Effectiveness of chitosan from crab shell as antibiotic for *Escherichia coli*

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Effectiveness of chitosan from crab shell as antibiotic for *Escherichia coli*

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Abstract. Background: Research in finding a safe, effective and natural ingredient against *Escherichia coli* continues to be carried out as a result of an increasing antibiotic resistance. Chitosan has been known as a food preservative but the research for its effectivity against Eschericia coli is still inadequate. Indonesia is rich of natural animal resources including crustaceans, like Crab. Objective: How effective are chitosans extracted from crab shells as an antibiotic against *Escherichia coli* when compared with ceftriaxone. Method : Experimental method with a *static group comparison* where *E. coli* in Mueller Hinton Agar (MHA) on petri dishes was given chitosan intervention with initiating concentrations starting from 4% to 8% and 30mcg of ceftriaxone given by diffusion. The antimicrobial of *E.coli* was measured with Whatman paper and standard calipers measured in millimeters. Result : Results of normality and homogeneity variance tests with (p>0,05) were inhomogenous and not distributed normally so it was analyzed with Kruskal Wallis Test continued with a Mann Whitney Test. Conclusion : Chitosan solution extracted from crab shells with concentrations of 4%, 5%, and 6% have an antibiotic effect against the growth of *E.coli*. The most effective concentration to inhibit *E.coli* is 4%.

1. Introduction

Infectious diseases are results of an increasing morbidity and mortality in human especially in developing countries including Indonesia [1]. The prevalence and distribution of infectious diseases in human gastrointestinal tract remains being a world-wide problem, especially the ones caused by poor hygiene and sanitation. Food contact with feces contamination is one of the reasons for human infection [2]. Digestive tract infection is caused by various living creatures, including microbes. Eventhough Eschericia coli (*E.coli*) is one of the digestive system's normal flora, as soon as it exists elsewhere, it will turn into a pathogen [3,4]. Based on the research on *E.coli*, it is also known as a common cause for urinary tract infection [5]. *E.coli* also causes various infections, like acute meningitis, pneumonia, intra-abdominal infection, and other organ infections [3,4,6,7].

A variety of research to find a new and effective antibiotic against *E.coli* are held as a result of a wide resistency it has [7,8]. A variety of research say that Enterobacteriae, including *E.coli* is resistant against a wide range of antibiotic classes, like penicillin, ampicillin, pipericillin, from quinolone, nalidixid acid and from the cephalosporin group which are cefalothin and cefuroxime [7–11]. The effectivity of cephalosporins which is ceftriaxone against gram negative bacteria is 82%-95% and against gram positive bacteria is 67%-90% [9]. Therefore, right now in hospitals, ceftriaxone is the mostly used drug as an antibiotic against UTI, but the massive and uncontrolled use of this drug could cause resistancy against *E.coli* [12]. Ceftriaxone is a broad-spectrum third generation cephalosporin

class antibiotic that could reach the central nervous system is stabile against beta lactamase and still believed to be effective against gram negative bacterias including Enterobacteriaceae [9,9,12–14]. Ceftriaxone has a long half-life and this is one of the special things about it. It also has a really high bioavailability in the form of injection, making it a primadona against meningitis, UTI and digestive tract infection [14,15].

Chitosan is one of the interesting things to do a deep research on as an alternative to antibiotic alternative, especially chitosan which comes from shells which is widely available in Indonesia. The results of the shell waste can be precessed into flour through various processes, resulting in Chitosan [16]. Nowadays chitosan is modified in physiochemistry and biological ways to turn it to a raw material in some fields like waste, water, agriculture, cloth and textile, cosmetics, nutritional supplement, and food processing [17,18]. Aside from its low toxicity, allergenity, biocompatibility, biodefradibility and bioactivity makes chitosan an interesting substance for a wide range of application as a biomaterial in pharmacy and medicine [19]. Chitosan is also widely known for fighting against microorganisms, where the antimicrobe mechanism works by ionic interaction through bacterial cell wall. This interaction causes bacterial cell wall peptidoglycan hydrolysis which then provocates intracellular electrolyte leakage resulting in the death of the microorganism. Chitosan's antibacterial activity is influenced by viscocity. The antibacterial activity of chitosan against *E.coli* is suspected to increase when chitosan's molecular weight decreases, it is because chitosans with a low molecular weight enters microbes easier through diffusion where it then disrupts the cell's metabolism [20]. Based on the description above, it is important to do a research about the difference antibiotic effectiveness between chitosans extracted from crab shells at concentrations of 4%, 5%, 6%, 7% and 8% with ceftriaxone against *E.coli* through the process of diffusion.

