Assessment of antimicrobial activity of lemon juice against *Acinetobacter baumannii*

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Bsc Biomedical Science (Hons)

This dissertation is presented in part fulfilment of course or degree for which the student is enrolled.
Contents

Declaration Page 3
Abstract Page 4
Introduction Page 5
Materials and Methods Page 7
Results Page11
Discussion Page14
Future study Page16
Conclusion Page16
References Page17
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Assessment of antimicrobial activity of lemon juice against *Acinetobacter baumannii*

Abstract

*Acinetobacter baumannii* is a gram negative pathogen that is a prolific nosocomial infection which causes infections such as cutaneous wound infection, respiratory tract infections, pneumonia, urinary tract infections as well as in rare cases necrotising fasciitis and endocarditis which is a major factor in its recently gained notoriety, and has developed from its recent proliferation around wounded soldiers coming back from the recent desert conflicts of Iraq and Afghanistan; gaining it the nickname ‘iraqibacter’.

The aim of this experiment was to prove that Lemon juice has an antimicrobial effect and may have a synergistic effect when used in conjunction with antibiotics to improve their function.

Tests were carried out on two bacterial strains of *A. baumannii* (E75U382259 and NCTC1216) with the former being a resistant strain to antibiotics, and the latter being susceptible to antibiotics. For the investigation of these effects, minimum inhibitory concentration, and minimum bactericidal concentrations were obtained using 96 well microtitre plates. These tests were carried out for gentamicin, lemon juice and a HCl control. Checkerboard assays were also carried out to determine if there was synergy between lemon juice and gentamicin.

Results obtained showed that there was a significant difference between the effect of lemon juice and the HCl control with P0.036 & P0.059 respectively. There was a significant difference between the two bacterial strains against gentamicin (P0.00) and no significant difference between the bacterial strain when cultured in lemon juice (P0.721).

These results show that lemon juice must act by a different mode of action to gentamicin as there was no significant difference between the two bacterial strains. Therefore, the factors that incur antibiotic resistance in E75U382259 does not provide any tolerance to withstanding lemon juices antimicrobial
effects. The significant difference between lemon juice and the HCl control also provides evidence that the lemon juice attributes its antimicrobial effects from a factor other than its acidity. Although, further study is needed to investigate this. These effects may be due to flavonoids present in the lemon juice which are a class of compound already known to possess antimicrobial properties.

Introduction

*Acinetobacter baumannii* is a gram negative opportunistic pathogen that has come to international attention over the last 15 years. (1-4) This notoriety has grown out of its propensity to survive in harsh environments which is aided by *A. baumannii* ’s ability to form biofilms. Furthermore *A. baumannii* ability to successfully colonise both biotic, but especially abiotic surfaces such as Polystyrene, glass and metal. (5-11)Which have made *A. baumannii* a major cause of nosocomial infections. Compounding its effectiveness to cause infections is its rapid development of antibiotic resistance to most first line and second line antibiotics. This rapid increase in antibiotic resistance has caused the United States Centre for Disease Control and Prevention (CDC) to designate *A. baumannii* as a serious hazard level pathogen, Meaning it requires close monitoring and active prevention and control activities. *A. baumannii* is known to cause a wide variety of disease conditions. Most commonly it causes cutaneous wound infections, respiratory tract infection, pneumonia, urinary tract infections and meningitis. However, cases of endocarditis and necrotising fasciitis have also been reported. (12-17)

*A. baumannii* is reported to account for 2-10% of all gram negative infections in the US and European intensive care units (ICU), according to reports from the World Health Organisation (WHO). Infection with multidrug resistant (MDR) *A. baumannii* has also been shown to increase mortality within ICU’s from 11% to 28% Which is a deeply concerning discovery, highlighting the importance of research into effective treatment options.(13) Furthermore, the drive to find effective treatments are the increased length of hospital stay associated with *A. baumannii* infections. (13)

