

THE APPRAISAL OF ANTIOXIDANT PROPERTIES OF *Citrus hystrix* LEAF EXTRACTS AND THEIR ANTIBACTERIAL ACTIVITIES AGAINST TWO PLANT PATHOGEN BACTERIA

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Abstract

The *Citrus hystrix* tree, known as the kaffir lime, is a small tropical evergreen plant with a strong citrus scent and a wide range of bioactivities. This study aimed to determine the antioxidant properties of *C. hystrix* leaf extracts and their antibacterial activity against two plant pathogenic bacteria, namely *Erwinia chrysanthemi* and *Erwinia carotovora*. The antioxidant activity was evaluated through a DPPH dot blot assay and DPPH radical scavenging activity with 2,2-diphenyl-1-picrylhydrazyl (DPPH) serving as the radical. The Kirby-Bauer disc diffusion method was selected to evaluate antibacterial properties. The leaves of *C. hystrix* were consecutively soaked in hexane, ethyl acetate, and methanol to obtain crude extracts. Based on the DPPH dot blot assay, the lowest concentration of the extract that started to scavenge the DPPH radical was at 0.012 mg/mL. According to the DPPH radical scavenging method, all extracts showed promising antioxidant activity in a concentration-dependent manner. The ethyl acetate extract exhibited the highest percentage activity at 83%. The IC₅₀ value of 125.732 µg/mL was recorded for the ethyl acetate extract of *C. hystrix* leaves, which led to promising antioxidant activity to scavenge 50% DPPH radicals. In antibacterial activity, *E. carotovora* was more susceptible towards all extracts compared to *E. chrysanthemi*. The ethyl acetate extract was found to be the most active antibacterial agent with an inhibition zone of 20.67 mm. Therefore, *C. hystrix* can be regarded as a prospective source of both antioxidants and antibacterial agents, which is crucial as the foundation for scientific data used in the pharmaceutical industry, as well as a plant-based antibacterial agent in the agricultural industrial sector.

Keywords: Antibacterial activity, antioxidant activity, *citrus hystrix*, plant pathogen bacteria

Introduction

Citrus hystrix, which belongs to the family of Rutaceae, is indigenous to all areas of Southeast Asia and India. The glossy, fragrant, double (two-parted) evergreen leaves of this tree are often shredded and used as a flavouring in a range of Southeast Asian cuisine dishes but unfortunately lack use in bioactivity studies. Various phytochemical compounds have been detected in the leaves of *C. hystrix*. Terpenoids are the primary compounds found in the plant's leaves, as well as phytosterols and flavonoids which possess high antioxidants (Suttisansanee *et al.*, 2021; Palaogiannis *et al.*, 2023).

An antioxidant is a chemical that helps protect body cells from damage by free radicals. Free radicals are unstable molecules produced during regular cell metabolism, such as chemical processes that occur inside the cell. They may accumulate in cells, causing harm to other molecules. This damage might raise the risk of cancer and other disorders. Studies have shown that methanolic extracts from *C. hystrix* leaves, like those from kaffir lime leaves, are very effective at getting rid of free radicals. This effect is attributed to the high concentration of flavonoids, including quercetin and kaempferol, as well as other phenolic components, that are present in this extract. It has been reported that quercetin, rhamnetin, and isorhamnetin may boost in vivo antioxidant activity while decreasing blood and liver cholesterol levels (Anggraeny *et al.*, 2024).

Plant pathogenic bacteria contribute significantly to agricultural losses worldwide by reducing crop quality and yield. Bacterial soft rot disease has become a tremendous epidemic affecting crops and plants in tropical and subtropical regions. It was reported that the disease caused by *E. coratovora* on potatoes led to a greater total loss of production, up to 30% of the harvested crops (Bhat *et al.*, 2010; Viswanath *et al.*, 2018). The use of synthetic substances to control the disease is not considered as safe regarding human health, as they will build up health hazards. Therefore, this study was undertaken to identify alternative natural extracts to control soft rot disease caused by *E. coratovora* and *E. chrysanthemi*, as well as antioxidant agent from natural sources.

