. A green-blue colour indicated the presence of cardiac glycosides. To test for tannins, 2mL of FeCl₃ was added to 2mL of extract and a blue-black precipitate indicated the presence of tannins. For detection of presence of terpenoids, the method described by Kumar *et al.*, [15] was used. Two mL of acetic anhydride and 2mL of concentrated H₂SO₄ were added to 2 mL of extract and a reddish-brown layer on interface indicated the presence of terpenoids.

Detection of antimicrobial activities of extracted compounds via TLC-bioautography

The chemical constituents of the extracts were fractionated on aluminium-backed thin layer chromatography (TLC) plates (Merck, silica gel 60 F254). The plates were developed under saturated conditions with the eluent systems, developed in our laboratory that gave good separations. The mobile phase used for extraction of metabolites in isolates TAR-1, ALR-13 and ALR-12 was Chloroform: Methanol (9:1 v/v) solvents while MSS-3 and ALR-2 was Chloroform: Methanol (4:1 v/v). The separations were detected under ultraviolet light (254 nm) and the R_f values were calculated. TLC-bioautography assay was carried out as described by Begue and Kline [16] using immersion method. To develop the chromatograms, 1 μL (20 mg mL⁻¹) of each extract was loaded onto TLC plates in a narrow band and eluted using the different mobile solvent systems in a covered chromatography jar.

The developed plates were dried under the laminar flow for 6 h to remove traces of solvent on the plates. One mL of 0.5 McFarland standard bacteria pathogens were mixed with molten Mueller Hinton agar in sterile petri dish. The

prepared chromatograms were immersed in the molten Mueller Hinton agar and removed to solidify. This process was carried out in a laminar flow cabinet (Labotec, SA). The plates were then incubated in petri dish overnight at $37 \pm 2^\circ\text{C}$ and 100% relative humidity in the dark. The chromatograms were then sprayed with a 5 mg mL^{-1} solution of tetrazolium compound (TTC) and incubated overnight. White bands indicate the area of inhibition of bacteria growth due to the presence of compounds that inhibited the growth of tested organisms and prevent the reduction of TTC to formazan (reddish coloration).

Statistical analysis

One-way ANOVA was used to analyse all data obtained. The analysis was carried out using the Statistical Package for Social Science (SPSS) version 16.0 and means were compared using Tukey's Studentized Range Test. A p value < 0.05 was considered statistically significant.

RESULTS

Preliminary screening of antimicrobial activity by disc diffusion assay

Of the 12 isolates tested, nine were positive for antimicrobial activity against at least two of the pathogens tested. Most of the isolates with antimicrobial activities were isolated from *T. aestivum*, *M. spicata* and *A. vera*, with inhibition zone between 6.3 to $16.6 \pm 0.6 \text{ mm}$. They were *Sphingomonas yabuuchiae* TAR-1, *Enterobacter asburiae* TAR-4, *Pseudomonas putida* TAL-5, *Delftia tsuruhatensis* TAL-1, *Sphingobacterium cladoniae* TAL-2, *tequilensis* ALR-2, *P. entomophila* ALR-12, *C. indologenes* ALR-13 and *P. putida* MSS-3. *T. aestivum* hosted the most number of endophytes with positive antimicrobial activities, with five of the eight isolates demonstrating antimicrobial activities towards four to eight pathogens tested. The antimicrobial activities were more prominent in four isolates: *S. yabuuchiae* TAR-1, *E. asburiae* TAR-4, *P. putida* TAL-5 and *D. tsuruhatensis* TAL-1, with zone of inhibition from 6.3 to $15 \pm 0.5 \text{ mm}$ (Table 2). However, four isolates: *B. tequilensis* ALR-2, *P. entomophila* ALR-12, *C. indologenes* ALR-13 and *P. putida* MSS-3 from *A. vera* and *M. spicata* respectively, inhibited seven to eight of the pathogens with zone of inhibition from 7.6 to $16.6 \pm 0.6 \text{ mm}$ (Table 2). The Gram-positive bacteria (*S. aureus* and *B. cereus*) and Gram negative bacteria (*P. vulgaris*, *K. pneumoniae* and *E. coli*) were more susceptible to the antimicrobial metabolites from the endophytic bacteria as they were strongly inhibited by seven isolates while the Gram-negative bacteria (*S. Typhimurium*) was less susceptible to the metabolites of the endophytic bacteria as it was inhibited by five isolates.

