# Phytochemical profiles and Antimicrobial activity of *Citrus hystrix* DC. (Kaffir lime) leaves extract against selected bacterial gastrointestinal pathogens

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## ABSTRACT

In the recent decade, search of complementary and alternative plant derived medicine has gained gradual interest among researchers due to side effects of the chemically synthesized drugs and antibiotic resistant bacteria. This study was carried out to determine the phytochemical compounds and antibacterial activity of methanolic leaves extract of *Citrus hystrix* on selected gastrointestinal pathogens (*Staphylococcus aureus*, *Bacillus cereus*, *Salmonella typhimurium* and *Shigella flexneri*) using the agar well diffusion method. The maximum inhibition zone was observed against *B. cereus* (17.33±0.58 mm) followed by *S. aureus* (15.67±0.58 mm). The extract showed moderate antibacterial activity against *S. flexneri* (11.67±0.58 mm) followed by *S. typhimurium* (11.33±0.58 mm). The strongest MIC value was observed against *B. cereus* with concentration 31.25 mg/ml. The extract showed good patterns of inhibition against *S. aureus* and *S. typhimurium* with concentration 125 mg/ml. MIC values were maximized against *S. flexneri* with concentration 250 mg/ml. The most effective bactericidal activity of the extract was observed against *B. cereus* with MBC at 125 mg/ml followed by *S. aureus* and *S. flexneri* with MBC at 250 mg/ml. *S. typhimurium* was the least sensitive to the extract with the MBC value at 500 mg/ml. Phytochemical screenings revealed the presence of alkaloid, flavonoid, terpenoid and tannin compounds.

Keywords: *Citrus hystrix*, gastrointestinal pathogens, MIC, MBC, phytochemical.

## INTRODUCTION

Bacterial gastroenteritis becomes a major global challenge with increased morbidity, mortality and significant public health and social implications (Wei et al. 2017) Bacteria are responsible for around 20 to 40% of diarrheal cases and they are likely to contribute more rapidly in developing regions causing the burden of this disease and increase children mortality (Barrett & Fhogartaigh 2017). Viruses, bacteria and parasites can cause gastroenteritis and bacteria including *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Escherichia coli and Staphylococcus* spp. can produce gastrointestinal infections. Furthermore, the use of antibiotics is the single most important factor leading to the development of resistant bacteria.

*Citrus hystrix* DC, commonly known as kaffir lime belonging to the family *Rutaceae* and genus *Citrus* grows all over India and Southeast regions of Asia, Southern China, Malaysia and Thailand (Arumugam et al. 2014). The plant has thorny bushy appearance about three to six meters tall with aromatic fruits and leaves and locally known as limau purut. The leaves of this plant are strongly aromatic and have a distinctive, wide petiole that makes them appear double lobed with a glassy, dark green sheen to the surface (Ali et al. 2015). *C. hystrix* is used as a key ingredient of cuisines in many countries. In Southeast Asian and Thai dishes, they use one or two fresh leaves as a spice and for various flavouring purpose. In Malaysia and Thailand, the leaves of *C. hystrix* used as a spice in preparation of famous dish, "tom yam".

*C. hystrix* contains a lot of vitamins and act as antioxidant that are important for health benefits. Previous study reported that *C. hystrix* exhibited various pharmaceutical effects such as antimicrobial, anti-inflammatory, anticancer, remedies in analgesic, sedative, spasmolytic and anaestheticn (Sauid & Aswandi 2018). Previous work also reported that the ethanolic extract of *C. hystrix* leaves contain many bioactive compounds and exhibited antimicrobial activity against several serotypes of *Salmonella* spp. and *Enterobacteriaceae* (Utami et al. 2017). This study aimed to evaluate the inhibitory effect of *C. hystrix* leaves against gastrointestinal pathogens and to determine the phytochemical compounds responsible for the inhibitory activity.

## METHODOLOGY

### Collection of Plant Sample

The leaves of plant samples were collected from Kampung Banggol Tok Esah, Manir with coordinates 5°19'15.3"N 103°03'25.0"E while some were bought at Chabang Tiga market in

Kuala Terengganu, Terengganu. The fresh leaves of *C. hystrix* were washed thoroughly and then air dried for 10 days by spreading evenly in the open drying area under the shade. The dried leaves were grinded into fine powder by using electric grinder and then store in air-tight container at 4˚C before extraction process.

