

STUDY OF ANTIMICROBIAL ACTIVITY OF LIME JUICE AGAINST *VIBRIO CHOLERA*

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Abstract: The antimicrobial effect of crude extract of lime (*Citrus aurantifolia*) was assayed against different bacterial species by agar well diffusion method. The highest inhibition zone of 28 mm was observed in *Vibrio cholerae* followed by *Enterobacter* species (9mm), *Citrobacter* species (8mm) and *Escherichia coli* (8mm). *Shigella*, *Salmonella* and *Klebsiella* species were found resistant. So the study was mainly focused on *V. cholerae*. Different concentrations of the crude extract such as 75%, 50%, 25% and 5% were tested to calculate the minimum amount that inhibits the bacteria which showed the zone of inhibition (ZOI) as 31mm, 24mm, 17mm and 9mm respectively. The minimum bactericidal concentration (MBC) ranged from 6.25 mg/l to 50 mg/l. This result suggests that citrus fruit like limes are effective in preventing infection with *Vibrio* species.

Key words: Agar well; Crude extract; Minimum bactericidal concentration; Zone of inhibition.

INTRODUCTION

Limes (*Citrus aurantifolia*) are a small citrus fruit either be sour or sweet, sour limes possess a greater sugar and citric acid content than lemons and feature an acidic and tart taste. The nutritional profile includes information on a full array of nutrients including carbohydrates, sugar, soluble and insoluble fiber, sodium, vitamins, minerals, fattyacids, amino acids and more. Limes contain unique flavonoid compounds that have antioxidant and anti-cancer properties. While these flavonoids have been shown to stop cell division in many cancer cell lines, they are perhaps most interesting for their antibiotic effects.

Cholera outbreak during summer hit different parts of the country including the capital city and causes an enormous loss of lives (DISVI, 1990). This disease still recognized as a major problem for Nepalese children, being recorded as the second most prevalent diagnosis in out- patient services. . *V. cholerae*, pathogen of the cholera bacteria that continues to cause large pandemics, occur naturally in the environment (Griffin *et al.*, 2001) and cause illness through contaminated food or water, can be inhibited by using lime juice. In several villages in West Africa where cholera epidemics had occurred, lime juice was also found to have a strong protective effect against cholera (Tomotake *et al.*, 2006).

Citric acid, the major organic acid in these juices, was found to be responsible for inhibiting the growth of *Vibrios*. The natural biocidal activity of lime juice was studied in order to explore its possible use as a disinfectant and inhibitor of

Vibrio cholerae in drinking water for areas lacking water treatment plants (Daquino and Teves, 1994). Owing to the support of national and international organization for the studies on treatment and prevention of these diseases based on traditional practices, medicinal plants are becoming hopeful source of antidiarrhoeal drugs (Lin *et al.*, 2002).

METHODOLOGY

Preparation of Standard Inoculum

Different cultures of bacteria such as *V. cholerae*, *E. coli*, *Enterobacter*, *Citrobacter*, *Klebsiella*, *Salmonella* and *Shigella* species were taken and sub-cultured on nutrient agar for pure culture. Standard inoculum of 0.1% was made by comparing with standard turbidity.

Selection and Extraction of Lime

Lime that was free of decay and mold was taken. It was washed with distilled water several times to remove soil and other extraneous matter so that any dirt or bacteria residing on the surface will not be transferred to the fruit's interior and sterilized with 70 % ethanol. Then pieces were made with sterilized knife. Juice was extracted using juice extractor and juice was placed in the sterilized bottle with screw cap.

Qualitative Screening and Determination of Antibacterial Activity

The crude extract was screen for its antibacterial activity i.e. determination of zone of inhibition against tested organism by agar well diffusion method as given by (Dingle *et al.*,

1953). Sterile Mueller-Hinton Agar plates were inoculated with prepared inoculum with sterile cotton swab. Then with the help of sterile cork borer no. 6, wells were made in the inoculated media plate. 50 µl of the working solution/suspension of different concentration was transferred into the well with the help of micropipette. The control was also placed in the separate well at the same time. After proper incubation, the plates were viewed for the zone of inhibition, which is suggested by clear areas without growth around the well.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The crude extract which showed antibacterial activity, were subjected to two-fold serial dilution method to determine minimum bactericidal concentration (MBC). For each bacterium, a set of 12 screw capped test tube containing 1ml nutrient broth was required. The test tubes were labeled as positive growth control, negative growth control and numbers 1 to 10. Different dilution of crude extract as 75%, 50%, 25% and 5% was made. Then two fold serial dilution of the extract was prepared each containing equal volume but decreasing concentration.

50µl of culture inoculum of test bacteria was added to each tube with the help of micropipette except negative control. All the tubes were incubated at 37°C for 24 hours and observed for turbidity by comparing with +ve and -ve control. The results were interpreted on the basis of the fact that growth occurs in the positive control and any other tube in which the concentration of the extract is not sufficient to inhibit growth and lowest concentration of the agent that inhibits growth of the organism as detected by lack of visible turbidity as designated the minimum inhibitory concentration (MIC). However, in some cases it was difficult to identify whether the turbidity was due to the growth of bacteria or due to the turbidity of extract itself, the tubes were sub-cultured on nutrient agar plate with proper label followed by incubation at 37°C for 24 hours. Then they were examined for the growth of bacteria. The tube with minimum concentration of extract in which the growth was completely stopped was also clearly notified. This determines the minimum bactericidal concentration (MBC).