Solvent extraction methods for high extract yield.

Extraction using ethylacetate solvent yielded more analytes than the use of diethylether and n-hexane solvents (Table 3). Ethylacetate gave the highest yield per isolate particularly for *C. indologenes* ALR-13 with 3.4 mg L^{-1} . In contrast, n-hexane gave the lowest yield per isolate (mean of 0.69 mg L^{-1}) except for the extraction of analytes from *P. entomophila* ALR-12 which appeared to be evenly distributed in the two solvents, ethylacetate and n-hexane with 1.2 and 1.3 mg L^{-1} yield respectively.

Phytochemical screening of the bioactive compounds

The phytochemical screening results of the isolates suggested the presence of alkaloids, flavonoids and tannins or their derivatives, though tannins were only detected in ALR-2 and TAL-1 (Table 4). Hence, *S. yabuuchiae* TAR-1, *P. entomophila* ALR-12, *B. tequilensis* ALR-2, *P. putida* MSS-3 and *C. indologenes* ALR-13 are promising isolates that produce bioactive compounds that are active against the human pathogens, hence they were selected for further investigations.

Antimicrobial activities of extracted compounds detected via TLC-bioautography

When the ethylacetate extracts were subjected to TLC, the metabolite of *P. entomophila* ALR-12 was best separated as the chromatogram gave 3 fractions as seen under UV light; A12-1, A12-2 and A12-3 of Rf values 0.85 , 0.78 and 0.60 (Figure 1). However, only one of the fractions (A12-1) indicated antimicrobial activity against *S. aureus*, *B. cereus* and *C. albicans*. A similar result was obtained for *C. indologenes* (ALR-13) chromatograms which was separated into 3 fractions: A13-1, A13-2, A13-3 with Rf values 0.88 , 0.75 and 0.55 respectively (Figure 1). Also, only one of the fractions (A13-1) indicated antimicrobial activity against *B. cereus*. However, *B. tequilensis* ALR-2 chromatogram indicated 2 fractions separation: A2-1 and A2-2 with Rf values 0.98 and 0.86 respectively (Figure 1). It appeared that the two fractions showed antimicrobial activities against *S. aureus* and *B. cereus* (Figure 2) as the zone of inhibition was wider and appeared to map the two fractions with Rf values 0.98 and 0.86 .

In addition, *S. yabuuchiae* TAR-1 and *P. putida* MSS-3 chromatograms produced two fractions each; T1-1 and T1-2 with Rf values 0.93 and 0.36 , and M3-1 and M3-2 of Rf values 0.96 and 0.84 respectively while only one fraction (M3-1) indicated activity while no activity was observed for the two fractions of *S. yabuuchiae* TAR-1.

Table 1: Similarity values of 16S rRNA gene sequences retrieved from the endophytic bacteria of *T. aestivum*, *M. spicata* and *A. vera* plants.

Isolates	Nearest relatives*	Accession No	Identity %	Reference
TAL-1	<i>Delftia tsuruhatensis</i>	KJ780828	99%	[28]
TAL-2	<i>sphingobacterium cladoniae</i>	KJ780824	99%	[28]
TAL-3	<i>Klebsiella variicola</i>	KJ780825	99%	[28]
TAL-5	<i>Pseudomonas putida</i>	KJ780826	99%	[28]
TAL-6	<i>Pseudomonas entomophila</i>	KJ780827	99%	[28]
TAR-1	<i>Sphingomonas yabuuchiae</i>	KJ780829	99%	[28]
TAR-4	<i>Enterobacter asburiae</i>	KJ780823	99%	[28]
TAR-5	<i>Bacillus weihenstephensis</i>	KJ780830	99%	[28]
MSS-3	<i>Pseudomonas putida</i>	KM280649	997%	[29]
ALR-2	<i>Bacillus tequilensis</i>	KJ689792	97%	[12]
ALR-12	<i>Pseudomonas entomophila</i>	KJ689793	98%	[12]
ALR-13	<i>Chryseobacterium indologenes</i>	KJ638982	99%	[12]

*Closest relative species in the 16S rRNA gene sequence database. TAL, TAR, ALR, MSS represent leaf, root and stem of *T. aestivum*, *A. vera* and *M. spicata* respectively

Table 2: Antimicrobial activity of endophytic bacteria metabolites from *T. aestivum*, *M. spicata* and *A. vera* plants.