Reports of infections in the US have shown that *A. baumannii* is responsible for around 7,000 hospital acquired infections and 500 deaths yearly.
Although traditionally found in aquatic environments it has become a serious problem in the recent desert conflicts of Afghanistan and Iraq where it has caused serious wound infections earning it the nickname ‘Iraqibacter’. (19) Antibiotic resistance in a wider context is also of major concern which has caused alarm recently with the detection of bacteria that are resistant to all currently used antibiotics. Antibiotic resistance has been labeled as one of the top 3 greatest threats facing human health by the WHO. (18) According to the CDC, in the United States alone antibiotic resistance causes an estimated 2 million infections and accounts 23,000 deaths per year. With the European Centre for disease control and prevention estimating that 25,000 people die annually in Europe from antibiotic resistant infections. Furthermore, it is feared that soon certain medical procedures will become unviable to perform due to the risk of incurable infection. Special attention has also been paid in a report by the current UK government on antimicrobial resistance to gram negative bacteria as they cause the most serious infections, whilst also being highly prevalent in healthcare settings where patients are vulnerable to infection. The report also noted that there was a ‘striking lack of new antimicrobial agents against MDR Gram-negative bacterial’

Due to the lack of development of traditional antibiotics a number of novel treatments have earned greater attention, notably nutraceuticals. Nutraceuticals already have a historical reputation of possessing antimicrobial properties. The nutraceuticals of note are citrus fruits such as lemon, lime and orange juices, manuka honey and garlic. Citrus juices have been shown to have a number of factors and components such as alkaloids, flavonoids, steroids, terpenoids, saponins, cardiac glycosides, and reducing sugars among others, that may contribute to their antimicrobial effects. Although, steroids appear to be absent from lemon juice. Manuka honey is also a well known natural antimicrobial. With literature suggesting this may be largely due to the low acidity of honey (pH 3.4-6.1). This hypothesis has also been put forward for citrus fruits efficacy as antimicrobial agents. For manuka honey in particular it is believed that the antimicrobial effect may be aided by the high presence of MGO. Garlic is believed to gain its antibiotic properties from organosulfate compounds with a direct link between antimicrobial efficacy and increased number of sulphur atoms.
Despite all of these nutraceuticals possessing antimicrobial effects there is a discrepancy in the amount of research carried out into the practical applications of these nutraceuticals. Medical grade manuka honey has been available in the US since 2010 in the form of honey infused wound dressings. However, the antimicrobial properties of citrus juices such as lemon is yet to be brought into the public domain and patient contact, it is therefore an area worthy of investigation.

The aim of this experiment was to prove that Lemon juice has an antimicrobial effect and may have a synergistic effect when used in conjunction with antibiotics to improve their function.

**Materials & Methods**

All tests bellow were carried out in triplicate for statistical significance.

Culture solutions.
For producing the stock cultures for each experiment 5ml of nutrient broth was placed into a 25ml sample tube. 3 separate colonies were then taken from the bacterial agar culture plates using a aseptic wire loop technique and placed into the nutrient broth. The culture medium was then mixed using a vortex before being placed into a incubator at 37 degrees celsius for 24hrs.

Before each experiment the incubated culture was then diluted using nutrient broth to an absorbance of 0.1 @ 620nm in a techan infinite.

MIC & MBC Gentamicin.
Microtitre plates were taken with lids marked and labeled appropriately for bacteria, antibiotic ,as well as location of positive and negative controls marked. 8 sample tubes were taken and 500ul of nutrient broth added to each. Then 500ul of gentamicin previously made in nutrient broth was added to tube 1. The tube was then mixed using a vortex, before 500ul was pipetted out and dispensed into the tube 2. 500ul was then taken from tube 2 and dispensed into tube 3. The process was repeated for all 8 tubes. 500ml is then removed from tube 8. 100ul of solution was then taken from each of the 8 tubes and deposited
in the corresponding wells. 100ul of nutrient broth was added to well 12 as this will served as the positive control and 200ul of broth was placed in well 11 to serve as the negative control. 100ul of inoculum was then placed in wells 1-8 and well 12.

A zero hour reading of the microtitre plate was taken using a Tecan infinite plate reader at 620nm.

The plate was then placed into a incubator at 37 degrees celsius for 24hours. After the 24 hour incubation the plate was read again in the Tecan infinite at 620nm. Following this 10ul was taken from each well that showed no growth and placed as a spot onto nutrient agar. The agar plates were then placed into the incubator for 24hours. After 24 hours it was recorded whether or not growth was shown on the plates and the concentrations of the solutions recorded.