Material and Methods

Plant Collection and Extraction

The leaves of *C. hystrix* were collected from supplier's backyard at Rantau Perintis Jengka, Pahang, Malaysia. The leaves were dried at room temperature in the UiTM Cawangan Pahang chemistry laboratory for two weeks and later were ground into small pieces. The ground leaves (200 g) were soaked consecutively for three weeks using organic solvents such as hexane, ethyl acetate (EA), and methanol in a ratio of 1:3 (sample: solvent) and followed by filtration using Whatman filter paper No. 1. All the filtrates were subjected to evaporation using a rotary evaporator at 40°C to yield three types of crude extracts (Rosli *et al.*, 2025). After evaporation, all extracts were transferred into small containers and kept in refrigerator prior to use.

Phytochemical Screening Test

The phytochemical test was conducted by means of screening some important phytochemicals in each extract of *C. hystrix* leaves. All phytochemical experiments were

modified from the previous studies reported by Rosli *et al.*, (2025).

Alkaloid Test

About 1.0 mL of 1% (v/v) hydrochloric acid (HCl) was added to 3.0 mL of extract. After 20 min of heating, the mixture was cooled and filtered. A white creamy precipitate indicated the presence of an alkaloid after adding two drops of Mayer reagent.

Tannin Test

About 1.0 mL of 10% (w/v) ethanolic potassium hydroxide (KOH) was added to 1.0 mL of extract. A cloudy white precipitate indicated the presence of tannin.

Saponin Test

About 2.0 mL of the extract was shaken vigorously with 2.0 mL of water for 2 min before being warmed. The stable formation of white froth indicated the presence of saponin.

Phenolic Test

Two drops of 5% (w/v) FeCl_3 were added to 1.0 mL of extract. A greenish precipitate indicated the presence of phenolic.

Flavonoid Test

About 1.0 mL of 10% (w/v) sodium hydroxide (NaOH) was added to 3.0 mL extract. A yellow coloration indicated the presence of flavonoids.

Steroid Test

Five drops of concentrated H_2SO_4 were added to 1.0 mL of extract. Red coloration indicated the presence of steroids.

Terpenoid Test

About 0.5 mL of chloroform and a few drops of concentrated H_2SO_4 were added to 1.0 mL of extract. A reddish-brown precipitate indicated the presence of terpenoids.

Antioxidant Screening and Dot Blot Assay

0.6% (w/v) DPPH solution was prepared by dissolving 0.6 g in 100 mL of methanol. This DPPH reagent was sprayed on the developed thin-layer chromatography of each extract to screen the presence of antioxidants. The appearance of a yellow color against a purple background indicated the presence of antioxidants.

A series of concentrations ranging from 400 mg/mL to 0.012 mg/mL was prepared from each type of extract. Then, about 5 μL of each extract concentration was dropped onto a square block of a TLC plate with a 1.5×1.5 cm dimension. After the drying process, the TLC plates were sprayed with 0.05% (w/v) DPPH solution. The appearance of a yellow color on the square block of the TLC plate against a purple background indicated the presence of antioxidant properties of the extracts. The first yellow color appeared on the block of the TLC

plate, indicating the lowest concentration of that extract scavenged the DPPH radical. The method was repeated in triplicate (Rosli *et al.*, 2025).

Quantitative Radical Scavenging Activity

All types of extracts were subjected to a twofold dilution process ranging from 400 µg/mL to 12.5 µg/mL. About 1.0 mL of each concentration was mixed with 3.0 mL of 0.004% DPPH solution. The mixture was gently stirred and stored in a dark place for 30 mins. After 30 mins, the absorbances of all mixtures and standard ascorbic acid were recorded at 517 nm using a UV/Vis spectrophotometer. The percentages of radical scavenging activity of the extracts were calculated as reported (Rosli *et al.*, 2025). The percentage data indicated the trend of antioxidant properties with the change of concentration. The absorbance data and the percentage values were used to plot the graph to determine the IC₅₀ of each extract. The lower the IC₅₀, the better the extract acts as an antioxidant agent (Aryal *et al.*, 2019).