Isolates	Annular zone of inhibition (mm)							
	<i>S. Typhimurium</i>	<i>P. vulgaris</i>	<i>K. pneumoniae</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>S. pyogenes</i>	<i>B. cereus</i>	<i>C. albicans</i>
TAR-1	12 ± 0.5 ^c	7.0 ± 0.5 ^a	8.7 ± 0.57 ^b	12 ± 0.5 ^c	15 ± 0.5 ^d	10 ± 0.5 ^b	10 ± 0.8 ^b	10 ± 0.5 ^b
TAR-4	neg	neg	11 ± 0.5 ^c	6.3 ± 0.6 ^a	8.0 ± 0.5 ^b	neg	9 ± 0.5 ^b	neg
TAR-5	neg	neg	neg	neg	neg	neg	neg	neg
TAL-1	neg	neg	9.7 ± 0.57 ^a	11 ± 0.5 ^b	11 ± 0.5 ^b	neg	10 ± 0.5 ^{ab}	neg
TAL-2	neg	7.7 ± 0.6 ^a	neg	neg	7.7 ± 0.6 ^a	neg	neg	neg
TAL-3	neg	neg	neg	neg	neg	neg	neg	neg
TAL-5	neg	7.0 ± 0.5 ^a	neg	neg	neg	9.0 ± 0.5 ^b	9 ± 0.5 ^b	9 ± 0.5 ^b
TAL-6	neg	neg	neg	neg	neg	neg	neg	neg
ALR-2	8.3 ± 0.57 ^a	9.6 ± 0.6 ^b	8.3 ± 0.57 ^a	10 ± 0.6 ^b	neg	10 ± 1.7 ^b	11 ± 0.57 ^c	11.6 ± 0.6 ^c
ALR-12	13.3 ± 0.57 ^b	10.3 ± 0.6 ^a	10.3 ± 0.57 ^a	13.7 ± 0.6 ^b	14 ± 1.0 ^c	16.6 ± 0.6 ^c	10 ± 0.57 ^a	15.6 ± 0.6 ^d
ALR-13	7.6 ± 0.57 ^a	11 ± 1.0 ^c	11.7 ± 0.57 ^c	7.6 ± 0.6 ^a	14.3 ± 0.5 ^d	14.3 ± 0.6 ^d	neg	8.3 ± 0.6 ^b
MSS-3*	11 ± 0.5 ^b	10 ± 0.8 ^b	14.3 ± 0.5 ^c	15.3 ± 0.5 ^d	11 ± 0.5 ^b	7.6 ± 0.5 ^a	9.6 ± 0.5 ^{ab}	11.6 ± 0.5 ^b
Chloramphenicol (30µg)	28 ± 0.5 ^d	24 ± 0.5 ^b	30 ± 0.5 ^c	26 ± 0.5 ^c	26 ± 0.5 ^c	26 ± 0.5 ^c	26 ± 0.5 ^c	16 ± 0.5 ^a
Gentamicin (10µg)	20 ± 0.5 ^a	26 ± 0.5 ^c	22 ± 0.5 ^a	20 ± 0.5 ^a	20 ± 0.5 ^a	30 ± 0.5 ^c	23 ± 0.5 ^b	28 ± 0.5 ^d

Data are mean ± SD values. One-way ANOVA was used to analyse data using Tukey's Studentized range test (alphabets a-e). Values are statistically significant at p < 0.05. TAR, TAL, MSS, ALR, ALS and ALL represent isolates from the root, stem and leaf of *T. aestivum*, *M. spicata* and *A. vera*, respectively. "neg" negative (means no inhibition zone observed). *reference [29].

Table 3: Extraction yields and R_f values of chromatograms of metabolites of endophytic bacteria.

