

Screening of Antibacterial Activity of *Goniothalamus calvicarpa* Extracts against *Xanthomonas axonopodis* pv. *citri* in vitro

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ABSTRACT

Citrus canker caused by *Xanthomonas axonopodis* pv. *citri* (Xac) is a serious disease in many parts of the world and is difficult to control. The use of copper-based pesticides is becoming a concern due to the accumulation of heavy metals in orchard soils and more benign treatment methods are needed. Eighteen Thai native plant extracts were screened for antibacterial activity against a Thai isolate of Xac and it was found that ethanolic extracts of *Goniothalamus calvicarpa* leaves showed the strongest antibacterial activity against Xac in vitro. The *G. calvicarpa* extracts were then sequentially dissolved with hexane, ethyl acetate and methanol and retested. A 70% aqueous ethanol extract and a methanol soluble extract produced strong inhibition zones against Xac. Although thin layer chromatographic profiles revealed the likely presence of flavonoids in the biologically active extracts of *G. calvicarpa*, the active compounds have yet to be identified. Work is proceeding to determine whether specific extracts of *G. calvicarpa* have biological activity against citrus canker in the field.

Keywords: Antibacterial activity, Plant extract, Citrus canker

INTRODUCTION

Citrus canker caused by *Xanthomonas axonopodis* pv. *citri* (Xac) is widespread in Asia and a biosecurity threat in many parts of the world affecting the global trade in citrus. The pathogen causes crater-like lesions on the fruit which are surrounded by a chlorotic halo with a water-soaked margin (Graham et al., 2004; Ngoc et al., 2007). Control is difficult. Currently, copper-based pesticides are intensively used to decrease disease incidence. However, the accumulation of Cu in orchard soils may affect root growth, nutrient uptake and allow copper-resistant strains of the causal organism to persist (Behlau et al., 2017). Lime, kaffir lime and pomelo are important orchard crops in many provinces in Thailand. Ngoc et al. (2007) first reported Xac infesting citrus in Thailand. During the monsoon season, producers spray copper-based pesticides if early stages of bacterial infection are observed. Where infestations are severe, diseased trees are destroyed to reduce crop loss in surrounding orchards. Alternative chemicals are therefore being sought to reduce copper loading into the environment. Plant based-bactericides may be one option if they can be shown to be effective.

Thailand has an abundance of plant species which possess potential bioactive compounds, some of which are effective against bacterial diseases. For example, plant extracts have been reported to be highly efficient in the control of some food-borne pathogens (Dholvitayakhun et al., 2012; Jarriyawattanachaikul et al., 2016) and against some plant pathogenic bacteria (Prakash and Karmegam, 2012; Bhagwat and Datar, 2014). Crude extracts obtained from leaves of nine Thai plant families were investigated for their herbicidal activity and allelochemical content (Poonpaiboonpipat and Jumpathong, 2019). Among these, extracts from *Goniothalamus calvicarpa* had the highest total phenolic content (143.7 mg gallic acid equivalent per g of crude extract). Further, Jewers et al. (1972) isolated Goniothalamine, a bioactive styryllactone, from four *Goniothalamus* species and Wiart (2007) reported that *Goniothalamus* species were a potential source of antibacterial agents.

In this study, we compared leaf extracts of *G. calvicarpa* with those from seventeen other Thai native plant extracts to ascertain whether there was any antibacterial activity against the causal agent of citrus canker. Among the plants selected for study, the presence of antimicrobial, anticancer and antioxidant activity have been reported in some species (Calixto et al., 2004; Uthairatsamee et al., 2011; Pukdeekumjorn et al., 2016). Also, the bark from *Garcinia dulcis*, *Zingiber zerumbet* and *Cinnamomum porrectum* have been used as folk medicine (Deachathai et al., 2005; Chien et al., 2008; Limsuwan et al., 2013). To study the plant extract chemical profiles, thin layer chromatography (TLC) was used to observe group of compounds. TLC is a qualitative method which can be used to aid in rapid chemical profile identification of plant extracts (Suphrom et al.,

2018). As an integral part of the study, we aimed to identify a suitable solvent for leaf extraction to obtain effective bioactive compounds.