RESULTS

Lime juice was assayed for their possible antibacterial activity against the different organisms by agar well diffusion method. It was found to be very effective against *V. cholerae* as shown by producing highest zone of inhibition (28mm), less effective towards *Enterobacter*, *Citrobacter*, and *E. coli* whereas ineffective against *Shigella*, *Salmonella* and *Klebsiella* species (Figure 1). So, the study was mainly focused on *V. cholerae*. Different concentrations of crude extract (lime juice) such as 75%, 50%, 25% and 5% were tested to calculate the minimum amount that inhibits the growth as well as to kill the bacteria. The ZOI was found as 31mm, 24mm, 17mm, and 9mm in dilution 75%, 50%, 25% and 5% of crude extract respectively.

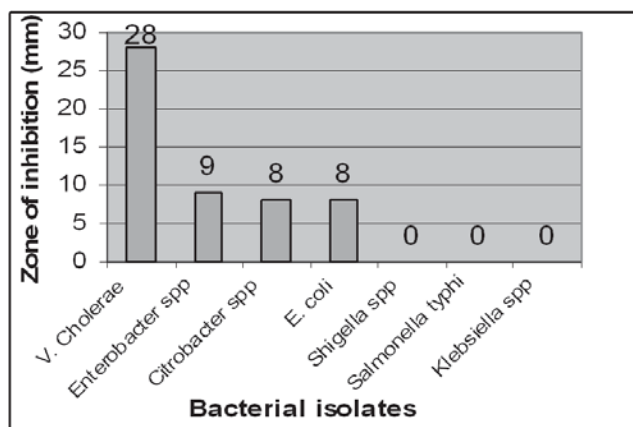


Figure 1: ZOI shown by crude extract against different bacterial isolates

Evaluation of Antibacterial Activity

Antibacterial activity was evaluated by two ways viz. measuring zone of inhibition and quantitative determination of extract for minimum bactericidal concentration (MBC). The diameter of zone of inhibition (ZOI) produced by extract on particular bacteria was measured for the estimation of potency of extract as depicted in Table 1. Similarly, the minimum amount of extract usually expressed in terms of microgram or milligram per milliliter of the bacterial broth solution required to inhibit the growth as well as to kill the bacteria altogether clearly expresses the better effectiveness of the extract against the corresponding bacteria. The MBC values of 6.25 mg/l, 6.25 mg/l, 12.50 mg/l and 50 mg/l was determined in 75%, 50%, 25% and 5% of crude extract respectively.

Table 1: ZOI at different concentration and MBC given by lime juice against *V. cholerae*

S. N.	Different concentration of crude extract	Antimicrobial activity	
		ZOI (mm)	MBC (mg/ml)
1.	75%	31	6.25
2.	50%	24	6.25
3.	25%	17	12.50
4.	5%	9	50.0

DISCUSSION

Lime juice was found very effective against *V. cholerae*. The zone of inhibition was observed highest in *V. cholerae* (28mm), in case of *Enterobacter* (9mm), *Citrobacter* (8mm), *E. coli* (8mm) but no zone of inhibition was observed in *Shigella*, *Salmonella* and *Klebsiella*. Similar results have been reported by (WHO, 2002). (Munoz *et al.*, 1995) tested four strains of *Vibrio cholerae* 01, *V. parahaemolyticus*, *Escherichia coli*, *Salmonella typhimurium*, *Shigella flexneri*, *Sh. sonnei*, and *S. enteritidis*. The greatest bactericidal effect was reported against *V. cholerae* 01. Other enterobacteria were reported to be resistant. Different concentrations of lime juice were found to inhibit *V. cholerae* as shown by zone of inhibitions 31 mm, 24mm, 17mm, 9mm and MBC as 6.25 mg/ml, 6.25 mg/ml, 12.50 mg/ml and 50 mg/ml was observed in dilution 70%, 50%, 25% and 5% respectively. This has been done by two methods via agar well diffusion and two fold serial dilution. The result

indicates the existence of antimicrobial compounds in crude extract and shows active constituents present in them.

Antimicrobial substance applied in agar well diffuses readily in concentric circles and inhibits or kills the microorganisms that are susceptible. This effect, manifested by a zone of clearing, is observed upto a point where the decreasing concentration of diffusing antimicrobial substance is still sufficient to inhibit or kill the organism. Beyond the point, the concentration is insufficient and hence growth starts. The diameter of zone of inhibition produced depends on several factors broadly classified as extrinsic and intrinsic parameters. The extrinsic parameters like pH of the medium, period and temperature of incubation, volume of well, size of inoculum etc. In 1994 Mata *et al.*, reported the effect of lime juice against *Vibrio cholerae* O1, El T or, Inaba. The lethal effect was evident within 5 min of exposure to lime juice. (Castillo *et al.*, 2000) reported that concentrated lemon juice and essential oils inhibited *V. cholerae* completely at all studied dilutions and exposure times.

In vitro antimicrobial screening methods provides the required preliminary observation to select among the crude plants products, those with potentially useful properties for further chemical and pharmacological studies. The present study disclosed in reducing cholera transmission as well as disease risk.

CONCLUSION

In recent years, special attention is being given on alternative safe natural bio-remedies to cure the infectious diseases because of their less or no side effects and resistance in microbes against them. The result reveals that lime juice was found very effective against *V. cholerae*. So it may provide new leads in development of new antimicrobial compound for the therapy of cholera.

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