Isolates	Ethylacetate % Yield (w/v)	Diethylether (mg L ⁻¹)	n-Hexane	Solvent System	Fraction & R _f value
TAR-1	2.4	1.32	0.67	CM (9:1)	TR1 (0.93), TR2 (0.36)
MSS-3	1.5	0.95	0.53	CM (4:1)	M3-1 (0.96), M3-2 (0.84)
ALR-2	2.3	1.03	0.6	CM (4:1)	A2-1 (0.98), A2-2 (0.86)
ALR-13	3.4	2.02	0.97	CM (9:1)	A13-1 (0.88), A13-2 (0.75), A13-3 (0.55)
ALR-12	1.2	0.87	1.3	CM (9:1)	A12-1 (0.85), A12-2 (0.78), A12-3 (0.60)

TAR, TAL, MSS, ALR, ALS and ALL represent isolates from the root, stem and leaf of *T. aestivum*, *M. spicata* and *A. vera* respectively. CM (Chloroform: Methanol) ratio.

Table 4: Phytochemical screening of the ethylacetate extracts

Isolates	Tannins	Alkaloids	Cardiac Glycosides	Steroids	Flavonoids	Terpenoids	Anthraquinone
TAL-1	+	+	-	-	-	-	-
TAL-2	-	-	-	-	-	-	-
TAL-3	-	-	-	-	-	-	-
TAL-5	-	+	-	+	-	-	-
TAL-6	-	-	+	-	-	-	-
TAR-1	-	+	-	-	+	-	-
TAR-4	-	+	-	-	-	-	-
TAR-5	-	-	-	-	-	-	-
MSS-3	-	+	-	-	+	-	-
ALR-2	+	+	-	-	+	-	-
ALR-12	-	+	+	+	-	-	-
ALR-13	-	+	-	+	-	-	-

(+) indicates the presence of compound (-) indicates absence of compound. TAR, TAL, MSS, ALR, ALS and ALL represent isolates from the root, stem and leaf of *T. aestivum*, *M. spicata* and *A. vera* respectively.

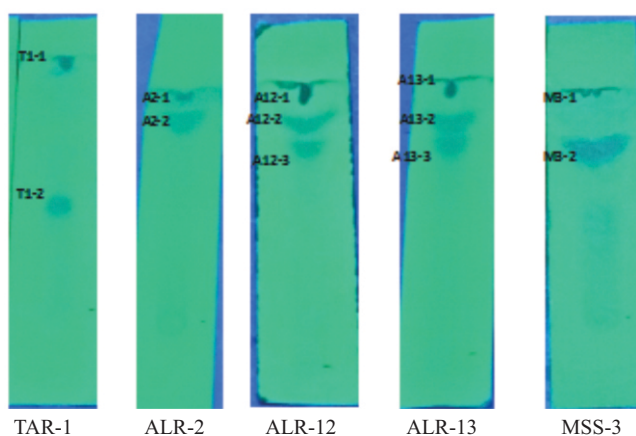


Figure 1: Bioautography plates. Chromatograms of ethylacetate extracts of TAR-1 showing fractions (T1-1, T1-2), ALR-2 showing fractions (A2-1, A2-2), ALR-12 showing fractions (A12-1, A12-2, A12-3), ALR-13 showing fractions (A13-1, A13-2, A13-3) and MSS-3 showing fractions (M3-1, M3-2) respectively. (Image scale x2)

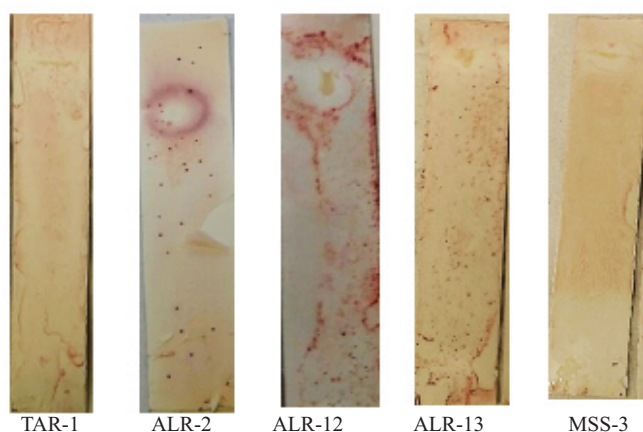


Figure 2: Clear areas showing inhibition zones of chromatograms when immersed in molten agar seeded with bacterial pathogens, incubated at $37 \pm 2^\circ\text{C}$ for 18h then sprayed with tetrazolium compound and then incubated at $37 \pm 2^\circ\text{C}$ for 24 h. (Image scale x2)