Antibacterial Activity Assay against Plant Pathogen Bacteria

Disc Diffusion Method

The cultured bacteria of *E. chrysanthemi* and *E. carotovora*, which were previously standardized with optical density (OD) 0.12 at 480 nm, were aseptically swabbed onto the solidified agar surface to initiate the bacterial growth. The impregnated disc of each extract at different concentrations ranging from 800 mg/mL, 400 mg/mL, 200 mg/mL, 100 mg/mL, and 50 mg/mL was aseptically and properly placed on the inoculated agar and incubated at room temperature for 24 hours. The experiment was done in triplicate and was repeated for positive standard ampicillin, negative control, and solvents such as hexane, ethyl acetate, and methanol. The diameter of the inhibition zone (in mm) was measured to evaluate the extract's effectiveness against the bacteria. The greater the zone, the more effective the extracts acting as antibacterial agents (Harun *et al.*, 2023).

Results and Discussion

Table 1 Phytochemical Screening of Leaf Extracts of *C. hystrix*

Phytochemicals	Type of extracts		
	Hexane#	Ethyl Acetate*	MeOH*
Alkaloid	√	x	x
Tannin	x	x	√
Saponin	√	x	√
Phenolic	x	√	x
Flavonoid	√	x	√
Steroid	√	x	x
Terpenoid	√	√	x

√=present; x = absent; * = antioxidative extracts after spraying with 0.6% DPPH solution; # =

not antioxidative after spraying with 0.6% DPPH solution

According to Table 1, all phytochemicals appeared in the hexane extract except tannins and phenolics. The ethyl acetate extract only contained phenolics and terpenoids. The methanol extract was screened to contain only tannin, saponin, and flavonoid. This demonstrates that extracts of varying polarities yield different types of phytochemicals, meaning that extract polarity influences the presence of phytochemicals. Generally, a more polar extract will contain a greater number of polar phytochemicals, and vice versa. All phytochemicals found in all extracts might contribute to their antioxidant and antibacterial properties of *C. hystrix* extracts. This study aligns with previous research in which antioxidant activity (Rosli *et al.*, 2025) and antibacterial activities (Harun *et al.*, 2023) are influenced by the presence of specific phytochemicals.

Antioxidant Activity

Semiquantitative DPPH Dot Blot Assay

This technique is the fastest way to determine the lowest concentration of extract to scavenge the DPPH radical by observing the first yellow color that appears on the square block of the TLC sheet. Table 2 illustrates the lowest concentration of each extract to scavenge the DPPH radical. Generally, all extracts scavenged DPPH radicals at lower concentrations. However, hexane extract and ethyl acetate extract started to scavenge DPPH radicals at concentration 0.012 mg/mL, whereas methanol extract did at a concentration of 1.56 mg/mL. These screening results indicated that hexane extract and ethyl acetate extract are more effective for scavenging the process of DPPH radicals compared to methanol extract. The main reason for this event is usually related to the presence of phytochemicals in the extracts. Usually phytochemicals such as phenolic acids, tannin, saponin, and terpenoid are the primary types of phytochemicals responsible for antioxidant activity. Therefore, all extracts have the capability to scavenge DPPH radicals, since they all contain the phytochemicals stated in Table 1. Moreover, all extracts have the potential to be a good antioxidant source since they scavenge DPPH radicals at lower concentrations.