MATERIALS AND METHODS

Plant extract preparation

Samples of seventeen plant species from nine families were collected from central Thailand in November 2016 (Table 1). Leaves and other parts from these species were washed in tap water and dried at 45 °C for 48 h in a hot air oven. The dried leaves were extracted with 70% aqueous ethanol in a ratio of 1:10 w/v for 72 h in the dark. The extract solutions were filtered through Whatman No. 1 filter paper, evaporated in a rotary evaporator, lyophilized, and then stored in the dark at 4 °C until use.

Table 1. Plants used to obtain extracts.

Family	Plant species	Plant part	Location [¥]
Annonaceae	<i>Anomianthus dulcis</i> (Dunal) J. Sinclair	Leaves	1
	<i>Goniothalamus calvicarpa</i> Craib	Leaves	1
	<i>Mitrephora keithii</i> Ridl	Leaves	1
	<i>Mitrephora wangii</i> Hu	Leaves	1
	<i>Orophea polycarpa</i> A. DC.	Leaves	1
Asteraceae	<i>Elephantopus scaber</i> L.	Roots	2
Bignoniaceae	<i>Pachyptera hymenaea</i> (DC.) A. Gentry	Leaves	2
Clusiaceae	<i>Garcinia dulcis</i> (Roxb.) Kurz	Bark	3
Eicaceae	<i>Agapetes lobbii</i> C.B. Clarke	Leaves	1
Lauraceae	<i>Cinnamomum loureiroi</i> Nees	Leaves	1
	<i>Cinnamomum porrectum</i> Kosterm.	Leaves	1
Magnoliaceae	<i>Michelia floribunda</i> Fin. et Gagnep	Leaves	1
	<i>Manglietia garrettii</i> Craib	Leaves	1
	<i>Magnolia henryi</i> Dunn.	Leaves	1
Moraceae	<i>Ficus microcarpa</i> L.f. var. <i>crassifolia</i> Sheeh Liao	Leaves	3
Zingiberaceae	<i>Zingiber zerumbet</i> (L.) Smith.	Stems	2
		Roots	2
	<i>Hedychium coronarium</i> J. Koenig	Leaves	1

Note: [¥]1 Romklao Botanical Garden under the Royal Initiative, Phitsanulok Province, Thailand.

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Antibacterial activity test

Test Microorganisms. Phytopathogenic bacterial strains of *Xanthomonas axonopodis* pv. *citri* (Xac), isolated from kaffir lime (strain number DOABC856), lime (strain number DOABC956), and pomelo (strain number DOABC992) were obtained from the Plant Protection Research and Development Office, Department of Agriculture in Thailand.

Preliminary screening for antibacterial activity. The antibacterial activity assay, that was used to test for effectiveness against Xac, used the paper disc diffusion method (Boonkaew and Camper, 2005). Bacterial suspensions, adjusted to 10^8 CFU/mL, were inoculated onto the surface of nutrient agar plates (Hi-media, India). The dried 70% ethanol extracts were re-dissolved in methanol and the samples were loaded onto 6 mm sterile discs (Whatman, GE) (4 mg/disc), air-dried for ca. 1 h, and placed on the surface of bioassay plates. Each experiment was carried out using three replicates, and chloramphenicol (1 mg/mL) was used as the positive control. Plates were then held in the dark for 24 h at 30 °C. The inhibition zone around the discs was measured in mm.

Secondary screening for antibacterial agents. From the preliminary screening results, crude extracts from *G. calvicarpa* were selected for more detailed study. Extracts were dissolved in hexane, ethyl acetate, and methanol before testing for antibacterial activity using the same paper disc diffusion method.

Chromatographic profile detected using thin layer chromatography (TLC)

Eighteen crude extracts were loaded onto a silica plate (TLC silica gel 60 F254 aluminum sheet, Merck, Damstadt, Germany). The mobile phase (5% methanol in dichloromethane) was prepared at ambient room temperature. The TLC plate was developed to a distance of 8 cm. After the development, the plate was observed under visible, short-wave UV at 254 nm and long-wave radiation at 366 nm. To detect essential oils, flavonoids, glycosides, terpenes, carbohydrates and steroids, the plate was sprayed with anisaldehyde-sulfuric acid (Nandhasri et al., 2005; Gerlacha et al., 2018). Anisaldehyde-sulfuric acid spray reagent has greater ability to detect classes of compounds than sulfuric acid alone (Gerlacha et al., 2018).