**Preparation of Extract**

500g of the leaves powder were soaked in 5000 mL of absolute methanol in the ratio 1:10 with frequent agitation at 100 rpm for 3 days. The schott bottle was covered with aluminium foil to prevent degradation of bioactive compounds in plant sample. After that, the macerate was filtered using Whatman No. 1 filter paper. The filtrate were concentrated using rotary evaporator at 40˚C. The crude extract obtained was stored at 4˚C until further use in phytochemical and antibacterial testing.

**Phytochemical analysis**

The methanol extract of *C. hystrix* leaves were subjected to preliminary screening of phytochemical compounds including alkaloids, flavonoids, terpenoids, tannins and saponins. The screening test was carried out by standard methods.

**Test for Alkaloids**

Two drops of Wagner's reagent were added to 1 mL of plant extract and appearance of reddish-brown precipitate indicates the presence of alkaloids (Madike et al. 2017).

**Test for Flavonoids**

One mL of 10% sodium hydroxide (NaOH) solution were added to 3 mL of plant extract and the formation of an intense yellow color indicates the presence of flavonoids (Madike et al. 2017).

**Test for Terpenoids**

Two mL of chloroform were added to 0.5 mL of plant extract followed by the addition of 3 mL of concentrated sulphuric acid to form a layer. Reddish brown coloration of the interface indicates the presence of terpenoids (Madike et al. 2017).

**Test for Tannins**

Two mL of ferric chloride were added to 1 mL of the plant extract. A dark green colour indicates the presence of tannins (Temitope & Olugbenga 2015).

**Test for Saponins**

Two mL of distilled water were added to 1 mL of the plant extract and the mixture were shaken vigorously and left to stand for 10 minutes. The development of foam on the surface of the mixture more than 10 mm indicates the presence of saponins (Temitope & Olugbenga 2015).

**Bacteria strains**

The antimicrobial activity of *C. hystrix* extract was tested on 4 gastrointestinal pathogens namely *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (ATCC 11778), *Salmonella typhimurium* (ATCC 13311) and *Shigella flexneri* (ATCC 12022). All of the test microorganisms were obtained from stock culture at Microbiology Laboratory, Centre of Medical Laboratory Technology, UiTM Puncak Alam Campus, Selangor, Malaysia.

To prepare the working cultures, the test microorganisms were sub-cultured in 4 mL Mueller-Hinton broth and incubated for 2 to 4 hours at 37˚C to reach the exponential phase. The working cultures were adjusted to 0.5 McFarland standard, in order to get bacterial density equivalent to approximately 1 to 2 X $10^8$ colony-forming units cfu/ml. The working cultures were directly used in antibacterial assay.

**Antibacterial assay**

The determination of antimicrobial activity was performed by using the agar well diffusion method as described by Lingaraju et al. (2016) with minor alterations. Sterile cotton swabs were dipped into prepared bacterial suspension (inoculum grown for 2 to 4 hours at 37˚C in Mueller-Hinton broth adjusted to 0.5 McFarland standard) were inoculated by rubbing over the entire surface of the Mueller-Hinton plate. Wells of 6 mm in diameter were created for each Petri-plate using sterile blue micropipette tips (1000 uL).

A total of 60 µL of *C. hystrix* extract and 10% DMSO served as negative control were added into the wells using sterile micropipettes. Simultaneously, the standard antibiotics (as positive control) are tested against pathogens. The positive control used for *S. aureus* was tetracycline (30ug) while for *B. cereus* was chloramphenicol (30ug). Ampicillin (10ug) was used for both *S. typhimurium* and *S. flexneri*. Plates were incubated at 37˚C for 24 hours. After the incubation period, the inhibition zones including the diameter of the well for each plate was measured in millimeter (mm) and recorded.

The tests were performed in triplicate to ensure the accuracy and reliability of the results.

### Statistical analysis

All analyses were run in triplicate. Data were analyzed by the SPSS package, version 21. Data were expressed as mean ± standard deviation using Independent T-Test. P value < 0.05 were considered statistically significant.

## RESULT AND DISCUSSION

### Plant extraction

The effectiveness of bioactive compounds extraction is greatly dependent on the solvent used during the extraction process. A good solvent's properties including rapid physiologic absorption of the plant extract, preservative action, ability to retain the effectiveness of bioactive compounds extraction (Sivanandham 2015). In the present study, methanol was chosen as the solvent of choice for the extraction of polar biologic active compounds. Methanol solvent can extract many bioactive compounds from plant material as compared to other solvents. In addition, methanol was used to extract active compounds in aromatic plants.

