

Research Article



Antimicrobial Activity of Lemon Peel Extract against the Bacterium Belonging to the Genus *Xanthomonas*

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ABSTRACT

It has been observed that there is hardly any bacterial disease of lemon fruit. The reason for this has been well established by many authors working on the antimicrobial properties of the extract of the peel of this fruit. Several gram negative and gram positive bacteria have been used for such studies. Several compounds have been found to be present in the rind of this fruit and shows marked antibacterial properties. However, one gram negative bacteria belonging to the genus *Xanthomonas* is capable of causing disease of the fruit called as citrus canker. This investigation has found that this organism is well adapted to grow at a low pH of 5 as the peel extract has been found to be highly acidic. It is also resistant to the antibacterial substance, which is a flavoprotein, containing coumarin and is responsible for inhibiting topoisomerase II or DNA gyrase found in bacteria and in many plants at high concentration like 100nM or more. It seems to be resistant to other antimicrobials found in the peel like tetrazene in high concentration.

Keywords: Lemon, *Xanthomonas*, citrus canker, topoisomerase II, flavoprotein.

INTRODUCTION

Citrus limon (L) or what is commonly called as lemon, belong to Rutaceae family, and its distribution is widespread from South East Asia, India to southern China. Lemon is a pale yellow, elliptically shaped berry fruit. Like all other citrus fruits, it too generally contains sugar, other polysaccharides, organic acids, lipids, vitamins, minerals and volatile compounds. Juice of lemon has been reported to exhibit antimicrobial activity against *Vibrio cholerae*¹.

The peel of this fruit is rich source of flavonoids, glycosides, coumarins, and volatile oils². Many polymethoxylated flavones have also been detected in the peel, having several important bioactivities, which are very rare in other plants³. Antimicrobial activity of the peel extract is directly concerned with the components that they contain. Certain essential oils, protopine and corydaline alkaloids, lactones, polyacetylene, hypeicin and pseudohypericin compound are reported to be effective toward various bacteria^{4, 5, 6}. Nevertheless, other active terpenes, as well as alcohols, aldehydes and esters contribute to the overall antimicrobial effects of the essential oils^{7, 8}. The antibacterial assay of lemon peel extract in different solvent such as ethanol, methanol and acetone were previously studied using different microorganisms^{9, 10}. Mathur *et al.* (2011)¹¹ discussed the possibility of using this peel extract in various application including food preservation¹².

MATERIALS AND METHODS

Preparation of Extract

The peel of lemon was homogenised in different solvent individually. The solvents used were: ethanol, acetone,

methanol, CHCl₃ and water. The extracts were stored separately for further study. The extractions were carried out by soxhlet method as described by Tu *et al.*, (2017)¹³ and by routine shake flask method at 28°C on a rotary shaker rotating at 150 rpm for two hrs.. These were then filtered and centrifuged at 9000 x g for 10 minutes and the clear supernatant obtained were dried at 60°C on a water bath for 4 to 6 hrs. Till a dry residue was obtained in each case. The weights of the residues were noted for further dilution and studies.

Effect of the extracts on the growth of *Xanthomonas*

The effect of peel extracts of *Citrus limon* (L) on growth pattern of test organisms was studied¹⁴. Colonies of the respective bacterial culture was incubated in nutrient broth and McFarland 0.5 turbidity standard was obtained. Then 5 ml of bacterial suspension was added to 100 ml of nutrient broth along with 5 ml of the respective extract. Simultaneously two sets of experiment were run with the test. One set of experiment where nutrient broth without extract, while in another set of experiment nutrient broth contains seed extract only. The flasks were incubated for 24 h at 28°C on an incubator shaker rotating at 150 rpm. Growth was monitored by measuring absorbance at 610 nm at every 30 minutes interval. The final concentration of the extracts was 500 µg/ 100mL. in all further studies, unless otherwise specified.

Two extracts that were showing minimum inhibitory effect, were selected from the results of above experiments, these were viz. acetone extract (prepared at 28°C on a rotary shaker) and aqueous soxhlet extract.



Effect of pH on the inhibitory effect of the extracts on growth pattern of the organism

Three pH values were taken at random and these were pH 5 (since citrus fruits are acidic), pH 7 (the optimum ph of growth of most proteobacter organism, on a artificial medium) and alkaline pH like 9. The experiments were carried out very similar to those mentioned above using the 2 extracts as mentioned before.

Effect of extract concentrations on the inhibitory effect of the extracts on growth pattern of the organism

The different concentrations of the 2 extracts used were, 500µg, 1500µg and 2000µg per 100 mL (final concentrations). The experiments were carried out similar to those mentioned above.

