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Research Article

# ANTIBACTERIAL ACTIVITY OF THE ETHANOLIC EXTRACT OF LEAVES OF *Citrus maxima* (Burm.) Merr. ON ESCHERICHIA COLI AND PSEUDOMONAS AERUGINOSA.

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**ABSTRACT**

**Objective:** The objective of the study was to evaluate the antibacterial activity of the ethanolic extracts of leaves of *Citrus maxima* (Burm.) Merr. *(EECM)* on *Escherichia coli* and *Pseudomonas aeruginosa.* **Methods:** The ethanolic extract of leaves of *Citrus maxima* (Burm.)Merr.(EECM) was prepared by percolation method. Pathological isolates *Escherichia coli* and *Pseudomonas aeruginosa* were obtained from the Department of Microbiology, Assam Medical College & Hospital. Disc diffusion method for antimicrobial susceptibility testing was performed according to the Kirby-Bauer method. The whatman-1 filter paper discs of 6mm sizes impregnated with the plant extract were placed on Mueller-Hinton agar plates seeded with bacterial cultures of 0.5 Mc Farland standards. The antibacterial activities were assessed by the presence or absence of inhibition zones after incubating the plates at 37$^0$c for 24 hours. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of EECM for the selected pathogens were determined by broth macrodilution method. **Results:** Maximum zone of inhibition in antibacterial susceptibility test was shown by *Pseudomonas aeruginosa.* MIC value of the extract for *Pseudomonas aeruginosa* (0.312mg/ml) was found to be lower than *Escherichia coli* but MBC value (1.25mg/ml) was found to be the same for both the bacteria. **Conclusion:** The plant extract of *Citrus maxima* (Burm.) Merr showed significant antibacterial activity against *Escherichia coli* and *Pseudomonas aeruginosa.*

**Keywords:** Pseudomonas aeruginosa, Escherichia coli, MIC, MBC, *Citrus maxima*.

## INTRODUCTION

Plants have been utilized as source of herbal medicines since ancient times and the presence of secondary metabolites in plants have implicated them for many therapeutic applications. The plants also act as a source of inspiration for development of novel drug compounds, as plant derived medicines have made large contributions to human health and well being [1]. According to the World Health Organization (WHO), almost 80% of the world's population relies on traditional medicines for their health needs due to better cultural acceptability, fewer side effects and better compatibility with the human body [2].

Emergence of pathogenic microorganisms that are resistant or multiresistant to major class of antibiotics has increased in recent years due to indiscriminate use of synthetic antimicrobial drugs. In addition, high cost and adverse effects are commonly associated with popular synthetic antibiotics are a major burning global issue in treating infectious diseases [3]. There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action for new and re-emerging infectious diseases. Therefore, researchers are increasingly turning their attention to folk medicine, looking for new leads to develop better drugs against microbial infections [4]. Antimicrobials of plant origin have enormous therapeutic potential. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials [5].

The plant, *Citrus maxima* (Burm.) Merr. (Rutaceae), is commonly known as shaddock or pomelo. The plant is indigenous to tropical parts of Asia. The fruit and pulp is cited as antitoxic, appetizer, cardiac stimulant and stomach tonic in ancient and medieval literature. Recently, leaves are found to exhibit antitumour activity [6]. Alcoholic extracts of the fruit also shows antidiabetic and anti hyperlipidaemic potential [7]. The essential oil of the fruit shows in vitro activity against *Staphylococcus aureus* and *Escherichia coli* [8]. Antibacterial activity of the leaves of *Citrus maxima* (Burm.) Merr. has not been evaluated so far. In this study, an attempt has been made to evaluate the antibacterial activity of the leaves of *Citrus maxima* (Burm.) Merr. against *Escherichia coli* and *Pseudomonas aeruginosa*.

## MATERIALS AND METHODS

### Collection, Identification and Extraction of Plant Materials

Fresh leaves of *Citrus maxima* were collected from the Assam Medical College & Hospital campus. The leaves of the plant were authenticated by Dr. L. R. Saikia, Professor, Life sciences, Dibrugarh University, Assam. A voucher specimen (No. DU/LS/208) was deposited at the Department of Life Sciences, Dibrugarh University. The cleaned materials were dried in shade, grinded to fine powder with the help of a mixer grinder and ethanolic extract was prepared using 90% ethanol by percolation method followed by steam evaporation in rotary flask evaporator [9]. The extract was transferred into clean and dried airtight vial and stored at 2$^0$-8$^0$C until ready for use. A net yield of 276 g (23% w/w) was obtained by percolating 1200g of dry powder of the leaves.

