ISOLATION AND CHARACTERIZATION OF *XANTHOMONAS AXONOPODIS* CAUSING CANKER DISEASE OF LIME TREE AND EVALUATION OF ABILITY OF EXTRACTION FROM STEM OF *EUPHORBIA TIRUCALLI* AGAINST *X. AXONOPODIS*

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Received: 19 June 2018; Accepted for publication: 30 August 2018

ABSTRACT

Bacterial citrus canker is one of the major causes of yield losses in citrus growing areas of the world. *Xanthomonas axonopodis* is the cause of this disease on lime tree which badly affects the plant health and fruit quality. In this study, 25 samples of lime tree canker were collected from Ben Luc district, Long An province in Vietnam. The bacteria were isolated from infected samples and identified base on the biochemical characteristics such as Gram staining, starch hydrolysis, Tween 80 hydrolysis, gelatin liquefaction, KOH test, Kovacs' oxidase and catalase test. The results showed that canker disease of lime tree caused by *X. axonopodis*. Furthermore, aqueous, ethanol, methanol, acetone and diethyl ether extracts from stem of *Euphorbia tirucalli* were used to evaluate antagonistic ability against *X. axonopodis* using agar well diffusion method. The results revealed that methanol stem extract showed highest antibacterial activity with zone of inhibition 25.67 ± 0.47 mm after 48 hours of incubation. The minimal inhibitory concentration for the methanol extract was 8-32 mg/mL for all of the bacteria. The diethyl ether extract did not show activity against the tested pathogenic bacteria. To our knowledge, this study was the basis for the application of plant extracts to manage lime tree canker and disease of other crops cause by *X. axonopodis*.

Keywords: Euphorbia tirucalli, canker disease, antibacterial activity, methanol extract.

1. INTRODUCTION

Citrus canker is an economically important disease in many tropical and subtropical countries. Several pathotypes have been described within the genus Xanthomonas primarily distinguished by their geographical origin and host range in addition to certain genotypic characteristics [1]. This disease occurs commonly in citrus growing regions. Infection causes lesions on the leaves, stem and fruit of citrus trees, including lime, orange and grapefruit. The primary symptoms of citrus canker are leaf and twig – spotting. Secondary rotting organisms invade lesions, causing fruit to rot. Owing to technological evolution, chemical substance is a necessary part in agricultural production processes. The use of chemicals is the best strategy for preventing pre and post-harvest crop losses caused by insect pests and diseases, but due to their residual toxicity, excessive use of pesticides are causing very serious health hazardous effects on human being, animal life and the whole environment. To avoid or reduce the deleterious effects of synthetic pesticides on ecosystem or the

environment, it is very necessary to find out alternative approaches for the management of plant pathogenic microorganism. The medicinal plant extracts is one of the interesting ways to substitute chemical substances for inhibition of plant pathogens which does not render wicked problems to environment and living being in the world. In the recent years antimicrobial properties of medicinal plants are being increasingly reported from different parts of the world [2-3].

Euphorbia tirucalli or known as San ho xanh, Xuong ca in Vietnam, belongs to Euphorbiaceae family and it is one of the most important trees known worldwide for its multiple uses. The plant is used to treat gonorrhea, whooping cough, asthma, leprosy, enlargement of spleen, jaundice, tumors and bladder stones. Stem latex is used to treat warts, tooth ache, cough, asthma, ear ache, leprosy, abdominal pain, tumors, rheumatism, skin diseases and intestinal worms. Root is used for colic pains [4]. Various studies indicated that this plant is a valuable source of medicinal compounds. Previous studies showed that active compound such as alkaloid, tannins and phenols in *E. tirucalli* was contributed their effectiveness in medicinal treatment [5]. This active compounds could inhibit the bacteria growth due to its ability to form complex with extracellular proteins of cell wall and discrupt microbial membrane, or was toxic to microorganisms, inhibit bacteria by inactive microbial adhension, enzymes and cell envelope transport proteins [6]. *E. tirucalli* is a good example, but not much information is available on the plant diseases, especially against phytopathogenic bacteria in Vietnam. Therefore, the objectives of this study was to characterize the causal agent of lime tree canker and subjected to antimicrobial activity by using stem extraction of *E. tirucalli* by methanolic, ethanolic, acetone, diethyl ether and aqueous which was the basis for the application of plant extracts to manage the lime tree canker and disease of other crops with similar pathogens.