MIC & MBC Lemon Juice

Microtitre plate was taken and lid marked and labeled appropriately for bacteria, Lemon juice percentage as well as location of positive and negative controls marked.

8 tubes sample tubes were taken. 500ul of double strength nutrient broth was dispensed into tube 1. Then 500ul of nutrient broth added to tubes 2-8. 500ul of gentamicin previously made in nutrient broth was then added to tube one. The tube was then mixed using a vortex before 500ul was pipetted out and dispensed into the tube 2. 500ul is then taken from tube 2 and dispensed into tube 3, process is repeated for all 8 tubes. 500ml is then removed from tube 8. 100 ul of solution was then taken from each of the 8 tubes and deposited in the corresponding wells. 100ul of nutrient broth was added to well 12 as this will serve as the positive control and 200ul of broth was placed in well 11 to serve as the negative control. 100ul of inoculum was then placed in wells 1-8 and well 12.

A zero hour reading of the microtitre plate was taken using a Tecan infinite plate reader at 620nm.

The plate was then placed into a incubator at 37 degrees celsius for 24hours. After the 24 hour incubation the plate was read again in the Tecan infinite at 620nm.

Following this 10ul was taken from each well that showed no growth and placed as a spot onto nutrient agar. The agar plates were then placed into the
incubator for 24 hours. After 24 hours it was recorded whether or not growth was shown on the plates and the concentrations of the solutions recorded.

HCl Control
For the acid control, first the pH of the lemon juice and HCl were equalised by adding sterilised water to the HCl until the pH was the same using a digital pH probe. Then the produced acid solution was used following the exact same method as that used for the lemon MIC above.

Checkerboard
9 sample tubes were taken and 500ul of nutrient broth added to all 2-8 tubes. Into tube one was placed 500ul of gentamicin. Then a 500ul of gentamicin was added to tube 2. Tube two was mixed using a vortex before 500ul was removed and placed into tube three. This process was repeated until all nine tubes had been mixed. 50ul of solution was then pipetted into all wells in the respective columns 1-9. 50ul of nutrient broth was added to column 10. Then 6 sample tubes were taken and 600ul of nutrient broth added to tubes 2-6. Into tube 1 was placed 600ul of double strength nutrient broth and 600ul of lemon juice. Tube one was then mixed using a vortex. 600ul was then taken from tube 1 and placed into tube 2. This process was then repeated with tubes 3-6. 50ul of each solution was then placed into each well in the rows A-F with row A being tube 1, row B tube 2, etc. Into row G was placed 50ul of nutrient broth. Following this 50ul of culture was added to all 70 wells that contain the varying concentrations of Lemon and gentamicin solution. Into column 11 was placed 150ul of inoculum and 150ul of nutrient broth was placed into column 12 to act as positive and negative controls respectively. Each plate will only take one bacterial strain at a time and therefore separate plates are required for each bacterial strain. A zero hour reading of the microtitre plate was taken using a Tecan infinite plate reader at 620nm.
The plate was then placed into an incubator at 37 degrees celsius for 24 hours. After the 24 hour incubation the plate was read again in the Tecan infinite at 620nm.

Following this 10ul was taken from each well that showed no growth and placed as a spot onto nutrient agar. The agar plates were then placed into the incubator for 24 hours. After 24 hours it was recorded whether or not growth was shown on the plates and the concentrations of the solutions recorded.

Biofilm Formation
To ascertain the biofilm growth the plates used for the MIC or Checkerboards were taken and the liquid carefully removed to avoid disturbing the biofilm. The wells were then washed three times by dispensing 250ul of PBS and removing it using a pipet.

The plate was then placed into an incubator at 60 degrees celsius for 60 minutes to fix the cells to the well surface. Following this all wells were filled with 200ul of crystal violet solution and left for 15 minutes to stain the biofilms within the wells.

Crystal violet solution was then removed and the wells washed with 250ul of PBS eight times or until all crystal violet had been removed and PBS solution remains clear.

Following this the plate was then dried by inverting the plate and tapping onto tissue after which it was left to air dry. Once dry the wells were then filled with 200ul of 7% acetic acid and left for 10 mins for the crystal violet held within the biomass of the biofilm to be released.