### Phytochemical analysis

EECM was subjected to qualitative phytochemical analysis for flavonoids, alkaloids, tannins, saponins, sterols and terpenoids by following standard procedures [10].

### Microorganisms

Pathogenic bacterial isolates of *Escherichia coli* and *Pseudomonas aeruginosa* were obtained from the Dept. of Microbiology, AMCH. The organisms were sub cultured and stored in a semisolid medium (Mueller Hinton agar plates) at 4$^0$C until needed.

### Preparation of the culture media

Mueller Hinton agar was used for the antimicrobial susceptibility test. 3.7% of Mueller Hinton agar was mixed with hot distilled water and autoclaved at 15 lb pressure for 15 minutes. After autoclaving, it was allowed to cool to 45$^0$C-50$^0$C. Then the medium was poured into sterilized Petri dishes with a uniform depth of approximately 4 mm.

**Preparations of plant extract impregnated discs**

Whatman no.1 filter paper was used to prepare discs of 6 mm in diameter. They were sterilized by autoclaving and subsequently dried at 80°C for an hour in a hot air oven. The discs were then impregnated with 10 mg/ml and 5mg/ml concentration of the ethanolic extracts of *Citrus maxima* (Burm.) Merr. to get the final concentration of 0.25mg/disc and 0.125mg/disc respectively. The plant extract impregnated discs were then dried and kept in sterile condition till further use.

**ANTIMICROBIAL SUSCEPTIBILITY TEST**

**Disc diffusion method**

Disc diffusion method for antimicrobial susceptibility test was carried out according to the standard method by Kirby-Bauer to assess the presence of antimicrobial activity of plant extract [11]. A bacterial suspension adjusted to 0.5 Mc Farland standard ($1.5\times10^{8}$ CFU/ml) was used to inoculate Mueller Hinton agar plates evenly using a sterile swab. The plates were left ajar for 5 minutes. Then, the discs impregnated with ethanolic extract of leaves of *Citrus maxima* (Burm.) Merr. were placed individually on the Mueller Hinton agar surface with flamed forceps and gently pressed down to ensure contact with the agar surface. The discs were spaced far enough to avoid both reflections waves from the edges of the petri dishes and overlapping of rings of inhibition.

A standard commercial disc of Ciprofloxacin (5µg/disc) was used as a positive control and an ethanol (90%) impregnated disc was used as a negative control. Each test plate contained four discs, one of which is a positive control i.e., a standard commercial antibiotic disc (Ciprofloxacin 5µg/disc) and other is a negative control i.e., 90% ethanol impregnated disc. Besides the controls, each plate had two extract impregnated discs at the concentration of 0.25mg/disc and 0.125 mg/disc placed about equidistant to each other. The plate was then incubated at 37°c for 24 hours in inverted position to look for zones of inhibition to ascertain antibacterial activity of the ethanolic extract of leaves of *Citrus maxima* (Burm.) Merr. against selected micro organisms.

Zones of inhibitions produced by the sensitive organisms were demarcated by a circular area of clearing around the plant extract impregnated discs and were compared with the zone of inhibition produced by the positive control (ciprofloxacin 5µg/disc) and the negative control (ethanol impregnated disc). The tests were repeated six times to ensure reliability.

**Determination of Minimum Inhibitory Concentration (MIC) - Broth macrodilution Method**

MIC of the extracts was carried out using broth dilution method as described in Ibekwe et al, 2001 [12]. The initial concentration (5mg/ml) of the plant extract was diluted using double fold serial dilution by transferring 5ml of the sterile plant extract stock solution into 5ml of sterile Nutrient broth to obtain 2.5mg/ml concentration. The above process was repeated several times to obtain other dilutions: 2.5mg/ml (1:2), 1.25mg/ml (1:4), 0.625mg/ml (1:8), 0.312mg/ml (1:16), 0.156 (1:32) and finally 0.078mg/ml (1:64). Having obtained the different concentrations of the extracts, each concentration was inoculated with 0.1ml of the standardized bacterial cell suspensions (0.5 Mc Farland) of *Escherichia coli* and *Pseudomonas aeruginosa* in separate sets of tubes and incubation was done at $37^{0}$ c for 24 hours. The growth of the inoculum in the broth is indicated by turbidity or cloudiness of the broth. The lowest concentration of the extracts that inhibits growth of the organisms, as detected by lack of visual turbidity, was designated as the minimum inhibitory concentration (MIC).