### Phytochemical screening

Investigations of the phytochemical screening of *C. hystrix* methanolic leaves extract revealed the presence of alkaloid, flavonoid, terpenoid and tannin compounds (Table 1). Nevertheless, saponin compound was absence in *C. hystrix* methanolic leaves extract. The findings in the present study were correlated with the earlier study revealed that *C. hystrix* leaves extract exhibited the phytochemical compound including alkaloids, flavonoids and tannins compound (Ali et al. 2015). However, the qualitative screening of saponins compound was not conducted in the previous work.

TABLE 1. Phytochemical compounds tested in *C. hystrix* methanolic leaves extract

| Phytochemical compounds | Methanol extract |
|---|---|
| Alkaloids | + |
| Flavonoids | + |
| Terpenoids | + |
| Tannins | + |
| Saponins | - |

+: Present
-: Absent

### Antibacterial activity

The antibacterial activity of *C. hystrix* methanolic leaves extract against selected enteric pathogens was determined by using agar well diffusion assay. The result of agar well diffusion method showed that the methanolic extract of *C. hystrix* leaves has broad spectrum antibacterial activity against Gram-positive bacteria and Gram-negative bacteria (Table 2). Predominantly, the maximum inhibition zone was observed against *B. cereus* (17.33±0.58 mm) followed by *S. aureus* (15.67±0.58 mm). Meanwhile, the *C. hystrix* methanolic leaves extract showed moderate antibacterial activity against *S. flexneri* (11.67±0.58 mm) followed by *S. typhimurium* (11.33±0.58 mm). The present finding was in agreement with a previous study revealed that the leaves of *C. hystrix* has antimicrobial activity against *S. aureus*, *K. pneumonia*, *E. coli*, *S. typhi* and *P.vulgaris* (Newton et al. 2012). However, methanol extracts of *C. hystrix* leaves was found significant inhibition activities against selected Gram-positive bacteria compared to Gram-negative bacteria with p value < 0.05

Antimicrobial activity of *C. hystrix* methanolic leaves extract more pronounced effect against Gram-positive bacteria as compared to Gram-negative bacteria (Table 2). In a recent study related to the antimicrobial effects of *Psidium guajava* leaves extract also showed significant antagonistic activities against Gram-positive bacteria, but the limited or lack activity against Gram-negative bacteria. The resistance of Gram-negative bacteria to methanolic leaves extract of *C.hystrix* leaves due to their hydrophilic outer membrane and lipopolysaccharide (LPS) content. The cell wall of Gram-negative bacteria contained the outer membrane that is composed of structural lipopolysaccharides to block the penetration of hydrophobic compounds into target cell membrane. Furthermore, Gram-negative bacteria prevent the accumulation of antibacterial agents within cell wall since they contained inherent overexpressed or multiple efflux pumps that expels the antimicrobial drugs before it reached its targets (Valle et al. 2015). In contrast, the absence of outer membrane in Gram-positive bacteria render the cell wall more permeable to any compounds. Gram-positive bacteria causing enhance ion permeability, leakage of vital intracellular components or impairment of the bacterial enzyme systems after the direct contact of the extract compounds with the phospholipid bilayer of the cell membrane (Khanam, et al. 2015).

The comparison between diameter of inhibition zone and positive control showed significant difference against the tested bacteria with p value < 0.05 except for *S. flexneri* (Table 2). The least significant difference was observed at pair 2 (*B. cereus* and chloramphenicol) followed by pair 1 (*S. aureus* and tetracycline) indicated by comparatively lower p value (0.05 and 0.02), respectively. Pair 3 (*S. typhimurium* and ampicillin) showed significant difference with p value was 0. Nevertheless, pair 4 (*S. flexneri* and ampicillin) exhibited negative p value because the standard error of mean was 0. In conclusion, the diameter of inhibition zone of the tested bacteria showed significant difference when compared to their positive control excluding for *S. flexneri*. The findings in the current study were slightly contradicted to the previous work revealed that the inhibition zone of *Citrus* leaves extract against the tested bacteria (*S. aureus* and *B. cereus*) has significant difference when compared to the positive control but others bacteria (*P. vulgaris*, *P. aeruginosa* and *K. pneumonia*) showed no significant difference when compared to the positive control (Abdallah 2016). Factors including different plant species, extraction process, AST method and standard (positive) antibiotic used were contributed to the different result in both studies.