2. MATERIALS AND METHODS

2.1. Samples collection and bacterial isolation

In this study, a total of 25 samples including leaves and fruits showing typical symptoms of lime canker were collected from Ben Luc district, Long An province of Vietnam. The bacteria were isolated by tissue culture method. The lesions and surrounding health tissues were cut into small pieces and washed in tap water before surface sterilizing in 1% sodium hydrochloride for 2-3 minutes. Following the pieces were immersed in 70% ethanol. These pieces were placed on nutrient agar (NA) media plates and incubated for 24 hours at 30 °C [7].

2.2. Morphological and biochemical tests

Colony morphology: The *X. axonopodis* colonies grown on yeast extract dextrose chloramphenicol agar (YDC) were yellow, mucoid, convex and differed in colony size (1-3 mm).

Gram staining: Gram's staining was performed to determine the size, shape, arrangement and Gram reaction of the isolates [8].

Catalase test: Catalase test was used to identify organisms that produce the enzyme catalase. A single colony was taken on a clean slide and hydrogen per oxide was added, smeared carefully. The catalase production was determined by adding the H_2O_2 (3% v/v) to a bacterial culture and the presence of catalase indicated by bubbles of free oxygen gas [9].

Starch hydrolysis test: Two gram rice starch enriched NA media was autoclaved and pured into petriplates. After cooling each isolate was transferred into media and incubate at

27 ºC for seven days. The plates were dispensed with 3% Lugol's iodine after scraping and without scraping the *X. axonopodis* culture on the media [10].

Tween 80 hydrolysis test: X. axonopodis culture was subjected to Tween 80 hydrolysis test [10]. 5 g NaCl, 0,1 g $CaCl_2.2H_2O$, 10 g peptone and 16 g agar to distilled water (1 L) with pH 7.4. Tween 80 was added to the molten media. The media was poured into petri plates. Each isolate was streaked on a medium. The culture was incubated at 27 ºC for seven days to observe opaque milky precipitate/milky crystal formation.

Kovacs' oxidase test: A drop of 1% Kovacs' reagent (1 g Tetramethyl-p-phenylenediamine dihydrochloride in100 mL distilled water) was placed on the center of Whatman filter paper No.1 and platinum loop full of *X. axonopodis* inoculum was gently rubbed on the filter paper. Positive control was also maintained [8].

Gelatin liquefaction test: Beef extract (3 g), peptone (5 g) and gelatin (120 g) in 1 L of water was poured into the test tubes 5 mL/test tube- plugged and autoclaved. The 24 hours old culture of each isolate was stab inoculated and incubated at 27 ºC. After 72 hours, tubes were placed at 40 ºC for 30 minutes prior to record the results. The same procedure was followed after 7, 14 and 21 days [11].

Milk proteolysis: Reactions were observed in reconstituted powdered skim milk containing 0.004% bromocresol purple (w/v) and sterilized for 30 minutes, was mixed at 48 ºC with sterile melted yeast extract nutrient agar (YNA) to obtain a 10% v/v concentration and poured over the surface of a thin layer of NA in petri plates. The plates should be dried, spot inoculated, and observed for a clear zone around the colonies after 3, 5, and 7 days [12].

KOH test: X. axonopodis aseptically removed from the agar medium with a toothpick placed on a glass slide into a drop of 3% KOH, and stirred for 10 seconds using a quick circular motion [13].