Table 2 Lowest Concentration of *C. hystrix*'s leaf extracts through Dot Blot assay

Extract/standard	Lowest concentration of DPPH Inhibition, mg/mL
Hexane	0.012
Ethyl acetate	0.012
Methanol	1.56
Standard ascorbic acid	0.012

Table 3 Quantitative Antioxidant Activity of *C. hystrix*'s leaf extracts through DPPH Radical Scavenging Method

Extracts/Standard	IC ₅₀ (ug/ml)
Hexane	234.046
Ethyl Acetate	125.732
Methanol	500.222
Ascorbic Acid	104.752

Quantitative DPPH Radical Scavenging Activity

This method was conducted to evaluate the trend of antioxidant activity percentages in relation to changes in extract concentration, as well as to determine the concentration of extract needed to scavenge 50% of the DPPH radical, as indicated by the IC_{50} value. According to Figure 1, generally, radical scavenging activity is in a concentration-dependent manner for all types of extracts. As the concentration of each extract increases, the levels of phytochemicals responsible for antioxidant activity also rise, which in turn enhances the overall antioxidant activity. Among tested extracts, ethyl acetate extracts exhibited higher activity compared to other extracts. This result might be due to the combination of phenolics and terpenoids, which is sufficient to create a synergistic effect that scavenges DPPH radicals. The combination led to more hydroxyl (OH) groups that are covalently attached to the base structure of both phenolic and terpenoid and excessively take part in the reduction activity towards DPPH radicals and, therefore, affect the scavenging activity (Saxena, 2012). The trend of IC_{50} in Table 3 for all extracts was not in a polarity-dependent manner. The ethyl acetate extract, which is considered a medium-polar extract compared to the more polar extract methanol, was more antioxidative with a lower IC_{50} at 125.732 $\mu\text{g/mL}$. The IC_{50} value indicated the ethyl acetate extract scavenged 50% of the DPPH radical at 125.732 $\mu\text{g/mL}$, more effective compared to methanol at 500.222 $\mu\text{g/mL}$ and the hexane extract at 234.046 $\mu\text{g/mL}$. It was also reported that the ethyl acetate extract of *C. vespertilionis* leaves exhibited a lower IC_{50} value compared to hexane and methanol extracts (Rosli *et al.*, 2025). The phenomenon might be attributed to the combination of phenolic and terpenoid, which enhances the suppression of the DPPH radical and increases antioxidant activity. On the other hand, the combination of phytochemicals in hexane extract and methanol extract does not involve more OH groups and is probably not causing a high scavenging effect (Harun *et al.*, 2024; Moncayo *et al.*, 2021).

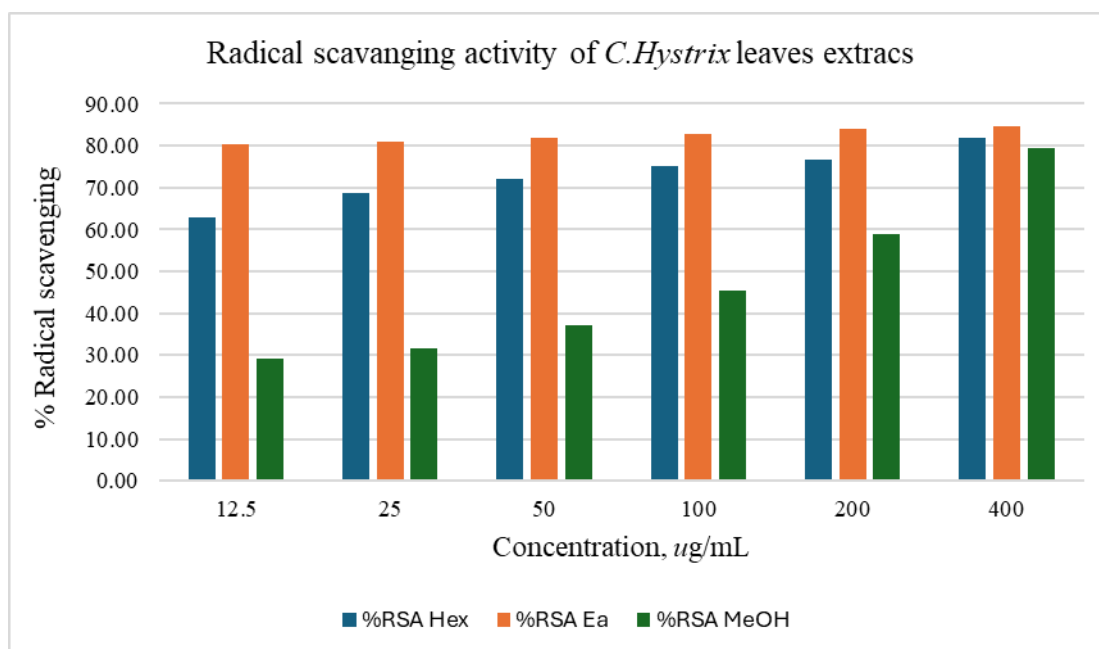


Figure 1 Radical scavenging activity of *C. hystrix* leaves extracts
RSA = Radical scavenging activity