Two control tubes were maintained for each test batch that included as antimicrobial control (tube containing extract and the growth medium without inoculum) and organism control (the tube containing the growth medium and the inoculum) [13]. The lowest concentration of the extract that completely inhibited bacterial growth (no turbidity) in comparison to control was regarded as MIC.

**Determination of Minimum bactericidal concentration (MBC)**

For calculation of minimum bactericidal concentration (MBC) of extracts, after the MIC has been determined, 0.1ml of inoculum from each of the tubes of broth was sub-cultured on Mueller-Hinton agar plates. The number of colonies on agar after incubation at $37^{0}$ for 24 hours is then counted and compared to the number of colony forming units/ml in the original inoculums. The lowest concentration of extracts that allowed less than 0.1% of the original inoculums to survive (i.e., 99% killing of bacterial isolates) were determined as a MBC [14].

**RESULTS**

The qualitative phytochemical analysis of EECM revealed presence of flavonoids, alkaloids, tannins and saponins. In the disc diffusion method, the zones of inhibition produced by the sensitive organisms selected for the study were measured using callipers and recorded after the incubation period. In the study, positive control (Ciprofloxacin) was found to produce zones of inhibition against all selected microorganism but the bacteria were insensitive to the negative control (ethanol impregnated disc); so no zone of inhibition was noted for the negative control. The zones of inhibitions shown by ethanolic plant extract impregnated discs and the positive control at different concentrations against the selected microorganisms are depicted in table 1 and the comparative activity of the plant extracts against the selected individual microorganism is represented with bar diagrams in figure 1 and 2. The MIC and the MBC of the plant extracts for the selected bacteria are depicted in table 2.

**Table 1: Zones of inhibitions as shown by ethanolic plant extract at different concentrations against selected microorganisms.**

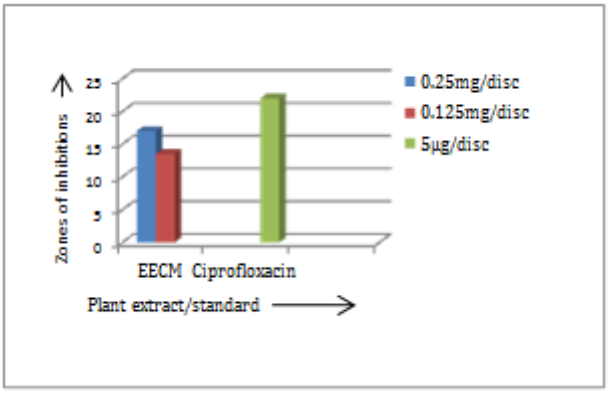
| **Extracts/ Positive control** | **EECM** | | **Ciprofloxacin** |
|---|---|---|---|
| **Concentration** | **0.25mg/disc** | **0.125mg/disc** | **5µg/disc** |
| Microorganisms | Zones of inhibitions (mm) | | |
| Escherichia coli | 17.0 ± 0.577 | 13.5 ± 0.428 | 22 ± 0.365 |
| Pseudomonas aeruginosa | 19.5 ± 0.428 | 14.83 ± 0.494 | 21 ± 0.577 |

**Data represents Mean ± Standard error of mean (n = 6).**

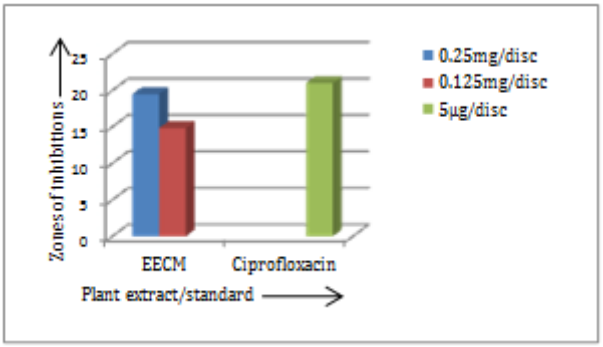
**Table2: MIC and MBC of ethanolic extract of leaves of *Citrus maxima* against *Escherichia coli* and *Pseudomonas aeruginosa.***

| **Microorganisms** | **Concentrations EECM (mg/ml).** | | | | | | | **MIC** | **MBC** |
|---|---|---|---|---|---|---|---|---|---|
| | 0.078 (1:64) | 0.156 (1:32) | 0.312 (1:16) | 0.625 (1:8) | 1.25 (1:4) | 2.5 (1:2) | 5 (1:1) | | |
| *Escherichia coli* | + | + | + | - | - | - | - | 0.625 | 1.25 |
| *Pseudomonas aeruginosa* | + | + | - | - | - | - | - | 0.312 | 1.25 |