2.3. Preparation of *Euphorbia tirucalli* stem extract

Fresh stems of *E. tirucalli* were collected from Ho Chi Minh city, in Vietnam. The stem was thoroughly washed under running tap water, air-dried and subsequently dried in hot air oven at 60 ºC for 72 hours. Dried stems of *E. tirucalli* were homogenized to a fine powder. 100 g of the powered stem was added with 400 mL methanol (75%), ethanol (75%), acetone (75%), ethyl acetate (75%) and aqueous separately at 37 ºC for 48 hours [14]. The resulting extracts were filtered using filter paper (Whatman No.1, USA) and each filtrate was concentrated with a rotary evaporator (Heidolph, Germany) at 60 ºC under vacuum condition. All extracts were kept at 4 ºC in airtight in bottles for further studies.

2.4. Agar well diffusion method

The assay for antibacterial activity of extracts was tested by agar diffusion method according to Toda *et al*. (1989) with some modifications [15]. Bacterial suspensions were cultured in peptone water for 18-20 hours (10^7 CFU/mL approximately) and 0.1 mL of this culture was spread on NA plates. Wells of 5.0 mm diameter were punched in to the agar medium and were filled 60 µL of extracts. Stem *E. tirucalli* extracts were dissolved in sterilized dimethyl sulfoxide (DMSO). Commercially Penicillin G (16 mg/mL concentration) was used as positive control while DMSO was taken as the negative control. These plates were allowed to stand for 5 minutes for the diffusion of extract to take place. The plates were then incubated at 37 ºC for 48 hours. Antibacterial activity was evaluated by measuring the

zones of inhibition (clear zone around each well) in millimeter (mm). Each combination of isolates and antimicrobial agent was repeated three times.

2.5. Minimum inhibitory concentration

Minimum inhibitory concentration (MIC) was determined by using the modified agar-well diffusion method where methanol, ethanol, acetone and aqueous extracts of *E. tirucalli* stem concentration ranged from 8 to 128 mg/mL [16]. MIC was determined as the lowest concentration that showed a clear zone of inhibition after incubation for 48 hours at 30 ±1 ºC.

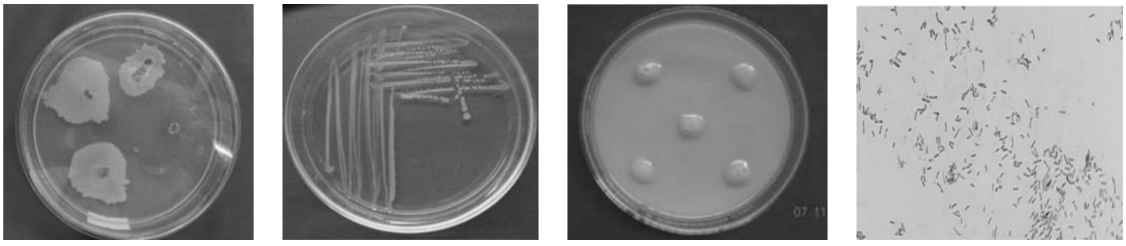
2.6. Statistical analysis

All experiments in the present study were carried out in triplicates and the results indicate their mean values. For statistical analysis, the standard errors of the means were calculated and the means with a significant difference ($p < 0.05$) were compared using Duncan multiple range test in Statgraphics Centurion XV.

3. RESULTS AND DISCUSION

3.1. Isolation and identification of bacteria

The samples of infected leaves and fruits were placed on Nutrient Agar plates. The bacterial colonies appeared after 48 hours of incubation at 30 ºC (Fig. 1A, B) and followed by inoculation on YDC media plates. The visual observation was identified the colony morphology of bacterium *X. axonopodis*. The *X. axonopodis* colonies grown on YDC were yellow and mucoid (Fig. 1C).