TABLE 2. Diameter of inhibition zone of *C. hystrix* methanolic leaves extract against selected gastrointestinal pathogens using agar well diffusion method.

| Tested | Mean of inhibition zone (mm)* | | | |
|---|---|---|---|---|
| | Gram positive | | Gram negative | |
| | *S. aureus* | *B. cereus* | *S. typhimurium* | *S. flexneri* |
| Extract | 15.67±0.58** | 17.33±0.58** | 11.67±0.58** | 11.33±0.58** |
| Positive control | 38.67±2.31** | 22.0±0** | 24.67±1.53** | 22.67±0.58 |
| Negative control | 0 | 0 | 0 | 0 |

*Inhibition zone is the mean of triplicates ± standard deviation
**Significant result (P<0.05)

TABLE 3. MIC and MBC values of *C.hystrix* methanolic leaves extract against the selected gastrointestinal pathogens

| Organisms | MIC values (mg/ml) | MBC values (mg/ml) |
|---|---|---|
| *S. aureus* | 125 | 250 |
| *B. cereus* | 31.25 | 125 |
| *S. typhimurium* | 125 | 500 |
| *S. flexneri* | 250 | 250 |

The MIC values of the *C. hystrix* methanolic leaves extract against the tested microorganisms were in the range of 31.25 to 250 mg/ml (Table 3). The strongest MIC value for *C. hystrix* methanolic leaves extract was exhibited against *B. cereus* with concentration of 31.25 mg/ml. This indicates the methanolic extract of *C. hystrix* leaves has better degree of inhibition against *B. cereus* compared to others selected enteric pathogens. Although the MIC values were much higher than *B. cereus*, methanolic extract of *C. hystrix* leaves showed good patterns of inhibition against *S. aureus* and *S. typhimurium* with concentration of 125 mg/ml for both bacteria. The MIC values for methanolic extract of *C. hystrix* leaves were maximized against *S. flexneri* with concentration of 250 mg/ml. These finding were found contradicted as compared with earlier report revealed that the *C. hystrix* leaves extract required lower MIC values to inhibit the growth of *S. aureus* and *S. typhimurium* (3.12 mg/ml and 25 mg/ml), respectively (Wanangkarn et al. 2018).

**Minimum Bactericidal Concentration**

The absence bacterial growth of the tested pathogens streaked from inhibition zone corresponding to their lowest MIC values indicated as the MBC. *C. hystrix* methanolic leaves extract showed potentially bactericidal activity against all the tested pathogenic bacteria ranging from 125 to 500 mg/ml (Table 3). In the present study, methanolic extract of *C. hystrix* leaves showed the most effective bactericidal activity against *B. cereus* with MBC of 125 mg/ml. Meanwhile, *C. hystrix* methanolic leaves extract showed moderate bactericidal activity

against both *S. aureus* and *S. flexneri* with MBC of 250 mg/ml. *C. hystrix* methanolic leaves extract showed least sensitive against *S. typhimurium* and its minimal bactericidal concentration reached to 500 mg/ml. The findings in the current study were contradicted to the previous work reported that *C. hystrix* leaves extract exhibited lower MBC values against *S. aureus* and *S. typhimurium* at concentration 6.25 mg/ml and 50 mg/ml, respectively (Wanangkarn et al. 2018). Factors including different extraction methods, type of solvents used and AST procedure have contributed to the different both MIC and MBC values.

## CONCLUSION

*C. hystrix* methanolic leaves extract possessed broadest antimicrobial agents against *S. aureus*, *B. cereus*, *S. typhimurium* and *S. flexneri*. The result from AST, MIC and MBC of methanolic extract of *C. hystrix* leaves showed varying strength against selected gastrointestinal pathogens. The results of present study suggest that the *C. hystrix* methanolic leaves extract exhibited strongest antibacterial activity against *B. cereus* with maximum inhibition zone and least of MIC and MBC values. The bioactive compounds detected in this herbal extract including alkaloids, flavonoids, terpenoids and tannin have contributed to the effective antibacterial activity which may be used as a potential antimicrobial agents to treat gastroenteritis caused by pathogenic bacteria.

Further investigations on identifying and quantifying the phytochemical constituents present in *C. hystrix* leaves extract need to be performed to obtain the specific bioactive metabolites that can be achieved using method such as High-Performance Liquid Chromatography (HPLC) and Gas Chromatography Mass-Spectrophotometry (GC-MS). Also, additional work is encouraged by using different extraction methods, testing the leaves of *C. hystrix* on wide range of microorganisms and against many infections to provide scientific validation that would further serve as a strong evidence for traditional medicine use of these herbs as potent natural antimicrobial agents. In conclusion, there is a need to investigate the *C. hystrix* leaves for individual bioactive compounds such as alkaloids, flavonoids, limonoids, phenolic and glycerophospholipids to obtain more precise and accurate results.

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## REFERENCES

Abdallah EM. (2016). Preliminary Phytochemical and Antibacterial Screening of Methanolic Leaf Extract of *Citrus aurantifolia*. Pharmaceutical Biotechnology: Current Research, 1:1.