DISCUSSION

Several bacterial endophytes found in this study are commonly known for their antimicrobial activities. Isolates such as *P. entomophila* ALR-12, *P. putida* MSS-3 and *B. tequilensis* ALR-2 are similar to other species in the studies of researchers prospecting for bioactive compounds of pharmacological importance.[17,18] The work of Li *et al*[2], suggested the ability of bacterial endophytes such as *Pseudomonas* sp. to produce metabolites capable of inhibiting the growth of other bacteria or pathogens. They isolated *Pseudomonas brassicacearum* subsp. *Neoaureantiaca* in *Salvia miltiorrhiza* plant and identified 11 cyclodipeptides with antifungi and antibacterial activities. In addition, surfactin analog, a bioactive compound was isolated from *Bacillus mojavensis* by Youcef-Ali, Kacem-chauouche.[19] The metabolites were found to inhibit phytopathogens and they suggested that the endophytic bacterium present in the roots of *S. miltiorrhiza* could protect the host by producing cyclodipeptides that inhibited the phytopathogens. Some *Bacillus* sp. was also reported by Shoji *et al.*,[17] to produce cerexins and polymyxin, some polypeptide antibiotics that inhibited bacteria pathogens. The study of Sun *et al.*,[20] also reported endophytic bacterium, *Bacillus amyloliquefaciens* from Chinese medicinal plant *Scutellaria baicalensis* and isolated two families of secondary metabolites, fengycins and surfactins, with broad spectral antibacterial and antifungal activities.

The results of the phytochemical screening showed compounds that have been identified by researchers to possess antimicrobial and antifungal properties, such as pyrrolnitrin, phenazines and phloroglucinol.[2,21,22] Endophytes are also known to produce a broad variety of bioactive secondary metabolites with unique structure, including alkanoids, benzopyranones, chinones, flavonoids, phenolic acid, quinones, steroids and terpenoids[23] some of which have been shown in this study. However, while finding the best extraction method, the ethylacetate could be said to be effective, compatible and useful in getting high yield of the analytes in these isolates. This agrees with earlier published results, that ethylacetate is often a favoured solvent for the

extraction of bioactive compounds from various microbes.[24,25] Also, only ethylacetate-derived extracts showed bioactivities in the TLC-bioautography, which suggested that the bioactive components of these isolates were present in the more polar solvent of ethylacetate (polarity index 4.4) than the non-polar solvents, diethylether and n-hexane (polarity index 2.8 and 0.1 respectively).

It was interesting to note that *B. tequilensis* ALR-2 and *C. indologenes* ALR-13 did not show activities during the preliminary screening assay against *S. aureus* and *B. cereus* respectively but produced activities with TLC-bioautography. This suggests that the separating technique and extraction process may have unmasked the bioactive compounds present into their active forms. However, the absence of activity in *S. yabuuchiae* TAR-1 extract could be due to evaporation of the active compounds, denaturation or photo-oxidation, or due to very little amount of the active compound as suggested by Masoko and Eloff.[26] A similar result was obtained by Eloff *et al*[27] and they suggested that the separated compounds that had no antimicrobial activities could be based on a synergy that may exist between the compounds present in the extract.

Hence, TLC-bioautography technique as presented gives insight to the interaction that exists between different compounds in the extract. These results enable us to rapidly identify the fractions of the extracts with the bioactive compounds of interest and the right separating mixture for the isolation of the bioactive compounds. Therefore, *B. tequilensis* (ALR-2), *P. entomophila* (ALR-12), *C. indologenes* (ALR-13) and *P. putida* (MSS-3) may be said to possess very promising antimicrobial compounds as revealed by the prominent zone of inhibition seen on their chromatograms. Hence, we propose further study on the isolation and characterization of some of these metabolites.

CONCLUSION

The antimicrobial activities of bioactive compounds produced by bacterial endophytes were determined in a rapid assay via TLC-bioautography. Several solvents were tested and ethylacetate was found to successfully extract higher yield of bioactive compounds. When TLC was performed, an average of two to three fractions were obtained. TLC-bioautography detected antimicrobial activities of the different fractions towards a series of test pathogens. This study contributes to methodological approach in rapid extraction of bioactive compounds and the determination of the antimicrobial activities of the fractions. Results here are also a milestone for studies related to antimicrobial activities of endophytic bacteria, and in bioprospecting for bioactive compounds of pharmacological and agricultural uses.

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Conflict of interest

The authors report no conflict of interest and are responsible for the content and writing of the manuscript.

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