A B C D

Figure 1. Leaf samples on NA plant (A), pure colony of *X. axonopodis* on NA plate (B) and YDC media plate (C). After Gram staining pinkish colony under micro scope (D).

3.2. Biochemical characterization of *X. axonopodis*

Citrus canker is an alarming hazardous threat to citrus fruits and for the citrus producing economy. In our study canker infected leaves and fruits samples from lime tree were collected and *X. axonopodis* was isolated and purified. A total of 25 isolates of *X. axonopodis* were compared for their biochemical characteristics (Table 1).

All the isolates were gram-negative as well as small size, rod shaped and pink color (Fig.1D), oxidase-negative, catalase and KOH positive. They were able to gelatin, Tween 80 and starch. In the present study, almost isolates were found to be milk proteolysis negative except four isolates (B1, B2, B3 and B23). All the isolates showed similar biochemical characteristics properties which those described previously for Asiatic form of *X. axonopodis* pv. *citri* [17, 18]. Besides, Vernière *et al*. (1998) reported that citrus canker pathotype A gave

positive results on the three tests (hydrolysis of gelatin and casein, the growth on 3% NaCl), while pathotype B had negative results for these tests. Pathotype C gave a positive result only on hydrolysis of casein (milk proteolysis) [19]. From the present study it was concluded that isolates collected belongs to those of *X. axonopodis* and these biochemical tests can be effectively help in deciding effective management practices for this devastating pathogen.

Table 1. Physiological and biochemical tests for identification of bacterial isolates causing canker disease

Samples code	Gram	Catalase	KOH	Gel	Kovac's	Tween 80	Starch	Protein
B1	-	+	+	+	-	+	+	+
B2	-	+	+	+	-	+	+	+
B3	-	+	+	+		+	+	+
B4	-	+	+	+	-	+	+	-
B5	-	+	+	+	-	+	+	-
B6	-	+	+	+	-	+	+	-
B7	-	+	+	+	-	+	+	-
B8	-	+	+	+	-	+	+	-
B9	-	+	+	+	-	+	+	-
B10	-	+	+	+	-	+	+	-
B11	-	+	+	+	-	+	+	-
B12	-	+	+	+	-	+	+	-
B13	-	+	+	+	-	+	+	-
B14	-	+	+	+	-	+	+	-
B15	-	+	+	+	-	+	+	-
B16	-	+	+	+	-	+	+	-
B17	-	+	+	+	-	+	+	-
B18	-	+	+	+	-	+	+	-
B19	-	+	+	+	-	+	+	-
B20	-	+	+	+	-	+	+	-
B21	-	+	+	+	-	+	+	-
B22	-	+	+	+	-	+	+	-
B23	-	+	+	+	-	+	+	+
B24	-	+	+	+	-	+	+	-
B25	-	+	+	+	-	+	+	-

3.3. Antibacterial activity of stem *E. tirucalli* extracts

In recent years, herbs have become effective means of treatment for recovery of various diseases for the prevention and control of the microbial diseases received increasing attention as alternative treatment of chemotherapeutics. In the present study, stem of *E. tirucalli* was extracted in four organic solvents (methanol, ethanol, acetone, diethyl ether)

and aqueous. The extracts were tested for antibacterial activity against isolated *X. axopodis*. The results revealed that methanol and ethanol extracts were found to be effective against test organisms as compared to other extracts, followed by aqueous and acetone extracts (Table 2).

