



## **Determination of total phenolic content and antimicrobial potential of different extracts of *Citrus hystrix* DC leaves**

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### **ABSTRACT**

Present study was intended to determine the total phenolic content and antimicrobial potential of different solvent extracts of *Citrus hystrix* leaves (CHL). The CHL were subjected to successive solvent extraction by Soxhlet extractor using different solvents (petroleum ether, chloroform, ethyl acetate, acetone, and methanol) to offer different CHL extracts. The total phenolic content (TPC) of each CHL extract was determined using Folin Ciocalteu method. The antimicrobial potential of each CHL extracts was determined using agar disc diffusion method against different bacterial strain such as *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The experimental results revealed chloroform extract of CHL to possess highest TPC (14.081 mg GAE/gram). The ethyl acetate, acetone and methanolic extracts of CHL exhibited good inhibitory potential against tested bacteria. Chloroform extract exhibited its antimicrobial potential only against *Escherichia coli*, whereas petroleum ether extract did not exhibit any antimicrobial activity. The MIC values were determined only for chloroform, ethyl acetate, acetone and methanolic extracts. Study concludes that except petroleum ether, all the CHL extracts possess substantial antioxidant and antimicrobial potential.

**Keywords:** *Citrus hystrix*, Total Phenolic Content, Antimicrobial, MIC.

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### **INTRODUCTION**

Oxidative damage (significant factor in human disease development or pathogenicity) and antibiotic resistance are the serious and widespread problems [1,2]. The global emergence of multidrug resistant bacteria threatens the current antibiotics therapy [2]. The antibiotic resistance has been commonly reported in *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* [3]. The extensive administration of conventional antibiotics against several infections results in prolonged treatment, multiple drug resistance, and high mortality risk [4,5]. This demands to explore the new antimicrobial therapeutic from plants origin (attributed to their less side and toxic effects). World Health Organization (WHO) estimates that 65–80% of world population of developing countries use plants for primary health care [6]. Evidence suggest herbal diets are associated with a decreased risk of infections, cardiovascular disease, and several form of cancers [7]. The 'antioxidant hypothesis' proposes polyphenols (dietary antioxidants) present in plants provide protection against oxidative damage (significant factor for development of human diseases) [1]. Exploration of antimicrobial activities from plants source, such as *Citrus* species might be a solution to combat the antimicrobial resistance and oxidative damage [3,4,8]. *Citrus hystrix* belongs to the family Rutaceae, and is commonly known as Kaffir lime, Limau purut in Malay [9]. Evidence suggest *Citrus hystrix* to possess antioxidant, antibacterial, antileukemic, antiinflammatory, and anticancer activities [3,10]. There is a lack of comparative studies over *Citrus hystrix* (Kaffir lime from province of Kedah state of Malaysia) leaves extracts (especially: petroleum ether, chloroform, ethyl acetate, acetone, and methanol altogether) total phenolic content and antimicrobial potential (against *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*). Hence present study was intended to explore the antibacterial activity and total phenolic content of different leaves extracts of *Citrus hystrix*.

## MATERIAL AND METHODS

### Material

The *Citrus hystrix* leaves (CHL) were collected from the campus of AIMST University. The voucher specimen no. AIMST/FOP/2018/21 of the same is submitted in the MDL4, Faculty of Pharmacy, AIMST university, Malaysia. The solvents, consumable and biological media were procured from Sigma Aldrich, SD Fine and Hi Media. The glass wares were cleaned using deionized water and dried at 160 °C for 2 hours and plastic wares procured were autoclaved before experiment.

### Preparation of CHL extracts

The dried CHL powder was subjected to successive extraction with petroleum ether, chloroform, ethyl acetate, acetone and methanol in an increasing order of polarity using Soxhlet apparatus to offer petroleum ether, chloroform, ethyl acetate, acetone and methanolic extracts of CHL.