RESULTS AND DISCUSSION

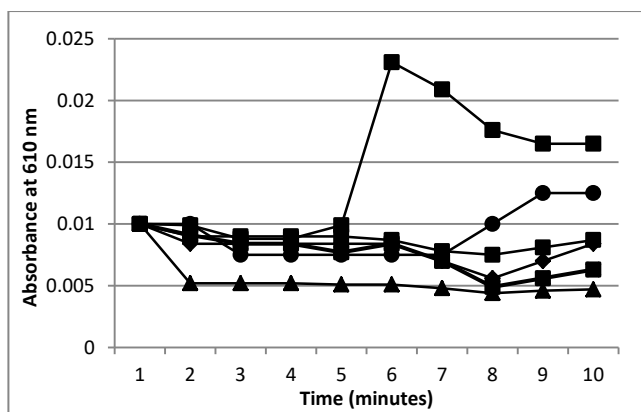


Figure 1: Growth pattern of *Xanthomonas* without any extract (●) and in presence of extracts carried out at 28°C on a rotary shaker: aqueous extract (◆), ethanol extract (■), methanol extract (▲), CHCl₃ extract (x) and acetone extract (x).

It can be noted from the above figure that there was significant growth in acetone extract as compared to the growth pattern without any extract, probably due to the fact that the inhibitory agents were not extracted by acetone. However, the methanol extract shows significant less growth.

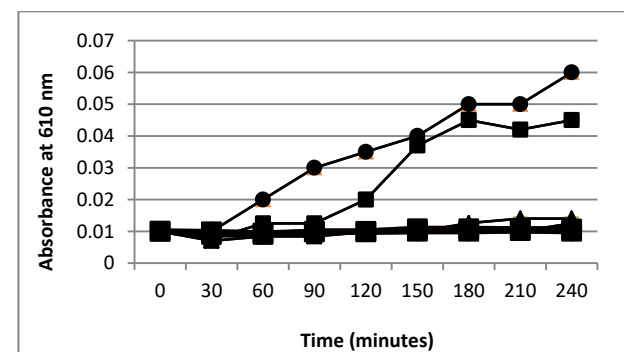


Figure 2: Growth pattern of *Xanthomonas* without any extract (●) in presence of soxhlet extracts: chloroform extract (◆), acetone extract (x), aqueous extract (■), ethanol extract (x), methanol extract (▲)..

It can be noted from the above figure that there was significant growth at soxhlet aqueous extract and least growth in all other extracts. This could be probably due to efficient extraction easily assimilable substances like certain carbohydrates or probably due to least extraction of inhibitory substances.

It was then decided to check the effect of different pH values, on the growth of the organism using the appropriate extract and from figure 1, acetone extract (at 28°C) was used. The results are as shown in figure 3.

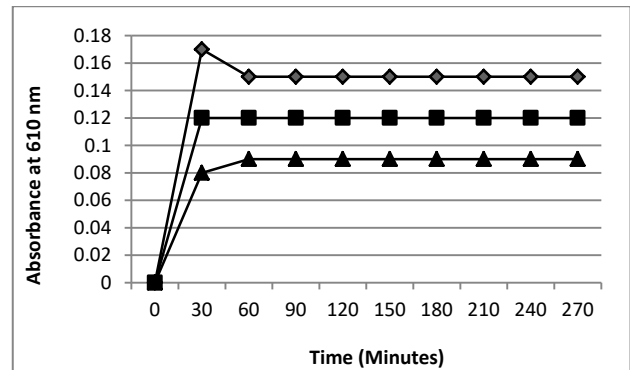


Figure 3: Growth pattern of *Xanthomonas* in presence of acetone (28°C extract) at pH 5 (◆), pH 7 (■) and pH 9 (▲).

It can be noted from the above figure that there was significant growth at pH 5 and least growth at pH 9. This establishes the fact that the organism is a potent plant pathogen as it has adapted to grow at a low pH like 5 due to the presence of acids like citric acid in the peel of the fruit. It shows significantly lesser growth at pH 7, at which most of the bacteria, including *Xanthomonas*, belonging to gamma proteobacter, prefers to grow. It was expected that there will be less growth at a slight alkaline pH like 9 since it showed less growth at neutral pH.

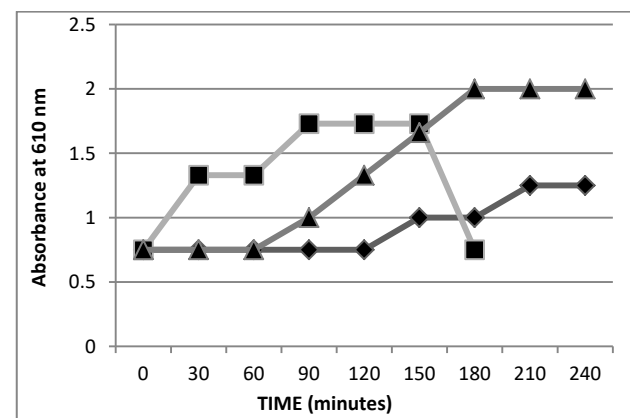


Figure 4: Growth pattern of *Xanthomonas* in presence of water (Soxhlet extract) at pH 5 (◆), pH 7 (■) and pH 9 (▲).