Table 2. Inhibitory activity of stem extracts of *E. tirucalli*

Bacteria	Zone of inhibition in mm					
	Aqueous	Methanol	Ethanol	Acetone	Ethyl acetate	Control Antibiotic
B1	11.67 ± 1.25^{a}	19.00 ± 0.82^{b}	17.00 ± 0.00^{b}	11.33 ± 1.25^{b}	-	16.67 ± 0.94^{c}
B2	12.67 ± 1.25a	14.00 ± 0.82^{b}	14.70 ± 0.94^{b}	10.67 ± 0.94^{a}	-	17.00 ± 0.00^{c}
B3	12.00 ± 0.82^{a}	17.00 ± 0.00^{b}	16.30 ± 0.47^{b}	11.00 ± 0.00^{c}	-	18.33 ± 0.47^{b}
B4	11.00 ± 0.82^{a}	14.67 ± 0.47^{b}	4.67 ± 0.94^{b}	9.00 ± 0.82^{a}	-	17.33 ± 0.47^{c}
B5	11.00 ± 0.00^{a}	17.00 ± 0.00^{b}	16.67 ± 0.94^{b}	10.33 ± 1.25^{a}	-	18.33 ± 0.47^{b}
B6	12.00 ± 0.82^{a}	16.33 ± 0.94^{b}	16.00 ± 0.00^{b}	11.00 ± 0.82^{a}	-	18.67 ± 0.47^{c}
B7	11.33 ± 0.94^{a}	14.00 ± 0.82^{b}	13.67 ± 0.47^{b}	8.67 ± 0.94^{c}	-	21.00 ± 0.82^{d}
B8	11.67 ± 0.94^{a}	14.33 ± 0.94^{b}	14.67 ± 0.94^{b}	11.33 ± 0.47^{a}	-	11.67 ± 0.47^{b}
B9	12.33 ± 1.25^{a}	16.00 ± 0.00^{b}	15.67 ± 0.94^{b}	11.00 ± 0.82^{a}	-	18.00 ± 0.00^{c}
B10	15.00 ± 0.82^{a}	13.67 ± 0.94^{a}	14.33 ± 0.47^{a}	10.00 ± 0.82^{b}	-	9.33 ± 0.47^{b}
B11	13.67 ± 0.47^{a}	17.67 ± 0.47^{b}	16.67 ± 0.94^{b}	13.00 ± 0.82^{a}	-	15.00 ± 0.00^{c}
B12	11.00 ± 0.82^{a}	13.00 ± 0.82^{b}	12.33 ± 0.94^{a}	10.33 ± 0.47^{a}	-	11.33 ± 0.47^{a}
B13	15.00 ± 0.00^{a}	15.33 ± 0.47^{a}	15.33 ± 1.25^{a}	12.33 ± 0.94^{b}	-	16.67 ± 0.47^{a}
B14	14.00 ± 0.00^{a}	17.33 ± 0.47^{b}	16.33 ± 1.25^{b}	12.67 ± 0.47^{a}	-	18.00 ± 0.82^{b}
B15	16.33 ± 1.25^{a}	20.33 ± 0.47^{b}	18.33 ± 0.47^{ab}	11.33 ± 1.25^{c}	-	23.70 ± 0.94^{d}
B16	15.33 ± 1.25^{a}	22.00 ± 0.82^{b}	20.00 ± 0.00^{b}	14.00 ± 0.82^{a}	-	22.00 ± 0.00^{b}
B17	11.33 ± 0.94^{a}	16.33 ± 0.94^{b}	16.67 ± 0.94^{b}	11.00 ± 0.82^{a}	-	19.33 ± 0.47^{c}
B18	12.00 ± 0.00^{a}	15.67 ± 1.25^{b}	17.33 ± 0.47^{b}	10.67 ± 0.47^{a}	-	17.00 ± 0.82^{b}
B19	15.00 ± 0.82^{a}	15.67 ± 1.25^{a}	15.67 ± 0.47^{a}	13.00 ± 0.82^{b}	-	17.67 ± 0.94^{c}
B20	12.33 ± 0.94^{a}	17.00 ± 0.82^{c}	15.00 ± 0.82^{b}	11.00 ± 0.00^{a}	-	17.67 ± 1.25^{c}
B21	16.67 ± 0.47^{a}	25.67 ± 0.47^{b}	24.67 ± 0.47^{b}	12.33 ± 0.47^{c}	-	28.33 ± 1.25^{d}
B22	12.00 ± 0.82^{a}	19.33 ± 0.94^{b}	19.33 ± 0.47^{b}	12.00 ± 0.00^{a}	-	15.00 ± 0.82^{c}
B23	15.00 ± 0.82^{a}	16.33 ± 0.94^{a}	15.67 ± 1.25^{a}	13.00 ± 0.82^{b}	-	18.33 ± 0.94^{c}
B24	8.33 ± 0.47^{a}	15.33 ± 0.47^{b}	14.00 ± 0.00^{b}	8.00 ± 0.00^{a}	-	18.33 ± 0.47^{c}
B25	10.00 ± 0.00^{a}	15.00 ± 0.00^{b}	14.33 ± 0.47^{b}	10.33 ± 0.47^{a}	-	17.33 ± 0.47^{c}