The plate is then read at 620nm in a tecan infinite using the biomass setting which shakes the plate for 10 seconds before reading to ensure accurate reading of the turbidity of the solutions in the wells caused by the released crystal violet.
Results

**Figure 1.** Graph showing the absorbance values of two bacterial strains of *A. baumannii* (blue E75U382259, Orange NCTC1216) when grown for 24 hours at 37 degrees Celsius at various concentrations of antibiotic gentamicin.

**Figure 2.** Statistical analysis using a 2 sample t test of the significance of the difference between the effects of gentamicin on two strains of *A. baumannii* (E75U382259, NCTC1216) using data shown in figure 1.
Figure 3. Graph showing the absorbance values of two bacterial strains of *A. baumannii* (blue E75U382259, Green NCTC1216) when grown for 24 hours at 37 degrees Celsius at various percentage concentrations of lemon juice.

Figure 4. Statistical analysis using a 2 sample t test of the significance of the difference between the effects of lemon juice on two strains of *A. baumannii* (E75U382259, NCTC1216) using data shown in figure 3.
Figure 5. Graph showing the absorbance values of two strains of *A. baumannii* (blue E75U382259, Orange NCTC1216) when grown for 24 hours at 37 degrees Celsius at pH concentrations equal to that of lemon juice at each dilute step. pH of solution was lowered to be equal to lemon juice using HCl.

![Graph showing absorbance values](image)

**Figure 6.** Statistical analysis using a 2 sample t test of the significance of the difference between the effects of HCl on two strains of *A. baumannii* (E75U382259, NCTC1216) using data shown in figure 5.

**Figure 7.** Statistical analysis using a 2 sample t test of the significance of the difference between the effects of HCl and lemon juice on two strains of *A. baumannii* (E75U382259, NCTC1216) using data shown in figure 3 and figure 5.

![Table of absorbance values](image)

**Figure 8.** Table showing the absorbance value of *A. baumannii* strain E75U382259 after incubation at 37 degrees celsius for 24 hours with a varying
concentration of both lemon juice and gentamicin. All wells in green denote growth with all cells in red denoting no growth.

<table>
<thead>
<tr>
<th>NCTC1216</th>
<th>Gentamicin Concentration mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>512</td>
<td>256</td>
</tr>
<tr>
<td>7.5</td>
<td>0.0413</td>
</tr>
<tr>
<td>6.25</td>
<td>0.0464</td>
</tr>
<tr>
<td>Lemon juice</td>
<td>3.175</td>
</tr>
<tr>
<td>concentration %</td>
<td>1.587</td>
</tr>
<tr>
<td>0.794</td>
<td>0.0421</td>
</tr>
<tr>
<td>0.397</td>
<td>0.023</td>
</tr>
</tbody>
</table>

| 0 | 0.001 | 0.058 | 0.1187 | 0.1773 | 0.6937 | 0.9576 | 0.9906 | 1.0426 | 0.9711 | 1.0047 |

Figure 9. Table showing the absorbance value of A. baumannii strain NCTC1216 E75U382259 after incubation at 37 degrees celcius for 24 hours with a varying concentration of both lemon juice and gentamicin. All wells in green denote growth with all cells in red denoting no growth.

Results for the checkerboard assays were carried out using the FIC index analysis which is the result of the sum of the FIC which is the equation below.

\[
\Sigma \text{FIC} = \text{FIC}_A + \text{FIC}_B = \left( \frac{C_A}{\text{MIC}_A} \right) + \left( \frac{C_B}{\text{MIC}_B} \right)
\]

MIC\text{A} and MIC\text{B} are the MIC of the Respective drugs alone and C\text{A} and C\text{B} are the drug concentrations in combination.

Discussion

As shown by Figure 1 it has been shown that gentamicin has a minimum inhibitory concentration of 256mg/ml and a minimum bactericidal concentration of 256mg/ml also for the Antibiotic resistant strain with the sensitive strain having a MIC and MBC of 64mg/ml. Gentamicin is the most commonly used aminoglycoside and is often used with gram negative bacterial infections due to its Safety rating, effectiveness and its cheap pricing. However as results in figure 2 show there is a significant difference between the effectiveness of the antibiotic against the two strains (p<0.00) with the declining effectiveness of the antibiotic decreasing due to increased resistance among the bacterial population novel treatments were investigated.