### Total phenolic content (TPC) of CHL extracts

The prepared CHL extracts were subjected to the determination of TPC using Folin-Ciocalteu method (11). Briefly Folin-Ciocalteu reagent (100 µl) and sodium bicarbonate (2 ml; 2.5 %) were added into each 100 µl of CHL extract. Each CHL extract mixture was completely mixed and allowed to stand at room temperature for two hours. Next, the absorbance of each CHL extract solution was measured at 750 nm using spectrophotometer. A similar procedure was repeated by replacing the CHL extract with Gallic acid. Quantification of TPC of each extract was done using standard curve of Gallic acid (2-10 µg/m), which was dissolved in methanol and expressed as mg Gallic acid per gram of plant material). For each CHL extract, the TPC measurement was done in triplicate and the mean was calculated. The Gallic acid equivalent (GAE) was determined using standard curve. The content of TPC was expressed as mg Gallic acid equivalent (GAE) of per gram dry weight of extract.

### Antimicrobial activity CHL extracts

The antibacterial activity of different CHL extracts was determined against *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* bacterial strains using disc diffusion method as per the reported protocol with slight modifications (12). Briefly, each CHL extract was dissolved in dimethyl sulfoxide (DMSO) with a concentration of 0.5 g/ml. The paper discs were infused with of herbal extracts and placed on Mueller Hinton agar plates which were inoculated with test organism. The disk without extracts served as control. Gentamicin was used as standard. All the plates were incubated at 37° C for 24 h, and all experiments were performed in triplicate.

### Determination of minimum inhibitory concentration.

The CHL extracts that exhibited strong antibacterial activity (zone of inhibition) were further explored their MIC values using disk diffusion method as per the reported protocol with slight modification (1, 12). The MIC value of CHL extracts was determined as the lowest concentration that completely inhibited bacterial growth after 24 hr of incubation at 37 °C. Different concentrations of each CHL extract (400, 200, 100, 50, 40, 30, 20 and 10 µg/mL) were prepared by dilution method. Dried and sterilized filter paper discs with 6 mm diameter were impregnated with the CHL extracts separately and placed over the agar surface that was inoculated with specific bacterial culture. Gentamicin (0.1 mg/ml) was used as standard for positive control and a blank disc (impregnated with respective solvents) was used as negative control. The plates were incubated at 37 °C for 24 hr. The diameters of the zones of inhibition by discs with different extracts were measured in mm (13). The disc with lowest concentration of extract that showed complete inhibition of growth of microorganism after incubation was noted and recorded as the MIC.

## RESULTS AND DISCUSSION

The high phenolic content in medicinal plants relates to their antioxidant potential that is beneficial in prevention of age related diseases caused by oxidative stress [14]. Facts suggest phenolic biomolecules are widely used in infectious and cardiovascular diseases, inhibition of bacterial pathogens, antioxidant, insecticides, virucides, fungicides, cosmetics, food preservative, antiparasitic, anticancer, and antidiabetic agents [15-18]. Phenolics are the bioactive compounds that are known for their antioxidant potential attributed to the presence of high activity as hydrogen or electron donor (8). Based on this fact, TPC value of different CHL extracts (petroleum ether, chloroform, ethyl acetate, acetone, and methanol) was determined by Folin-Ciocalteu method using standard curve of Gallic acid (Figure 1), and the resultant data for the same is given in table 1.