** Values in the same row with different superscript letters were significantly different at $p < 0.05$ (mean ± SD, n = 3)*

The methanol and ethanol extracts could inhibit all tested pathogenic bacteria with inhibition zones ranging from 13 ± 0.82 mm to 25.67 ± 0.47 mm and 12.33 ± 0.94 mm to 24.67 ± 0.47 mm, respectively. There was not a statistically significant difference in antibacterial activity of methanol and ethanol extracts ($p < 0.05$). MIC value of methanol and ethanol extracts was determined 8-32 mg/mL against the tested microorganisms (Table 3). Meanwhile, the diethyl ether extract did not show activity against the tested pathogenic bacteria. The lowest antibacterial activity and MIC were observed for acetone extract with inhibition zones ranging from 8 ± 0.05 mm to 14 ± 0.08 mm and 32-128 mg/mL. In this study, *E. tirucalli* extracts exhibited low antibacterial activity as compared to control antibiotic for most the tested pathogen bacteria except *X. axopodis* B10, probably due to the difference in the bactericidal mechanism. With *X. axopodis* B10, inhibition zones and MIC of control antibiotic was observed for 9.33 ± 0.47 mm and 128 mg/mL, respectively. Among the isolated *X. axopodis*, *X. axopodis* B21 was highly susceptible with all of extracts followed by *X. axopodis* B16 and *X. axopodis* B15, respectively.

Table 3. The MIC values of stem *E. tirucalli* extracts to microdilution assay

Bacteria	MIC (mg/mL)				
	Aqueous	Methanol	Ethanol	Acetone	Penicillin G
B1	128	8	16	64	16
B2	128	32	32	128	16
B3	64	8	8	64	8
B4	64	32	32	128	8
B5	128	16	16	128	8
B6	64	16	16	64	8
B7	64	32	32	128	8
B8	64	16	16	64	64
B9	64	16	16	128	8
B10	16	16	32	128	128
B11	32	8	8	32	16
B12	64	16	6	64	64
B13	16	6	8	32	8
B14	16	8	8	32	8
B15	8	8	8	32	8
B16	16	8	8	32	8
B17	16	8	8	16	8
B18	32	16	16	64	16
B19	16	16	16	64	16
B20	64	16	16	128	8
B21	32	8	8	64	8
B22	64	8	8	64	8
B23	16	16	16	64	8
B24	128	32	32	128	16
B25	64	16	16	64	16

This result is similar with a report of Jadhav *et al*. (2010) that antibacterial activity of ethanol and methanol extracts with *X. citri* was higher than aqueous extract [14]. According to Sultan *et al.* (2016), there was a difference in antibacterial activity for difference extracts of *E. tirucalli* which could be due to the biologically active phytochemical constituents [20]. These phytochemical constituents screened namely alkaloids, tannins and steroids were reported the in the present study through methanol and aqueous extracts of *E. tirucalli* Linn. Other researches showed that the presence of various phytochemicals such as alkaloids, tannins, polyphenol and triterpenes in *E. tirucalli* stems extracts which possess antimicrobial property may contribute to the formation of inhibition zone [5]. They also found alkaloid has been reported able to inhibit nucleic acid synthesis of bacteria, whereas the tannins able to give toxic to bacteria by increased their hydroxylation proses [21, 22]. Besides, polyphenol disturbs the growth of bacteria by inhibition of c-di-AMP that controls various functions in bacteria [6]. The present study is an important step in developing plant based pesticides which are ecofriendly for the management of the plant pathogenic bacteria and development of commercial formulation of botanicals.