It can be noted that above figure that there was significant growth at pH 5. The inhibiting compounds from lemon peel showing maximum inhibition of the log phase of the growth of the bacteria at this pH. However, the growth continues till 4hrs into the stationary phase. The bacteria show a typical diauxic growth. At pH 7 and 9,

the diauxic pattern is not observed. The lag phase is barely for 60 mins, followed by a log phase of another 30 mins in case of pH 7 and then a stationary phase of 60 mins. This is followed by a death phase in the next 30mins. At pH 9, the log phase goes on for 2 hrs. But at a slower rate than the one observed at pH 7, followed by stationary phase for the next 60 mins. The reason for this could be that at pH 7, the organism grows rapidly utilising all metabolisable compounds under stress of pH and certain inhibitory compounds like tetrazene (Dhanavade *et al.*, 2011), which can remain active at pH 7 in aqueous solution unlike coumarin. However, it appears that at pH 9, tetrazene tends to lose its biological activity possibly due to deprotonation.

Similar attempt was made to check the effect of different concentrations of the extracts on the growth pattern of the organism. It is known that a substance like coumarin will not be readily soluble in water at 28°C. However, other inhibitory substances like tetrazene might have some antibacterial effect.

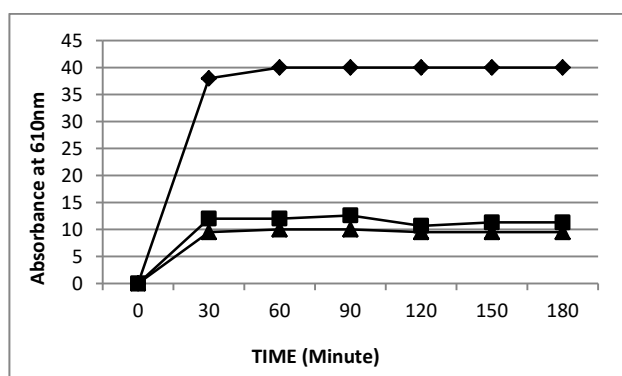


Figure 5: Growth pattern of *Xanthomonas* in presence of aqueous (soxhlet extract) at concentration 0.5 mg/100ml (◆), 1.5 mg/100ml (■) and 2 mg/100ml (▲).

It is evident from Figure 5, that in all the 3 concentration of the aqueous extract, the organism was under stress of the inhibitory compounds. This is seen from the short log phase of 30 mins., followed by stationary phase for the remaining 150 mins.

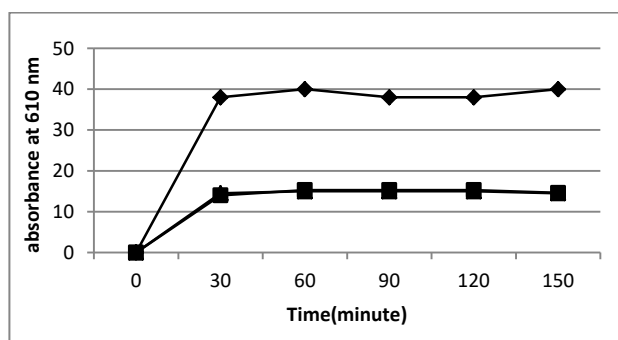


Figure no 6; Growth pattern of *Xanthomonas* in presence of acetone extract carried out at 28°C. Concentration 0.5 mg/100ml (◆), 1.5 mg/100ml (▲) and 2 mg/100ml (■).

It can be noted that above figure that was there significant growth at - Concentration 0.5 mg/100ml. The inhibiting compounds from the lemon peel seem to be

minimal at this concentration. In the other 2 concentrations there was no doubt a short log phase of 30 mins, but the organisms survived at stationary phase.

CONCLUSIONS

It has been established that the lemon peel contains an antibiotic called coumarin⁵, which is in the form of a flavoprotein and acts as a defence mechanism against pathogenic bacteria. It inhibits DNA gyrase i.e. topoisomerase II – the enzyme responsible for negative super coiling of DNA needed before its translation. It primarily affects the enzyme in bacteria and in other parasitic plants but not in animals and in fungi (where topoisomerase IV is predominantly present). In fact it is one of the main reasons for citrus fruit plants show resistance to bacterial infection during maturity of fruits. DNA gyrase of *Xanthomonas* is highly resistant to coumarin (at 100 nM concentration) and the bacterium is also known to produce another such antibiotic compound called albicidin which is twice as potent as compared to coumarin. The bacteria itself is resistant to albicidin as it produces it. Its main role is to block the DNA gyrase of the plant which in turn is believed to weaken the disease resistance of the plant so that an organism can invade its defence mechanism and cause the infection. This is in accordance with a report of Hashimi *et al* (2008)¹⁵, in case of *Xanthomonas albilineans*, which infects sugarcane to cause the leaf scald disease.

Secondly from most of the results mentioned above, it may be noted that the growth of the organism is restricted (as seen from a prolonged stationary phase). However, whether the population of the organism at this phase could bring about an infection or not is very doubtful as it could be a mixed population of virulent and avirulent strains. May be this is one of the reason, why citrus canker is not observed covering the entire lemon rind but is scattered all over the surface.

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