Ali, M., Akhter, R., Narjish, S. N., Shahriar, M., & Bhuiyan, M. A. (2015). Studies of Preliminary Phytochemical Screening, Membrane Stabilizing Activity, Thrombolytic Activity and In-Vitro Antioxidant Activity of Leaf Extract of *Citrus hystrix*. International Journal of Pharmaceutical Sciences and Research, 6(6), 2367–2374. https://doi.org/10.13040/IJPSR.0975-8232.6(6).2367-74

Arumugam, A., Gunasekaran, N., & Perumal, S. (2014). The Medicinal And Nutritional Role of Underutilized Citrus Fruit- *Citrus hystrix* (Kaffir Lime): A Review. Drug Invention Today, 6(1), 1–5.

Barrett, J., & Fhogartaigh, C. N. (2017). Bacterial gastroenteritis. Medicine (United Kingdom), 45(11), 683–689. https://doi.org/10.1016/j.mpmed.2017.08.002

Khanam, Z., Wen, C. S., & Bhat, I. U. (2015). Phytochemical screening and antimicrobial activity of root and stem extracts of wild *Eurycoma longifolia* Jack (Tongkat Ali). Journal of King Saud University - Science,27(1), 23-30. doi:10.1016/j.jksus.2014.04.006

Lingaraju, K., Raja Naika, H., Manjunath, K., Basavaraj, R. B., Nagabhushana, H., Nagaraju, G., & Suresh, D. (2016). Biogenic synthesis of zinc oxide nanoparticles using *Ruta graveolens* (L.) and their antibacterial and antioxidant activities. Applied Nanoscience (Switzerland), 6(5), 703–710. https://doi.org/10.1007/s13204-015-0487-6

Madike, L. N., Takaidza, S., & Pillay, M. (2017). Preliminary Phytochemical Screening of Crude Extracts from the Leaves, Stems, and Roots of *Tulbaghia violacea*. International Journal of Pharmacognosy and Phytochemical Research, 9(10), 1300–1308. https://doi.org/10.25258/phyto.v9i10.10453

Newton, I., Ajithkumar, P., & Panneerselvam, R. (2012). Effect of *Citrus hystrix* and *Citrus limon* extracts on antibacterial activity against

human pathogens. Asian Pacific Journal of Tropical Biomedicine, 1–4. Retrieved from www.elsevier.com/locate/apjtb

Sauid, S. M., & Aswandi, F. A. (2018). Extraction methods of essential oil from kaffir lime ( *Citrus hystrix* ): A review. 1, 56–64.

Sivanandham, V. (2015). Phytochemical techniques - a review. World Journal of Science and Research, 1(2), 80–91. https://doi.org/10.4028/www.scientific.net/AMR.1055.182

Temitope, O. O., & Olugbenga, O. B. (2015). Phytochemical screening of ten Nigerian medicinal plants. International Journal of Multidisciplinary Research and Development, 2(4), 390–396. Retrieved from http://www.scopus.com/inward/record.url?eid=2-s2.0-0026355037&partnerID=40&md5=31098e8c7eb4e1d22a94c8db72f30e12

Utami, R., Kawiji, Khasanah, L. U., & Nasution, M. I. A. (2017). Preservative effects of kaffir lime (*Citrus hystrix DC*) leaves oleoresin incorporation on cassava starch-based edible coatings for refrigerated fresh beef. International Food Research Journal, 24(4), 1464–1472.

Valle, D. L., Andrade, J. I., Puzon, J. J. M., Cabrera, E. C., & Rivera, W. L. (2015). Antibacterial activities of ethanol extracts of Philippine medicinal plants against multidrug-resistant bacteria. Asian Pacific Journal of Tropical Biomedicine, *5*(7), 532–540. https://doi.org/10.1016/j.apjtb.2015.04.005

Wanangkarn, A & Tan, F.J. & Fongsawad, K & Tirasaros, M. (2018). Bioactivity screening of Thai spice extracts for applying as natural food preservatives. International Journal of Agricultural Technology. 14. 783-796. Retrived from http://www.ijat-aatsea.com/pdf/v14 n5...

Wei, L., Ratnayake, L., Phillips, G., McGuigan, C. C., Morant, S. V., Flynn, R. W., Mackenzie, I.S., & MacDonald, T. M. (2017). Acid-suppression medications and bacterial gastroenteritis: a population-based cohort study. British Journal of Clinical Pharmacology, 83(6), 1298–1308. https://doi.org/10.1111/bcp.13205

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