2. Method

This experiment is designed by laboratorium experimental with *post test only control group design* in a *static group comparison* which observation is done after the treatment group which is *E.coli* inside a Mueller Hinton Agar (MHA) is given chitosan intervention with concentrations starting from 4%-8% and 30 mcg of ceftriaxone. Chitosan from crab shells are obtained from Indonesia Cirebon Bio Chitosan Company in a form of pure powder. Chitosan powder is taken from 4 gr, 5gr, 6 gr, 7 gr, 8 gr and diluted with 1% acetic acid 100 ml each, stirred with a magnetic stirrer, then the result will be a gel chitosan with 4%, 5%, 6%, 7% and 8% concentration. Ceftriaxone antibiotic is obtained from pharmacies in a form of injection powder, then diluted with a pro injection aqua until it is 30 mcg by weight [21].

E.coli ATCC 25922 bacterial colony are obtained from microbiology lab of Faculty of Medicine, Univesity of Muhammadiyah Sumatera Utara. The culture of *E.coli* is done by taking the colony with an ose method which was sterilized with a Bunsen burner and had been cooled down, then it is inserted inside a petri dish with MHA media. Then it is incubated at 37^{o}C for 24 hours. The measurement of antimicrobial sensitivity (diffusion) is done by preparing an agar plate and petri dish which contains *E.coli* colony. Preparing a 6,28mm disc paper made out of Whatman. Each disk is heated at 70c for 15 minutes in an oven for sterilization. Then the sterilized disc paper is inserted in a testing material with a volume of 1ml for 1-5 munites so that the solution will be absorbed by the disc paper. Then prepare the agar plate which contains *E.coli* colony. Dip a cotton swab in a liquid media filled with *E.coli* colony. Then spread equally on an MHA surface. Disc paper with 4%, 5%, 6% 7%, and 8% concentrations are placed on the surface of the agar that has been planted with *E.coli*, with a sterilized pipette, then it is placed on MHA while pressing it a little in order for it to stick well, incubate at 37C for 18-24 hours, then measure the antimicrobial zone in a disc paper with a caliper in millimeter unit [22].

Sample is then taken with a Federer formula : (n-1) (t-1) >_ 15, where n is the sample size and t is the amount of experimental groups [23]. t in this experiment is 6 groups made up of *E.coli* colony. *E.coli* is treated with 4% chitosan as a first treatment (P1), *E.coli* colony is given 6% chitosan treatment of 5% as a second treatment(P2), *E.coli* colony is given a 6% chitosan treatment as a third

treatment(P3), *E.coli* colony is given a 7% chitosan treatment fourth(P4), *E.coli* colony is given 8% chitosan fifth (P5), then it is given ceftriaxone for the sixth treatment (P6). Sample for each group is obtained:

$(n-1)\ (t-1) \geq 15$
$(n-1)\ (6-1) \geq 15$
$N \geq 4$

Where each groups are made at least 4 samples, the total sample used for this experiment are 24 samples.

2.1. Experimental design

The study has been designed and developed as shown in figure 1. It consists of chitosans extracted from crab shells at concentrations of 4%, 5%, 6%, 7% and 8%, *E.coli* ATCC 25922 bacterial colony, *E. coli* culture, *E. coli* incubation, grouping, incubation at 37ºC for 24 hours, measurement of antimicrobial sensitivity, diffusion, prepare the agar plate which contains *E.coli* colony, incubate again at 37C for 18-24 hours, then measure the antimicrobial zone in a disc paper with a caliper in millimeter unit.