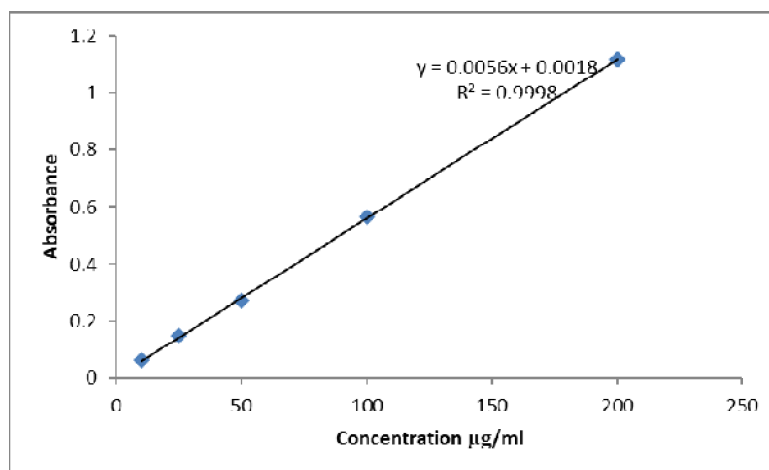


Figure 1. Standard curve of gallic acid

The resultant data given in table 1, suggest that among all CHL extracts, the chloroform extract exhibited high total phenolic content (14.081 mg GAE/gram), followed by methanol (13.826 mg GAE/gram), petroleum ether (13.231 mg GAE/gram) and acetone (11.769 mg GAE/gram) extracts, and ethyl acetate extract showed lowest total phenolic content (11.326 mg GAE/gram). From table 1, it can be clearly assessed that polarity of solvent influences the distribution of phenolic compounds (19).

TABLE 1: TOTAL PHENOLIC CONTENT OF EXTRACTS OF *CITRUS HYSTRIX* LEAVES

| Petroleum ether extract | Chloroform extract | Ethyl acetate extract | Acetone extract | Methanol extract |
|-------------------------|--------------------|-----------------------|-----------------|------------------|
| 13.23                   | 14.08              | 11.32                 | 11.76           | 13.82            |

Evidence suggest that emergence of resistance microorganisms against conventional antibiotics is a major factor that influence investigators to search the new antimicrobials from plants origin (3). Based on this fact present study was intended to determine the antibacterial potential of different CHL extracts. *Citrus hystrix* DC (Kaffir lime) commonly found in Malaysia. Several other investigations also supported antioxidant (20, 21), and antimicrobial (22-25) potential of *Citrus hystrix* DC plant. The present study evaluated antibacterial activity of CHL extracts by agar disk diffusion and MIC against various pathogenic bacteria. The different CHL extracts were investigated for their antimicrobial activity (using disc diffusion method) against different microorganism such as *E. coli*, *K. pneumoniae*, *S. aureus* and *P. aeruginosa*. Gentamicin used as standard in this study. The resultant data for the same is given in table 2. Ethyl acetate, acetone and methanolic extracts showed good inhibitory effect (zone of inhibition) against all the four strains of bacteria. Chloroform extract of CHL exhibited the antimicrobial activity only against *E. coli*, whereas petroleum ether extract did not exhibit any antimicrobial activity. The comparison of zone of inhibitions of different extracts (11-17 mm) and gentamicin (24.7-25 mm) against four bacterial strains at concentration of 100 µg/mL, established that antibacterial potential of CHL extracts were possessed substantial antibacterial activity when compared with gentamicin. Among all CHL extracts, the methanolic extract exhibited maximum antibacterial potential against *E. coli* and *P. aeruginosa*.

TABLE 2: ZONE OF INHIBITION OF CHL EXTRACTS

| Microorganism        | Zone of inhibition of CHL extracts in (mm) |             |               |              |             |             | Control |
|----------------------|--------------------------------------------|-------------|---------------|--------------|-------------|-------------|---------|
|                      | Petroleum ether                            | Chloroform  | Ethyl acetate | Acetone      | Methanol    | Gentamicin  |         |
| <i>E. coli</i>       | -                                          | 11.3 ± 0.47 | 11.3 ± 0.47   | 13           | 17.3 ± 0.50 | 24.7 ± 0.47 | -       |
| <i>K. pneumoniae</i> | -                                          | -           | 8.7 ± 0.47    | 11           | 13.7 ± 0.47 | 25          | -       |
| <i>S. aureus</i>     | -                                          | -           | 8.7 ± 0.47    | 11           | 10          | 25          | -       |
| <i>P. aeruginosa</i> | -                                          | -           | 10.7 ± 0.57   | 12.66 ± 0.57 | 13.7 ± 0.57 | 24.7 ± 0.57 | -       |

