ISSN 0974 – 5211

Journal of Natural Products
Volume 4 (2011)
www.JournalofNaturalProducts.com

Research Paper

Antibacterial Efficacy of Chitosan, Manuka Honey and Chlorophyll against *Klebsiella Pneumoniae*

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(Received 13 January 2011; Revised 13 January-09 March 2011; Accepted 12 March 2011)

ABSTRACT

Klebsiella pneumoniae is one of the common gram-negative bacteria causing community and hospital acquired infections. Multi drug resistant *K. pneumoniae* has been a persistent threat; this compels the search for alternate antibacterial agents. Chitosan, manuka honey and chlorophyll have received much attention after extensive research was conducted to investigate antibacterial properties and applications in the field of biomedicine and pharmaceutics. A preliminary study was undertaken to compare the antibacterial effect of chitosan, manuka honey and chlorophyll against *K. pneumoniae.* Stock solutions of chitosan, manuka honey and chlorophyll were prepared at 1% w/v concentration. The primary methods used in this investigation were minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). From the study, it was shown that chitosan exhibited MIC breakpoint at 175µl/ml and MBC at 425µl/ml. against *K. pneumoniae.* Manuka honey and chlorophyll however, did not show significant antibacterial effect at 1% w/v due to the degree of dilution but were found to be effective at 100% w/v. Chitosan demonstrated promising results to use as the alternate antibacterial agent against multi-drug resistant *K. pneumoniae.* Chitosan can also be incorporated in hand gloves, coating agent on medical equipments and invasive devices to discourage the adherence of *K. pneumoniae*.

Keywords: Chitosan; Biofilm; *K.pneumoniae*; Honey; Chlorophyll.

INTRODUCTION

Klebsiella pneumoniae is among the most common gram-negative bacteria known medically to be an important pathogenic bacterium that is opportunistic in nature (Wen-Chien Ko, et al., 2002). The majority of *K. pneumoniae* infections are hospital acquired causing urinary tract infection, bacteremia, nosocomial pneumonia, diarrhea and intraabdominal infection (Podschun and Ullmann, 1998). In addition, *K. pneumoniae* is reported as a potential community acquired pathogen (Anita, et al., 1985). The extended spectrum beta lactamase (ESBL) contributes to the multi drug resistance of *K. pneumoniae.* Christian Giske, (2008) highlighted that *K. pneumoniae* is resistant to third generation cephalosporin *viz.*, ceftizoxime, cefotaxime, ceftriaxone, and ceftazidime. Chitosan was first discovered by Rouget in 1859 as a

naturally occurring substance that is omnipresent in crustaceans mainly shrimps and crabs. Chitosan is uniquely abundant and has exceptional properties that are biocompatible, biodegradable and non toxic (Eldin, et al., 2008). Antimicrobial activity of chitosan has been proven against fungi, virus and bacteria (Dina, et al., 2008). Chitosan has more significant activity towards gram-negative bacteria compared to gram-positive bacteria (Chung, 2004).

Manuka honey is derived from the Manuka bush (*Leptospermum scoparium*) in New Zealand. It is known to be among the well-known honey for its antibacterial activity (Atrott and Henle, 2009). In contrast, to conventional honey, Manuka honey has unique antibacterial activity contributed by its non-peroxide factors resulting in high levels of antibacterial activity. Manuka honey is known to retain its antimicrobial activity despite the presence of heat and catalase, thus, making it known as non-peroxide honey (Molan, 1992).

Chlorophyll is a photosynthetic pigment commonly found in green plants and is extracted using acetone from green plants specifically alfalfa due to the high content of chlorophyllin (Chernomorsky, and Segelman, 1988). It is proven over the years to have antibacterial and bacteriostatic properties against Staphylococci, Streptococci and anaerobic spore forming bacteria (Smith, 2008).

The aim of this study was to compare the efficacy of chitosan, manuka honey and chlorophyll against *K. pneumoniae* using the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) method. The three naturally occurring compounds could be of great importance as it can be used as an effective alternate antibacterial against multi-drug resistant *K. pneumoniae*.

MATERIALS AND METHODS

Bacterial Strain: *K. pneumoniae* was obtained from Consolidated Laboratory Malaysia.