Antibacterial Activity of *Citrus hystrix* Leaf Extracts against *Erwinia chrysanthemi* and *Erwinia caratovora*

Plant pathogenic bacteria contribute significantly to agricultural losses worldwide by reducing crop quality and yield. This study evaluated the antibacterial effectiveness of different solvent extracts of *Citrus hystrix* leaves against plant pathogenic bacteria, *E. chrysanthemi* and *E. caratovora*. The antibacterial activity of hexane, ethyl acetate, and methanol extracts was evaluated using the agar well diffusion assay at concentrations of 50, 100, 200, 400, and 800 mg/mL. All treatments were conducted in triplicate. Data were analyzed using one-way ANOVA followed by the LSD test at $p \leq 0.05$.

Table 4 Mean Inhibition Zone (mm) against *Erwinia chrysanthemi*

Concentration (mg/mL)	Hexane	Ethyl acetate	Methanol
800	9.00 ± 2.00 a	14.33 ± 1.15 a	11.00 ± 4.58 a
400	7.33 ± 0.58 ab	10.00 ± 1.00 b	10.00 ± 2.65 a
200	6.00 ± 0.00 b	7.00 ± 1.00 c	9.67 ± 3.06 a
100	6.00 ± 0.00 b	7.17 ± 1.61 c	6.67 ± 0.58 b
50	6.00 ± 0.00 b	6.00 ± 0.00 c	6.00 ± 0.00 b

Means followed by different letters within the same column are significantly different at $p \leq 0.05$ according to LSD test.

Based on Table 4, among the three extracts tested, ethyl acetate extract exhibited the strongest antibacterial activity against *E. chrysanthemi*. At 800 mg/mL, the ethyl acetate extract produced the highest inhibition zone of 14.33 ± 1.15 mm, which differed significantly compared to other concentrations. The strong antibacterial effect of ethyl acetate extract suggests that semi-polar bioactive compounds were efficiently extracted using this solvent. Ethyl acetate is known to extract phytochemicals such as flavonoids, terpenoids, and phenolic compounds, which possess antibacterial properties through membrane disruption and inhibition of microbial metabolic activity (Cushnie & Lamb, 2011). The result of the ethyl acetate extract demonstrated that higher extract concentrations contain greater amounts of active compounds capable of suppressing bacterial growth. Similar concentration-dependent trends have been reported in previous studies involving medicinal plant extracts (Negi, 2012).

Methanol extract demonstrated moderate antibacterial activity against *E. chrysanthemi*. The inhibition zones ranged from 6.00 ± 0.00 mm to 11.00 ± 4.58 mm. Statistical analysis showed that concentrations between 200 and 800 mg/mL did not differ significantly, indicating that antibacterial activity remained relatively stable at higher methanol concentrations. The antibacterial activity might be associated with polar phytochemicals such as alkaloids, glycosides, and tannins.

Hexane extract showed the lowest antibacterial activity among the extracts tested. Although the inhibition zone reached 9.00 ± 2.00 mm at 800 mg/mL, antibacterial activity decreased substantially at lower concentrations and remained constant at 6.00 mm from 50 to 200 mg/mL. For hexane extracts, 400 mg/mL and 800 mg/mL concentrations did not show any significant differences. Lower concentrations were not significantly different from one another. This finding suggests that non-polar compounds extracted using hexane contribute less effectively to antibacterial action against *E. chrysanthemi*. Similar observations were reported by Dorman and Deans (2000), who found that antibacterial effectiveness depends strongly on solvent polarity and phytochemical composition.