Statistical analysis

Experimental data for antibacterial activity (inhibition zone size) were compared with the control using Duncan's new multiple range test ($P < 0.05$, $n = 3$).

RESULTS

Antibacterial activity

Primary screening used the 70% aqueous ethanol extracts from seventeen different plant species. The challenge test revealed that only the *G. calvicarpa* leaf extract, at a concentration of 100 mg/mL (4 mg/disc), exhibited an inhibition zone. For the secondary screening, the extracts were taken up in a range of solvents and the antibacterial activity against *X. axonopodis* pv. *citri* (Xac) was then evaluated on bioassay plates for phytopathogenic bacterial isolates isolated from kaffir lime, lime and pomelo. When chloramphenicol was used as the positive control and the different solvents as a negative control, chloramphenicol had the highest activity at a concentration of 1 mg/mL (20 µg/disc) when tested against Xac from the three citrus hosts (Figure 1).

The methanolic extract of *G. calvicarpa* had significantly higher antibacterial activity than other extracts when tested against Xac in lime and kaffir lime. However, the 70% aqueous ethanol extract and the methanol soluble extract both had high activity when tested against Xac from pomelo (Table 2). In contrast, the discs with added solvents alone produced no zone of inhibition. In this experiment, methanol soluble extract and 70% aqueous ethanol extract exhibited greater antibacterial activity on Xac from pomelo than Xac from pomelo from lime and kaffir lime regarding the inhibition zone presented on bioassay plate using paper disc diffusion assay. In contrast, Xac from pomelo is the least sensitive on antibacterial activity test when using chloramphenicol. The hexane soluble extract was not assayed due to the small content after evaporated to dryness.