**+ Growth; - No growth.**




**Graph 1: Antibacterial activity of ethanolic plant extract against *Escherichia coli* at different concentrations.**




**Graph 2: Antibacterial activity of ethanolic plant extracts against *Pseudomonas aeruginosa* at different concentrations.**

## DISCUSSION

Both *Escherichia coli* and *Pseudomonas aeruginosa* were found to be susceptible to the ethanolic extract of *Citrus maxima* (Burm.) Merr. at both the concentrations (10mg/ml & 5mg/ml). Maximum zone of inhibition was shown by *Pseudomonas aeruginosa.* MIC value of the extract for pseudomonas aeruginosa (0.312mg/ml) was found to be lower than Escherichia coli (0.625mg/ml) but MBC value was found to be the same for both the bacteria (1.25mg/ml).

Between the bacteria selected, the *Pseudomonas aeruginosa* showed a higher sensitivity to the plant extract compared to Escherichia coli. This signifies potent antibacterial activity of the extract because *Pseudomonas aeruginosa* is the most common gram-negative bacterium responsible for the nosocomial as well as community acquired infections. The development of multidrug resistant *Pseudomonas aeruginosa* is currently one of the greatest challenges to the effective management of infections [15].

The antibacterial activity of the plant extract can be attributed to the different phytochemicals present in the leaves of *citrus maxima* (Burm.) Merr. There is growing interest in correlating the phytochemical constituents of a medicinal plant with its pharmacological activity. Phytochemicals are non-nutritive plant chemicals that may have protective or disease preventive antimicrobial activities. Because of their structural differences from those of the more studied antimicrobial sources, their mode of action may too differ [16]. Flavonoids, alkaloids and saponins are found to be associated with antimicrobial effects in various studies using plant extracts [17]. These phytochemicals have been found to be present in the leaves of *Citrus maxima* (Burm.) Merr. in our laboratory and also in other scientific studies [5]. Flavonoids have been found to exhibit antimicrobial activity through various mechanisms like inhibition of nucleic acid synthesis, inhibition of cytoplasmic membrane function and energy metabolism [18]. The mode of action of antibacterial effects of saponins seems to involve membranolytic properties [19]. Saponins possess detergent like activity and might increase the permeability of bacterial cell membrane without destroying them. In theory, this activity might facilitate antibiotic influx through the bacterial cell wall membrane [20]. The mechanism of action of highly aromatic quaternary alkaloids is attributed to their ability to intercalate with DNA [21]. These may explain the probable mechanism of antibacterial activity of the plant.

A synergistic relationship between antioxidant status and antimicrobial effects of the plants are coming into light nowadays. Belofsky et al. demonstrated an increase in the antimicrobial activity of pure compounds when they are combined with antioxidants. Therefore, we can consider that if both antimicrobial and antioxidant compounds exist in the extract, they could interact and enhance the antimicrobial activity [22]. In a recent study, the antioxidant activity of the leaves of *Citrus maxima* (Burm.) Merr. has been shown against paracetamol induced hepatotoxicity in rats [23]. Flavonoids have been shown to have potent antioxidant and free radical scavengering activity [24] which is also found in *Citrus maxima* (Burm.) Merr. leaves that may potentiate its antibacterial effects. In recent years, ethnobotanical and traditional uses of natural compounds, especially of plant origin received much attention as they are well tested for their efficacy and generally believed to be safe for human use. They obviously deserve scrutiny on modern scientific lines such as phytochemical investigation, biological evaluation on experimental animal models, toxicity studies, investigation of molecular mechanism of actions of isolated phytoprinciples and their clinical trials [25].

## CONCLUSION

The study reveals the antibacterial activity of the ethanolic extract of *Citrus maxima* (Burm.) Merr. Leaves against *Escherichia coli* and *Pseudomonas aeruginosa*. The present study can pave a way for further research to determine the lead compounds in the leaves to develop newer antimicrobial agent in this era of antimicrobial resistance.

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**Conflict of interest** – Nil

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