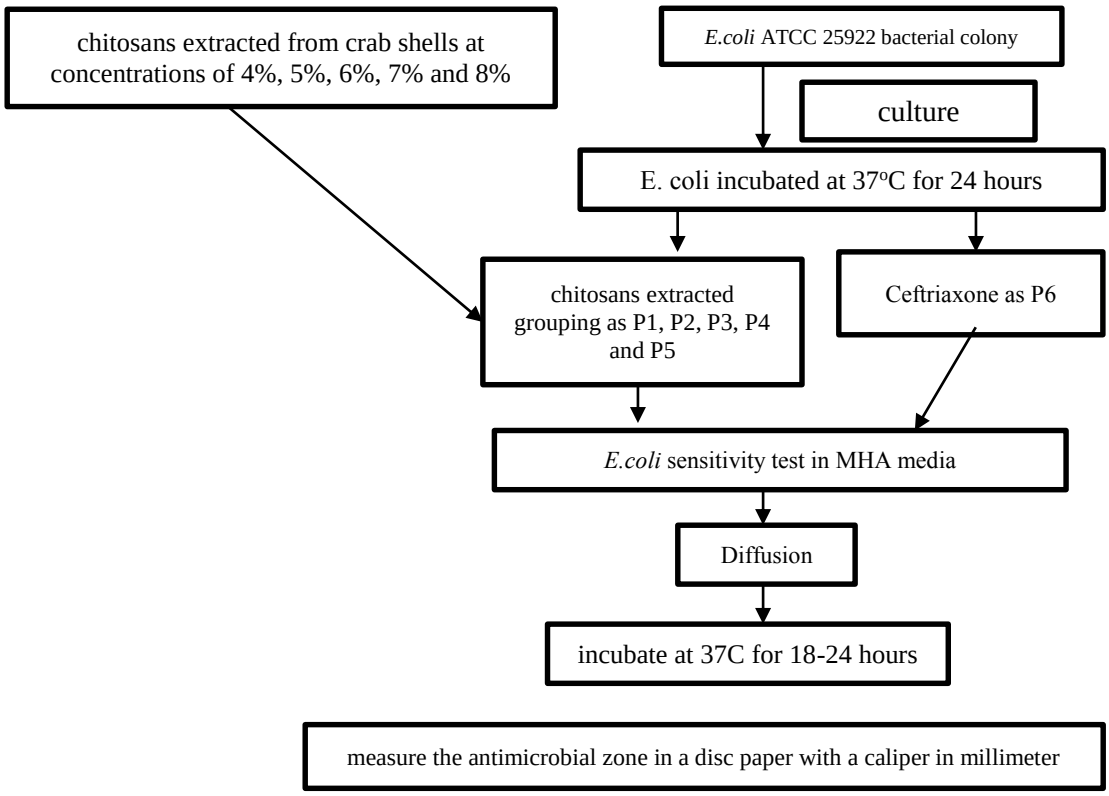

Figure 1. Experimental apparatus

2.2. Data Analysis

The ability of *E.coli* inhibition is measured by the length of clear zone surrounding the disc paper on every group. Chitosan's experiment effectivity which results from crab shell against *E.coli* is analyzed with a computer statistics, to see a significant effectivity from each disc with 30 mcg of ceftriaxone and paper disc which contains chitosan with concentrations of 4%, 5%, 6%, 7%, and 8%. The data on this research is an unpaired category variables which are variables made out of two unpaired groups. Data obtained will be tabulated then normality and varians homogeneity tests are done with ($p>0,05$). If the data obtained are homogenous and distributed normally then parametric test is done with a One

Way Analysis of Variant (ANOVA) test. While if the data isn't distributes normally or it is not homogenous, then the data is analyzed with a non parametric test which are Kruskal Wallis Test continued with Mann Whitney Test.

3. Results

This experiment is done in chemistry and microbiology labs of University of Muhammadiyah Sumatra Utara. Measurement is done with a caliper in millimeter unit.

Table 1. The result of antimicrobial *E.coli* by chitosan and ceftriaxone

Repetition	antibacterial activity (mm)					
	chitosan and ceftriaxone					
	4%	5%	6%	7%	8%	ceftriaxone
1	12.85	12.64	11.00	7.58	8.10	12.04
2	16.04	12.59	12.13	10.20	8.83	10.75
3	12.60	10,71	10.67	8.85	8.95	10.59
4	15.42	11.85	10.98	10.12	9.21	11.29

In table 2 it is concluded that giving a variety of chitosan concentration from crab shell gives different results clear zones.

Table 2. Analysis result of Normality Shapiro-Wilk test and Homogeneity test

Group	Normality Shapiro-Wilk	Homogeneity
Chitosan 4%	0.202	0.004
Chitosan 5%	0.273	
Chitosan 6%	0.146	
Chitosan 7%	0.344	
Chitosan 8%	0.447	
Ceftriaxone	0.532	

Note: 0,004 is obtained from the data homogeneity test which means the data isn't distributed normally and not homogenous.