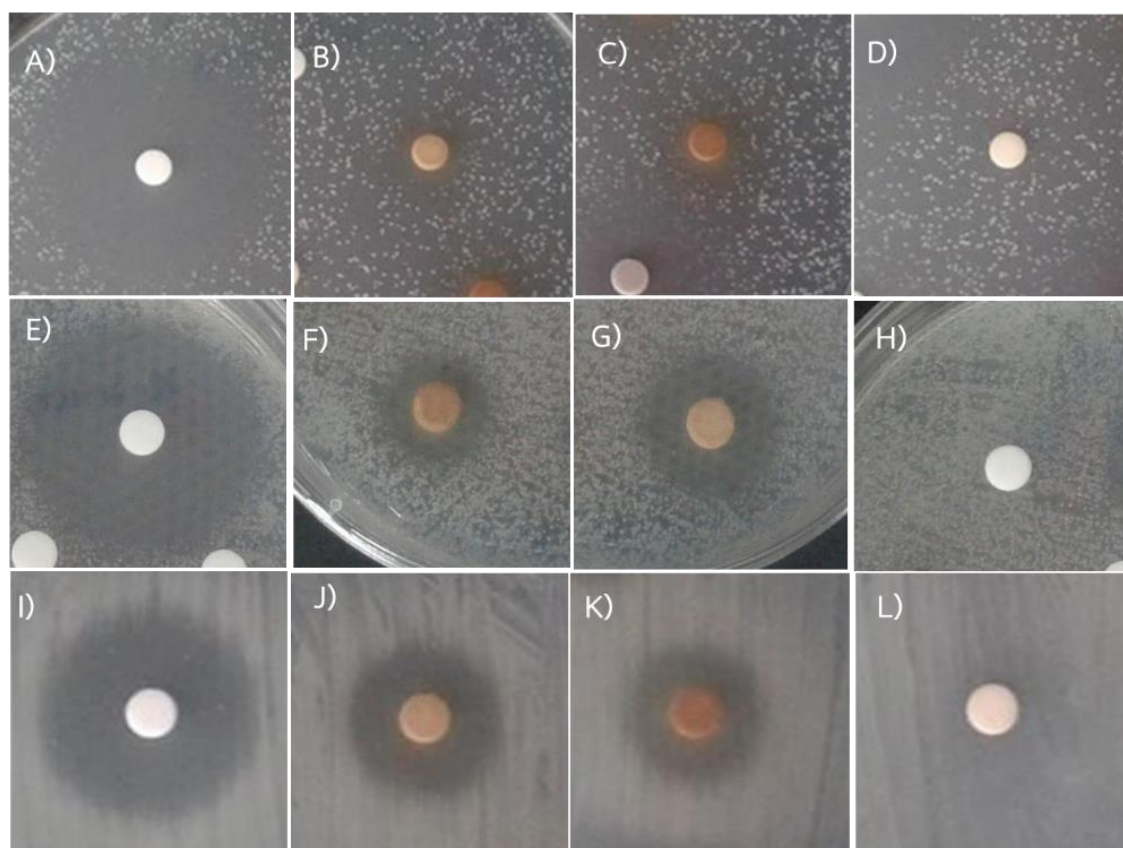


Figure 1. Growth inhibition zones of *Xanthomonas axonopodis* pv. *citri* (Xac) in challenge tests using *Goniiothalamus calvicarpa* crude leaf extracts. The paper disc diffusion assay tested against Xac from kaffir lime using **A:** chloramphenicol, **B:** methanol soluble extract, **C:** 70% aqueous ethanol extract, and **D:** ethyl acetate soluble extract. The paper disc diffusion assay tested against Xac from lime using **E:** chloramphenicol, **F:** methanol soluble extract, **G:** 70% aqueous ethanol extract, and **H:** ethyl acetate soluble extract. The paper disc diffusion assay tested against Xac from pomelo using **I:** chloramphenicol, **J:** methanol soluble extract, **K:** 70% aqueous ethanol extract, and **L:** ethyl acetate soluble extract.

Table 2. Antibacterial screening of leaf extracts (4 mg/disc) from *Goniothalamus calvicarpa* against *Xanthomonas axonopodis* pv. *citri* (Xac) cultures isolated from three citrus hosts.

Crude leaf extract	Inhibition zone (mm) [‡]		
	Xac (kaffir lime)	Xac (lime)	Xac (pomelo)
Ethyl acetate soluble extract	0±0.00 ^{A,d*}	0±0.00 ^{A,d}	0±0.00 ^{A,c}
Methanol soluble extract	10.67±1.15 ^{B,b}	14.00±1.73 ^{B,b}	18.33±2.08 ^{A,b}
70% aqueous ethanol extract	8.33±0.58 ^{C,c}	11.67±1.53 ^{B,c}	16.67±1.15 ^{A,b}
Chloramphenicol	30.67±1.15 ^{A,a}	29.67±2.52 ^{A,a}	25.33±1.53 ^{B,a}

Note: [‡] Values are presented as mean ± standard deviation.

Chloramphenicol at a concentration of 20 µg/disc for Xac in lime, and at a concentration of 40 µg/disc for Xac in kaffir lime and pomelo.

* Different small letters in each column indicate significant differences from Duncan's multiple range test ($P < 0.05$, $n = 3$).

* Different capital letters in each row indicate significant differences from Duncan's multiple range test ($P < 0.05$, $n = 3$).

Chromatographic profile

There was a diversity of bands that were clear under UV light at 254 nm and long wave UV light at 366 nm. The developed TLC (5% methanol in dichloromethane) had a number of separate dark spots within each lane indicating that the structures of the extracts contained chromophores (i.e., C=C, C=O bonds) (Figure 2-A). Long-wave UV radiation at 366 nm revealed a number of different fluorescent compounds exhibited as bright orange, yellow, purple and blue bands. Aromatic compounds and highly conjugated systems were visualized under short-wave UV at 254 nm (Figure 2-B). After staining with *p*-anisaldehyde-sulfuric acid, the compounds which did not contain chromophores, appeared purple, orange and yellow (Figure 2-C). There were distinct differences between species in the TLC patterns particularly under UV.