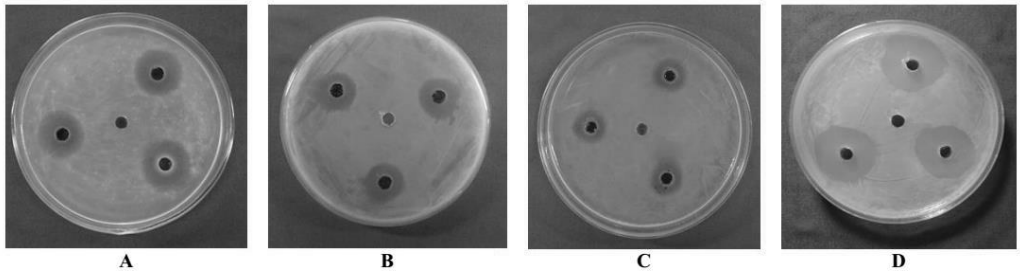


Figure 2. In vitro evaluation of stem *Euphorbia tirucalli* extracts through disc diffusion method. A: Methanol extract; B: Ethanol extract, C: acetone extract and D: control antibiotic**.**

4. CONCLUSION

Morphological and biochemical characteristic viz., shape, colony colour, Gram staining, starch hydrolysis, Tween 80 hydrolysis, gelatin liquefaction, KOH test, Kovacs' oxidase and catalase test were performed and confirmed to identify the bacterium as *X. axonopodis*. From this study, it can be concluded that stem extract of *E. tirucalli* could be an effective measure for controlling plant diseases like lime canker.

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TÓM TẮT

PHÂN LẬP, ĐẶC ĐIỂM CỦA TÁC NHÂN GÂY BỆNH LOÉT TRÊN CÂY CHANH VÀ ĐÁNH GIÁ HOẠT TÍNH ỨC CHẾ CỦA DỊCH CHIẾT CÂY *EUPHORBIA TIRUCALLI*

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Các mẫu bệnh loét từ cây chanh được phân lập và định danh vi khuẩn bằng các đặc tính sinh hóa như: nhuộm Gram, thủy giải tinh bột, thủy giải Tween 80, hóa lỏng gelatin, thử nghiệm KOH, Kovacs' Oxidase. Kết quả cho thấy, *X. axonopodis* là vi khuẩn gây bệnh. Chiết xuất nước, ethanol, methanol, axeton và dietyl ete từ thân cây *E. tirucalli* được sử dụng để đánh giá hoạt tính ức chế vi khuẩn *X. axonopodis* thông qua phương pháp khuếch tán thạch. Kết quả cho thấy, chiết xuất methanol cho thấy hoạt tính kháng khuẩn cao nhất với vùng ức chế 25,67 ± 0,47 mm sau khi ủ 48 giờ. Nồng độ ức chế tối thiểu của chiết xuất methanol là 8-32 mg/mL cho tất cả các vi khuẩn. Chiết xuất dietyl ete không cho thấy hoạt tính ức chế vi khuẩn gây bệnh được thử nghiệm. Kết quả nghiên cứu là cơ sở cho việc áp dụng các chiết xuất thực vật để quản lý bệnh hại cây có múi và các loại cây trồng khác có tác nhân gây bệnh tương tự *X. axonopodis* trong tương lai.

Từ khóa: Euphorbia tirucalli, bệnh loét, hoạt tính kháng khuẩn, chiết xuất methanol.