Table 3. The result of *Kruskall-Wall* analysis with the average and deviation standard

Group	n	Deviation average	P
Chitosan 4%	4	14.22±1.75	0.002
Chitosan 5%	4	11.94±0.90	
Chitosan 6%	4	11.19±0.64	
Chitosan 7%	4	9.18±1.27	
Chitosan 8%	4	8.77±0.47	
Ceftriaxone	4	11.16±0.65	

P<0,05 is obtained from the Kruskall wallis test which proves that every treatment tested has a different antimicrobial zone resulting from 4%, 5%, 6%, 7%, and 8% chitosan concentrations and ceftriaxone. With Mann Whitney test, the result between ceftriaxone and chitosan 4% -8% with ceftriaxone shows that ceftriaxone compared to 8% chitosan p<0,05 is obtained which means there is a difference in the antimicrobial effect between ceftriaxone and chitosan 8%

Table 4. Result from Mann Whitney test chitosan with ceftriaxone

Group	n	P	Remark
Chitosan 4%	4	0.021	Signifikan
Chitosan 5%	4		
Chitosan 6%	4		
Chitosan 7%	4		
Chitosan 8%	4		
Ceftriaxone	4		

From the data processing and data analysis result it is shown that there is a significant difference between ceftriaxone and 4% -8% chitosan.

4. Discussion

In this experiment, the highest average antimicrobial zone is the one with a concentration of 4% which is 14.22 mm, categorized as effective. In 5% chitosan concentration, antimicrobial zone obtained is 11.94 mm categorized as intermediate. In 6% chitosan concentration 11.19mm antimicrobial zone is obtained, categorized as intermediate. In 7% chitosan concentration, 9.18% antimicrobial zone is obtained, categorized as uneffective. In 8% chitosan concentration, 8.77% antimicrobial zone is obtained, categorized as uneffective. Those results show that chitosan could inhibit the growth of *E.coli* if the smallest concentration is used, which is 4%, with an antimicrobial zone of 14.22% which is categorized as sensitive. It is suspected that the lower the chitosan solution concentration, the lower the solution viscocity, resulting in an easier diffusion process in the *E.coli*-cultured media [24,25].

That statement is strengthened by the experiment done on chitosan concentration effect on the antibacterial activity with an agar diffusion method which shows that the antimicrobial zone obtained from the effects of chitosan on *E.coli* growth are 0,2%, 0,4%, 0,6%, and 0,8% is 31,53mm, 21,57mm, 16,97mm, and 14,23mm. That is because the molecular size of higher viscosity is larger as the concentration is higher [24]. 0,2% chitosan solution has the highest inhibitive ability. That is because the viscosity of the solution is still low, resulting in an easier diffusion through the media which has been cultured by E-coli. While 0,8% chitosan has the lowest antimicrobial ability. It is because of the higher viscosity of the solution, making it unable to diffuse well [25].

The amine group (-NH2) which is contained inside the chitosan could give antimicrobial effects because it has a highly reactive postive ion, making it able to bind with bacterial cell wall which has a negative ion. Aside from that, -NH2 also has a free electron pair, resulting in this group being able to pull Ca2+ mineral which can be found in bacterial cell wall [26]. The outer layer of lipopolysaccharide found in gram negative bacteria has an extremely reactive pole against chitosan [20,26].

5. Conclusion

Chitosan solution from crab shells with concentrations of 4%, 5%, 6%, has antibiotic effects against the growth of *E.coli*. While in 7%, 8% chitosan solution concentrations have no antibiotic effects because there are no inhibitive respond against *E.coli*. Each chitosan solution affects *E.coli* growth. Minimal concentration of the solution which is 4% has an antimicrobial zone of 14.22 mm. The most effective concentration in inhibiting *E.coli* growth is 4% with an average of 14.22 mm with a high antimicrobial response compared to the other concentrations. 5% chitosan solution with an antimicrobial zone of 11.94 mm has no inhibitive response. 6% chitosan solution with an antimicrobial zone of 11.19mm has no antimicrobial response. 7% chitosan with an antimicrobial zone of 9.18mm has no inhibitive response and 8% chitosan with an antimicrobial zone of 8.77mm has no antimicrobial zone.