The 70% aqueous ethanol derived extract from *G. calvicarpa* was dissolved in hexane, ethyl acetate and methanol, and developed on TLC (5% methanol in dichloromethane) to detect the chemical components that had antibacterial activity against gram-negative bacteria. The developed TLC showed that the different solvents used in this study produced similar chemical profiles and the most intense profile was with ethyl acetate. Components which contained chromophores were visible under UV at 254 nm (Figure 3-A), while fluorescent compounds were visible as orange, yellow and purple regions when observed under UV at 366 nm (Figure 3-B). The TLC profile also clearly exhibited a yellow compound when dissolved in ethyl acetate. Previously, the total flavonoid content in *G. calvicarpa* extract was found to be 3.16 mg quercetin equivalent g⁻¹ of crude extract (Poonpaiboonpipat and Jumpathong, 2019). Moreover, the purple colored compounds dissolved very well in hexane, while the gray colored ones

could be dissolved in the other solvents that were used in this study (Figure 3-C). However, structure determination of these compounds are required using more advanced analytical methods such as liquid chromatography-mass spectrometry (LC-MS), Nuclear magnetic resonance (NMR) and Infrared spectroscopy (IR spectroscopy).

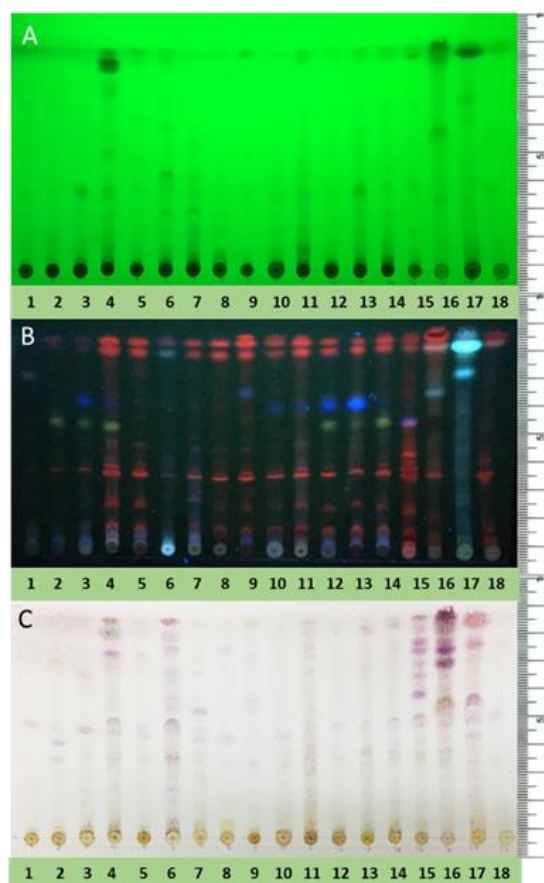


Figure 2. TLC fingerprints of 18 extracts from plant species that were investigated. The TLC plate was developed with 5% methanol in dichloromethane. **A:** TLC plate viewed under ultraviolet radiation at 254 nm, **B:** TLC plate viewed under ultraviolet radiation at 366 nm, **C:** TLC plate heated to 105°C and sprayed with *p*-anisaldehyde-sulfuric acid. **Lane 1:** *Anomianthus dulcis*, **2:** *Goniothalamus calvicarpa*, **3:** *Mitrephora keithii*, **4:** *Mitrephora wangii*, **5:** *Orophea polycarpa*, **6:** *Elephantopus scaber*, **7:** *Pachyptera hymenaea*, **8:** *Garcinia dulcis*, **9:** *Agapetes lobbii*, **10:** *Cinnamomum loureiroi*, **11:** *Cinnamomum porrectum*, **12:** *Michelia floribunda*, **13:** *Manglietia garrettii*, **14:** *Magnolia henryi*, **15:** *Ficus microcarpa* L.f. var. *crassifolia*, **16:** *Zingiber zerumbet* (stem), **17:** *Zingiber zerumbet*(root), **18:** *Hedychium coronarium*