Table 5 Mean Inhibition Zone (mm) against *Erwinia carotovora*

Concentration (mg/mL)	Hexane	Ethyl acetate	Methanol
800	20.00 ± 0.00 a	20.67 ± 4.93 a	15.33 ± 1.53 a
400	16.67 ± 2.89 ab	17.00 ± 3.00 ab	14.33 ± 3.51 a
200	16.00 ± 1.00 ab	15.00 ± 1.00 bc	14.33 ± 1.15 a
100	12.67 ± 1.15 b	14.33 ± 3.06 bc	12.00 ± 2.65 ab
50	13.00 ± 1.73 b	12.67 ± 2.52 c	8.33 ± 4.04 d

Means followed by different letters within the same column are significantly different at $p \leq 0.05$ according to LSD test.

Table 5 presents the antibacterial activity of *Citrus hystrix* leaf extracts against *E. carotovora* at different concentrations. At 800 mg/mL, the ethyl acetate extract produced the highest inhibition zone of 20.67 ± 4.93 mm, followed closely by the hexane extract (20.00 ± 0.00 mm). The results showed that the 800 mg/mL treatment differed significantly from lower concentrations, confirming the strong antibacterial effect at higher extract concentrations. The superior activity of ethyl acetate extract may be associated with the presence of semi-polar phytochemicals such as flavonoids, phenolic compounds, and terpenoids, which are widely reported to possess antimicrobial properties (Cushnie & Lamb, 2011).

Hexane extract also demonstrated relatively strong antibacterial activity against *E. carotovora*, particularly at higher concentrations. The inhibition zones at 800, 400, and 200 mg/mL showed no significant differences among these concentrations. This observation suggests that non-polar compounds extracted using hexane might contribute more effectively against *E. carotovora* compared with *E. chrysanthemi*.

For methanol extract, the inhibition zones remained relatively consistent between 800 and 200 mg/mL, with values ranging from 14.33 to 15.33 mm. The concentrations were not significantly different, indicating relatively stable antibacterial activity at moderate to high concentrations. However, the inhibition zone decreased markedly at 50 mg/mL (8.33 ± 4.04 mm), which differed significantly from all other treatments.

The differences in antibacterial activity among extracts may be attributed to variations in solvent polarity and phytochemical composition. Ethyl acetate is capable of efficiently extracting semi-polar compounds, while methanol extracts more polar compounds, such as alkaloids and tannins. Hexane, as a non-polar solvent, primarily extracts lipophilic compounds. The effectiveness of these phytochemicals against bacterial pathogens depends on their ability to disrupt membrane integrity, inhibit enzymatic activity, and interfere with bacterial metabolism (Burt, 2004).

Overall, the results indicate that *C. hystrix* leaf extracts possess promising antibacterial activity against *E. carotovora*, with ethyl acetate extract showing the greatest potential as a natural antibacterial agent for plant disease control.

Conclusion

The qualitative analysis confirmed that the *C. hystrix* leaf extracts contain alkaloids, tannins, phenolics, terpenoids, and flavonoids. Based on the dot blot assay, the ethyl acetate extract showed the lowest concentration to scavenge the DPPH radical (0.012 mg/mL). The radical scavenging activity revealed that the ethyl acetate extract was the most antioxidative with an

IC₅₀ value of 125.732 µg/mL. The ethyl acetate extract also served as the most active antibacterial agent, inhibiting *E. carotovora* with an inhibition zone of 20.67 mm. This result suggested that there is a strong relationship between phytochemicals present with antioxidant activity and antibacterial activity.

Ethics Statement

The research does not require research ethics approval.

Authors' Contributions

“Writing -methodology and formal analysis of data, Mohd Amran, N.A.S: Literature Review, Daud, S, Baharuddin, W.N, Harith, S; Abdul Aziz, N; Writing – Review and editing, Harun, A.”

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Conflict of Interest Statement

The authors declared no conflicts of interest regarding this research work.

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