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References

[1] World Health Organisation (WHO) 2014 Antimicrobial resistance: global report on surveillance (2014) *Bull. World Health Organ.*
[2] Nerín C, Aznar M and Carrizo D 2016 Food contamination during food process *Trends Food Sci. Technol.* **48** 63
[3] Fegan N, Gobius K S and Dykes G A 2014 Pathogenic *Escherichia coli Encyclopedia of Meat Sciences*
[4] Poolman J T 2016 *Escherichia coli International Encyclopedia of Public Health*
[5] Fasugba O, Gardner A, Mitchell B G and Mnatzaganian G 2015 Ciprofloxacin resistance in community- and hospital-acquired *Escherichia coli* urinary tract infections: A systematic review and meta-analysis of observational studies *BMC Infect. Dis.* **15** 545
[6] Croxen M A, Law R J, Scholz R, Keeney K M, Wlodarska M and Finlay B B 2013 Recent advances in understanding enteric pathogenic *Escherichia coli Clin. Microbiol. Rev.* **26** 822
[7] Von Baum H and Marre R 2005 Antimicrobial resistance of *Escherichia coli* and therapeutic implications *Int. J. Med. Microbiol.* **295** 503
[8] Peubez I, Chaudet N, Mignon C, Hild G, Husson S, Courtois V, De Luca K, Speck D and Sodoyer R 2010 Antibiotic-free selection in *E. coli*: New considerations for optimal design and improved production *Microb. Cell Fact.* **9** 65
[9] Niranjan V and Malini A 2014 Antimicrobial resistance pattern in *Escherichia coli* causing urinary tract infection among inpatients *Indian J. Med. Res.* **139** 945
[10] Vernaz N, Huttner B, Muscionico D, Salomon J L, Bonnabry P, López-Lozano J M, Beyaert A, Schrenzel J and Harbarth S 2011 Modelling the impact of antibiotic use on antibiotic-resistant *Escherichia coli* using population-based data from a large hospital and its surrounding community *J. Antimicrob. Chemother.* **66** 928
[11] Reinthaler F F, Posch J, Feierl G, Wüst G, Haas D, Ruckenbauer G, Mascher F and Marth E 2003 Antibiotic resistance of *E. coli* in sewage and sludge *Water Res.* **37** 1685
[12] Hsu L Y, Tan T Y, Tam V H, Kwa A, Fisher D A, Koh T H, Krishnan P, Chiew Y F, Jureen R and Tee N 2010 Surveillance and correlation of antibiotic prescription and resistance of gram-negative bacteria in Singaporean hospitals *Antimicrob. Agents Chemother.* **54** 1173
[13] Lee S G, Su Z Z, Emdad L, Gupta P, Sarkar D, Borjabad A, Volsky D J and Fisher P B 2008 Mechanism of ceftriaxone induction of excitatory amino acid transporter-2 expression and glutamate uptake in primary human astrocytes *J. Biol. Chem.* **283** 13116
[14] Kim B N, Peri A M and Paterson D L 2017 Ceftriaxone *Kucers the Use of Antibiotics: A Clinical Review of Antibacterial, Antifungal, Antiparasitic, and Antiviral Drugs, Seventh Edition*
[15] Scarborough M, Gordon S B, Whitty C J M, French N, Njalale Y, Chitani A, Peto T E A, Lalloo D G and Zijlstra E E 2007 Corticosteroids for Bacterial Meningitis in Adults in Sub-Saharan Africa *N. Engl. J. Med.* **357** 2441
[16] Nur Ramadhana, Dewi Safitri S D and M F 2016 Isolasi Kitosan Dari Limbah Cangkang Kepiting Bakau (*Scylla serrata*) Dan Aplikasinya Terhadap Penyerapan Trigliserida *Fak. Farm. Univ. Muslim Indones. Makassar* **8** 20
[17] Croisier F and Jérôme C 2013 Chitosan-based biomaterials for tissue engineering *Eur. Polym. J.* **49** 780
[18] Zhao L M, Shi L E, Zhang Z L, Chen J M, Shi D D, Yang J and Tang Z X 2011 Preparation and application of chitosan nanoparticles and nanofibers *Brazilian J. Chem. Eng.* **28** 353
[19] Şenel S and McClure S J 2004 Potential applications of chitosan in veterinary medicine *Adv.*

Drug Deliv. Rev. **56** 1467
[20] Goy R C, Morais S T B and Assis O B G 2016 Evaluation of the antimicrobial activity of chitosan and its quaternized derivative on *E. coli* and *S. aureus* growth *Brazilian J. Pharmacogn.* **26** 122
[21] Bigelow P 2010 How To Make Standard Solutions for Chemistry *Hell Creek Life*
[22] Shiloach J and Fass R 2005 Growing *E. coli* to high cell density - A historical perspective on method development *Biotechnol. Adv.* **23** 345
[23] Taylor J E 2006 A Gaussian kinematic formula *Ann. Probab.* **34** 122
[24] Rinaudo M, Pavlov G and Desbrières J 1999 Influence of acetic acid concentration on the solubilization of chitosan *Polymer (Guildf).* **40** 7029
[25] Liu X F, Guan Y L, Yang D Z, Li Z and Yao K De 2001 Antibacterial action of chitosan and carboxymethylated chitosan *J. Appl. Polym. Sci.* **79** 1324
[26] Martins A F, Facchi S P, Follmann H D M, Pereira A G B, Rubira A F and Muniz E C 2014 Antimicrobial activity of chitosan derivatives containing N-quaternized moieties in its backbone: A review *Int. J. Mol. Sci.* **15** 20800