Note: (-) - No inhibition

The minimum inhibitory concentration (MIC) is defined as the lowest concentration of antimicrobial that required to completely inhibit the growth of microorganism (13). In present study, all CHL extract were evaluated for MIC values using (disc diffusion method) except petroleum ether extract of CHL (with no zone of inhibition against any tested bacteria). The data for the same is given in table 3. The chloroform, ethyl acetate, acetone and methanolic extract of CHL exhibited lowest MIC value of 30 µg/mL (against *E. coli*), 40 µg/mL (against *E. Coli*), 20 µg/mL (against *K. pneumoniae* and *P. aeruginosa*), and 20 µg/mL

(against *S. aureus*) respectively. The results of present study were also in agreement with the results of other studies (26).

TABLE 3: MIC OF CHL EXTRACTS

| CHL Chloroform Extract Concentration in (µg/mL)    |    |     |     |      |      |      |      |      | Gentamicin | Control |
|----------------------------------------------------|----|-----|-----|------|------|------|------|------|------------|---------|
| Microorganism                                      | 10 | 20  | 30  | 40   | 50   | 100  | 200  | 400  |            |         |
| <i>E. Coli</i>                                     | -  | -   | 8.3 | 9    | 10.3 | 11.3 | 14.7 | 21   | 24.7       | -       |
| CHL Ethyl acetate Extract Concentration in (µg/mL) |    |     |     |      |      |      |      |      | Gentamicin | Control |
| <i>E. coli</i>                                     | -  | -   | -   | 8    | 10.3 | 11.3 | 12.7 | 15.3 |            |         |
| <i>K. pneumoniae</i>                               | -  | -   | -   | -    | -    | -    | 5.3  | 8.7  | 25         | -       |
| <i>S. aureus</i>                                   | -  | -   | -   | -    | -    | -    | 4.3  | 7.3  |            | -       |
| <i>P. aeruginosa</i>                               | -  | -   | -   | -    | -    | -    | -    | 5.7  |            | -       |
| CHL Acetone Extract Concentration in (µg/mL)       |    |     |     |      |      |      |      |      | Gentamicin | Control |
| <i>E. coli</i>                                     | -  | -   | -   | 8.7  | 11.7 | 13   | 14.3 | 17   |            |         |
| <i>K. pneumoniae</i>                               | -  | 7.3 | 9   | 10.7 | 14   | 15.3 | 19   | 20.7 |            | -       |
| <i>S. aureus</i>                                   | -  | -   | 7.3 | 8.7  | 10.3 | 11   | 15.7 | 18   | 25         | -       |
| <i>P. aeruginosa</i>                               | -  | 7.3 | 8.7 | 12.3 | 15   | 16.7 | 18.7 | 22   |            | -       |
| CHL Methanol Extract Concentration in (µg/mL)      |    |     |     |      |      |      |      |      | Gentamicin | Control |
| <i>E. coli</i>                                     | -  | -   | 5.7 | 9.3  | 13.3 | 17.7 | 19.3 | 21.7 |            |         |
| <i>K. pneumoniae</i>                               | -  | -   | -   | 9    | 11.7 | 13.7 | 15   | 17.3 |            | -       |
| <i>S. aureus</i>                                   | -  | 7.7 | 9   | 10.7 | 12   | 16   | 16.7 | 18.7 |            | -       |
| <i>P. aeruginosa</i>                               | -  | -   | -   | -    | 8.3  | 13.7 | 15.3 | 17.3 | 24         |         |

Note: (-) - No inhibition

## CONCLUSION

Present study concludes that all *Citrus hystrix* leaves (CHL) extracts possess high total phenolic content, that signals its high antioxidant potential. Also, all CHL extracts possess substantial inhibitory potential against tested micro-organisms except petroleum ether. The data of present study will serve as a preclinical data for further investigations.

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## CONFLICT OF INTEREST

Authors have no conflict of interest.

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