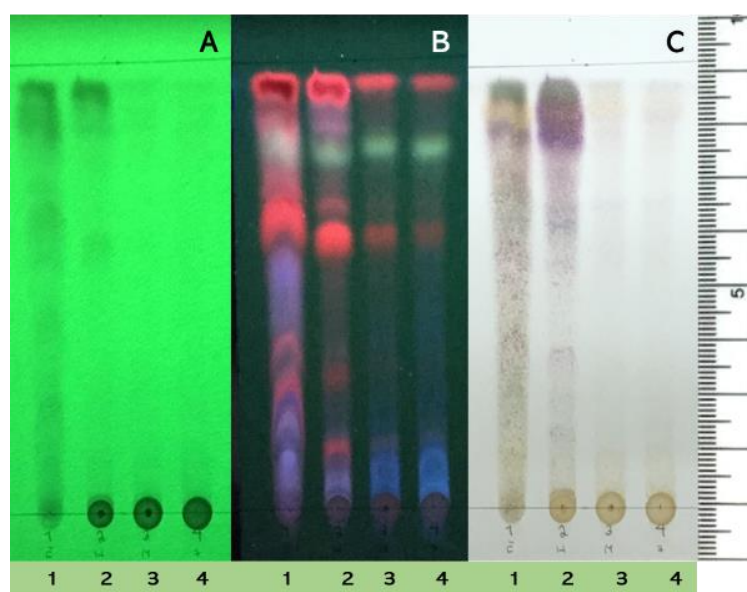


Figure 3. *Goniiothalamus calvicarpa* extracts: the chemical profile on TLC when dissolved in ethyl acetate (1), hexane (2), methanol (3) or 70% aqueous ethanol extract (4) and developed in 5% methanol in dichloromethane. TLC visualized under UV at 254 nm (A), UV 366 nm(B) and *p*-anisaldehyde – sulfuric acid stain (C).

DISCUSSION

Citrus canker is a devastating disease which occurs in more than 30 countries (Gottwald et al., 2002). At present, a combination of both chemical and biological methods are applied to control this disease which indicates the need for sustainable crop protection methods. The plant kingdom has been identified as a potential source for biological active substances (Bhagwat and Datar, 2014). Plant-based extracts contain natural molecules, some of which have the capacity to inhibit the growth of bacteria. Plant extracts and plant essential oils are also alternative sources for bio-pesticide production as a number of studies have established that they are efficient in controlling phytopathogenic bacteria and help in reducing disease incidence in crops (Leksomboon et al., 2000; Leksomboon et al., 2001; Omidbeygi et al., 2007).

Plant extracts have been reported in citrus canker control (Leksomboon et al., 2000; Leksomboon et al., 2001). In Thailand, lime is one of the most important citrus plants as it has a number of different uses and is in demand year round (Issarakraisila, 2016). In the monsoon season, canker disease is wide spread in every region of the country. Several studies have been undertaken to determine the potential of plant extracts in controlling canker in various orchards. According to Leksomboon et al. (2001), an aqueous extract from *Tamarindus indica* Linn. reduced the number of cankers on lime leaves in a field study when

compared with other plant extracts that were evaluated. In another study, antibacterial activity was tested *in vitro* against *Xanthomonas axonopodis* pv. *citri* (Xac) using fractions of leaf extract from *Polyalthia longifolia* and *Achyranthes aspera*. The active fractions, identified as unknown proteins, were found to have molecular weights of 19 and 30 kDa, respectively (Manonmani et al., 2009). In addition, essential oils from thyme were reported to have antibacterial activity in a greenhouse experiment and it was suggested that thyme essential oils be used as an alternate spray with copper (Samavi et al., 2009).

In general, procedures for the isolation of antibacterial agents from plants, and preliminary screening of those compounds using agar well or paper disc diffusion methods, have been used for selecting potential plant extracts that might be effective against bacterial pathogens. Further investigation, using a typical protocol such as bioassay-guided fractionation and step-by-step plant extract separation are required in order to obtain pure samples of the extracted chemical compounds before their structure can be determined.

The results obtained in this study indicate that separation of the antibacterial agents from *Goniothalamus calvicarpa* leaf extracts was possible using the chromatographic separation technique. Although spots were visualized readily on the TLC plates, the actual compounds remain to be characterized and identified. In the future, fractionation of compounds by HPLC and GC in association with MS would facilitate identification. The use of split columns would allow the collection of samples which could then be evaluated for their efficacy against Xac *in vitro*. Only after robust evaluation in greenhouse and field trials could a commercial product be developed.

CONCLUSION

Plants are important sources of antibacterial compounds. Eighteen plant extracts from this study had a diverse range of metabolites when evaluated using thin layer chromatography. A crude leaf extract from *Goniothalamus calvicarpa* provided promising results when tested against Xac using the paper disc diffusion method. Further study is required to identify the activity and effectiveness of antibacterial compounds.

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