Chitosan powder: Chitosan powder was purchased from Eastern Global Limited Malaysia.

Manuka honey: Commercially prepared manuka honey MG 100+ was purchased from Summer Pacific Limited Malaysia.

Chlorophyll: 100% liquid chlorophyll was purchased locally from Natural Health Farm Limited Malaysia.

***Preparation of inoculum*:** The standard bacterial strain of *K. pneumoniae* was inoculated in sterile nutrient broth and was incubated at 37^0C degree Celsius for 24 hours. The turbidity of the broth was matched with McFarland's standard solution to obtain a bacterial inoculum of 10^8 (colony forming unit) CFU/ml.

***Preparation of chitosan stock solution*:** Chitosan stock solution was prepared by adding 2.5g of powdered chitosan to 50ml of acetic acid and left overnight. After chitosan was dissolved, 200ml of methanol was added to dilute the dissolved chitosan. Further, the solution was stirred for several hours and filtered to produce 1% w/v chitosan solution (Marc, et al., 1980).

***Preparation of manuka honey stock solution*:** Honey stock solution was prepared by adding 2.5g of honey to 250ml of water and stirred to dissolve honey.

***Preparation of chlorophyll stock solution*:** Chlorophyll stock solution was prepared by adding 2.5g of chlorophyll to 250ml of water and stirred to dissolve chlorophyll.

***Minimal Inhibitory Concentration (MIC*):** The MIC is defined as the lowest concentration of chitosan, manuka honey and chlorophyll that is able to inhibit the

growth of *K. pneumoniae*. A series of 20 test tubes were prepared. Each test tube was added with 5ml of Muller Hinton broth and calibrated with 100µL of microbial suspension. Different concentration of chitosan, manuka honey and chlorophyll ranging from 25µl/ml to 500µl/ml was added to each test tube. The wide dilution range was considered to compare the efficacy. The tubes were incubated at 37^0C for 24h. After incubation, turbidity of each tube was visually inspected. Clear test tube indicated break point (Mackie and McCartney, 1996).

***Minimal Bactericidal Concentration (MBC)*:** Suspension from selected incubated test tubes was inoculated on nutrient agar plate using a sterile cotton swab. The plates were incubated at 37^0C for 24h and bacteriostatic and bactericidal effect was recorded.

RESULTS

In this study, the efficacy of chitosan, manuka honey and chlorophyll against *K. pnuemoniae* was determined using the MIC and MBC values.

Break point of chitosan was observed at 175µL/ml. 1% Manuka honey and Chlorophyll did not exhibit efficacy from 25µl/ml to 500µl/ml but exhibited efficacy at 100% concentration (Table 1).

Table–1: Minimal Inhibitory Concentration (MIC) against *K. pneumoniae*.

Samples	Minimal Inhibitory Concentration (µl)									
	25	**50**	**75**	**100**	**125**	**150**	**175**	**200**	**225**	**250**
Chitosan	+ -	+ -	+ -	+ -	+ -	+ -	- -	- -	- -	- -
Manuka honey	+ -	+ -	+ -	+ -	+ -	+ -	+ -	+ -	+ -	+ -
Chlorophyll	+	+	+	+	+	+	+	+	+	+
Samples	**Minimal Inhibitory Concentration (µl)**									
	275	**300**	**325**	**350**	**375**	**400**	**425**	**450**	**475**	**500**
Chitosan	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
Manuka honey	+ -	+ -	+ -	+ -	+ -	+ -	+ -	+ -	+ -	+ -
Chlorophyll	+	+	+	+	+	+	+	+	+	+

- (+) indicates turbid test tubes; (-) indicates clear test tubes.

Chitosan exhibited MBC at 425µL/ml. No significant results were recorded for 1% manuka honey and chlorophyll (Table 2).

Table-2: Minimal Bactericidal Concentration (MBC) against *K. pneumoniae*.