Lemon juice appeared to be highly effective against A. baumannii as it inhibited both bacterial strains at the same concentration of 3.175% and it was proven that there was no statistical difference between the effect of lemon juice on the
two strains. As there was no significant difference in lemon juice’s effect against the two strains of A. baumannii unlike with gentamicin, it can be assumed that lemon juice’s antimicrobial mode of action must be different from that of gentamicin as the mechanism of resistance does not work against lemon juice. Therefore as gentamicin works against the cell wall it can be concluded that this is not the site at which lemon juice effects bacteria. Given lemon juice’s known possible antimicrobial agents it may well be that this is caused by the flavonoids present and studies suggest that DNA gyrase among other proteins involved in bacterial genetic replication and function. However the Low pH of lemon juice could be an influencing factor in its antimicrobial effects as many studies have suggested that pH may be an influencing factor in many nutraceuticals from honey to lemon juice who all show considerably decreased pH levels. When a HCl control was made up however the bacteria survived extremely well living in pH equivalent of 12.5% lemon juice concentration, 9.325% greater then with lemon juice itself. This variation between lemon juice and HCl was proven to be statistically significant using a 2 sample T test which showed a p value of 0.036 for E75U382259 the resistant strain and 0.059 for NCTC 1216 which was the susceptible strain showing that lemon juice is causing the effect by a mode of action other than acidity.

Lemon juice was then carried out on a checkerboard analysis to see if there was any synergistic effects between lemon juice and gentamicin especially as gentamicin is known to have a synergistic effect with other antibiotics such as amoxacillin, vancomycin and ceftriaxone.

As lemon juice may be acting by a method which affect RNA synthesis then it follows a similar mode of action to those tradition antibiotics and is therefore a good candidate to help boost the efficacy of gentamicin.

Further to this is the synergistic effects shown with gentamicin with the growing resistance to gentromycin as well as many of the antibiotics traditionally used in combination therapy with gentamicin. lemon juice if developed into a safe form for application could provide a valuable therapy especially for those surfing already from nephropatholgical conditions which give them a lesser ability to handle high doses of gentamicin. Further to this is that the highest incidence rate of gram negative bacteria is in children of 1 year of age which can themselves only handle reduced dosing of antibiotics and therefore this synergy may prove highly beneficial for infections in for this group of the population.
Future study

From the information and results analysed above there are a number of areas that would benefit from future study to further investigate the antimicrobial effect of lemon juice and its synergistic effect of lemon juice of gentamicin. As the antimicrobial effect of lemon juice was not due to the acidic environment it would be wise to investigate which of the constituent parts of lemon juice itself has the antimicrobial effect. This would most likely require analysis with atomic absorption and or mass spectrometry to ascertain the exact composition of the lemon juice. It has also been suggested that the antimicrobial effects of lemon juice although not caused by the acidic conditions directly may be activated by the acidic conditions of the lemon juice and therefore if more time had been available then it would have attempted to neutralise the acid to investigate and change in antimicrobial activities. Similar to honey it has been seen that honey that looses its acidic properties has a significantly declined antimicrobial effect. However if it was found that lemon juice maintained antimicrobial properties without the acidic conditions would it be possible to apply this to a wound directly either as solution on infused into a wound dressing as has been tested in the US for Manuka honey.

Conclusion

In conclusion it has been shown that there is a significant difference between the antimicrobial effect of lemon juice and the HCl control on both strains, E75U382259 and NCTC1216. This proves that the anti microbial effect is due to factors other then pH. With the significant difference between the bacterial strains showing no significant difference in response to lemon juice but a significant difference in response to gentamicin it shows that lemon juice acts via a different mode of action to gentamicin. However, still shows synergistic effects which show promise in future, for helping with vulnerable patient groups such as very young children and those with kidney damage or disease. With future research it is hoped that a clinical use for lemon juice can be found similar to that of Manuka honey in wound dressings for cutaneous wound
infections. In all, our hypothesis has been proved that lemon juice has an antimicrobial effect and does show synergy when combined with the antibiotic gentamicin.

References