Samples	Minimal Bactericidal Concentration (µl)									
	25	**50**	**75**	**100**	**125**	**150**	**175**	**200**	**225**	**250**
Chitosan	+	+	+	+	+	+	+	+	+	+
Manuka honey	+	+	+	+	+	+	+	+	+	+
Chlorophyll	+	+	+	+	+	+	+	+	+	+
Samples	**Minimal Bactericidal Concentration (µl)**									
	275	**300**	**325**	**350**	**375**	**400**	**425**	**450**	**475**	**500**
Chitosan	+	+	+	+	+	+	-	-	-	-
Manuka honey	+	+	+	+	+	+	+	+	+	+
Chlorophyll	+	+	+	+	+	+	+	+	+	+

- (+) indicates turbid test tubes; (-) indicates clear test tubes.

DISCUSSION

Chitosan inhibits bacteria activity by penetrating the nucleus of the bacteria and inhibiting RNA and protein synthesis. Chitosan also exerts its antibacterial activity by acting as a chelating agent. It removes metals, trace elements or essential nutrients from bacteria causing distortion in cell growth and eventually death (Gristina, 1987).

Degree of deacetylation, molecular weight, pH value, reaction temperature and salts is the key in determining the degree of inhibition or antibacterial activity of chitosan (Nan Liu, et al., 2005). According to Chen (2002) high degree of deacetylation and chitosan concentration contributes to higher antibacterial activity. Higher degree of deacetylation directly increases electrostatic binding to the membrane and permeability effects (Tasi, et al., 1999). In the present study, degree of deacetylation of chitosan used was in the range of 85-90%. This property may have contributed to chitosan antibacterial efficacy towards *K. pneumoniae* and thus giving a significant minimal inhibitory breakpoint of 175µl/ml.

According to Molan (1992) dilutions of honey ranging from 25 to 0.25%, >50 to 1.5%, 20-0.6% and 50-1.5% showed significant MIC Thus, 1% w/v of manuka honey preparation was considered however, no significant antibacterial activity against *K. pneumoniae* was observed but exhibited efficacy at 100%. Antibacterial activity of manuka honey is contributed by the synergistic effect between methylglyoxal and non-antibacterial components in honey; Antibacterial activity methylglyoxal was proven to be tremendously reduced when it is in water (Molan, 2008). Hence, in this study, manuka honey samples did not exhibit significant antibacterial activity due to the degree of dilution. The volume of water used to obtain 1% w/v of manuka honey stock solution may have reduced the potency of the honey to act as an antibacterial agent.

100% chlorophyll extract was diluted to obtain a 1% w/v stock solution. The concentration used did not show any significant results for both MIC and MBC. Previous studies have stated that the bacteriostatic effect of chlorophyll is minimal (Sheila, 1957). Degree of dilution in this study was at a ratio of 1:100. Inhibition of gram negative bacteria was successful at a lower degree of dilution, this may have led to a decrease in active components which usually act against bacteria and thus, no significant results were obtained at 1% but exhibited efficacy at 100%. This leads to a conclusion that chlorophyll is not effective against gram-negative bacteria at lower concentrations.

Biofilm is threatening in the hospital arena as it evades host immune system and reduces susceptibility to antibiotics (Carlson, et al., 2008). Antibiotic based coatings of devices have been formulated however, efficacy was limited. There is a tremendous increase in the number of antibiotic resistant bacteria. Carlson (2008) found that chitosan coated surfaces showed a decline in biofilm viable cells as much as 95% to 99.9997% compared to control surfaces.

CONCLUSIONS

Manuka honey and chlorophyll did not exhibit antibacterial activity at 1%w/v concentration compared to chitosan. *K. pneumoniae* is susceptible to chitosan in vitro at 175µl/ml while bactericidal activity of chitosan was at 425µl/ml. Cholorphyll is ineffective against gram negative bacteria.

There is an increase in trend of extended spectrum beta lactamases *K. pneumoniae* infection that is worrisome as the number of morbidity and mortality is

increasing. Chitosan can be incorporated into coating of varied medical devices to reduce nosocomial infections especially biofilm. Chitosan may be also incorporated in hand sanitizer and as powdered coating in gloves as it has shown great antibacterial efficacy at low concentration.

ACKNOWLEDGMENTS: We thank the authorities of Management and Science University, Consolidated Laboratory (M) Sdn Bhd. Shah Alam, Selangor. Malaysia for extending requested facilities in commencement and completion of this research.

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