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INHIBITORY TEST OF GENTAMICIN ANTIBIOTICS AGAINST *Escherichia coli* AND *Staphylococcus aureus* BACTERIA USING DISC METHOD

UJI DAYA HAMBAT ANTIBIOTIK GENTAMISIN TERHADAP BAKTERI Escherichia coli DAN Staphylococcus aureus MENGGUNAKAN METODE CAKRAM

Fajar Husen[1)*], Nuniek Ina Ratnaningtyas[2)]

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Authors affiliation:

[1)]Department of Medical Laboratory of Technology, Bina Cipta Husada College of Health Science, Banyumas, Central Java, Indonesia

[2)]Department of Biology, Faculty of Biology, Jenderal Soedirman University, Banyumas, Central Java, Indonesia

Correspondence email:

*fajar@stikesbch.ac.id

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ABSTRACT

Escherichia coli and Staphylococcus aureus are bacteria that often cause health problems and diseases in humans, such as digestive (diarrhea) and skin (boils) disorders. Gentamicin is one of the most widely used antibiotics, which belongs to the aminoglycoside group. It is often used for treating minor bacterial and moderate fungal infections. Therefore, this study aims to determine the inhibition and effectiveness of gentamicin in inhibiting the growth of E. coli and S. aureus as well as to assess the pattern of sensitivity of these bacteria to the antibiotic. A completely randomized design method was used with one-way ANOVA, and further testing was carried out using Duncan's test. A total of 10 experimental units were used for each bacterium with two replications, after which the data were analyzed using SPSS v.25.0 software. The results showed that gentamicin could inhibit the growth of E. coli with the lowest and highest ZH values of 17 mm and 22.5 mm, respectively. It also had an inhibitory effect on S. aureus with the smallest ZH value of 15.5 mm and highest of 18.5 mm. The percentage of gentamicin inhibition against E. coli and S. aureus was 90% and 60% sensitive. Furthermore, the percentage of intermediate inhibition category for E. coli was 10% and 40% for S. aureus.

Keywords: *antibiotics, gentamicin, Escherichia coli, Staphylococcus aureus, disc method*

ABSTRAK

Bakteri Escherichia coli dan Staphylococcus aureus merupakan bakteri yang seringkali menyebabkan gangguan kesehatan dan penyakit pada manusia, seperti gangguan pencernaan (diare), atau gangguan kulit (bisul). Gentamisin merupakan salah satu antibiotik yang banyak digunakan dari golongan aminoglikosida. Gentamisin banyak digunakan sebagai antibiotik baik untuk infeksi ringan karena bakteri atau infeksi sedang akibat infeksi jamur. Tujuan penelitian ini adalah untuk mengetahui daya hambat dan efektivitas gentamisin dalam menghambat pertumbuhan E. coli dan S. aureus serta mengetahui pola kepekaan bakteri tersebut terhadap gentamisin. Metode penelitian ini adalah rancangan acak lengkap dengan one way ANOVA, dan uji lanjut menggunakan Duncan's test, dengan total unit percobaan adalah 10 untuk masing-masing bakteri dengan 2 ulangan, kemudian data dianalisis dengan software SPSS v.25.0. Hasil penelitian menunjukkan bahwa antibiotik gentamisin dapat menghambat pertumbuhan E. coli dan S. aureus. Gentamisin menghambat pertumbuhan E. coli dengan nilai zona hambat (ZH) terkecil 17 mm, dan tertinggi 22,5 mm, serta menghambat pertumbuhan S. aureus dengan nilai ZH terkecil 15,5 mm dan tertinggi 18,5 mm. Persentase inhibisi gentamisin terhadap E. coli 90% sensitif, dan teradap S. aureus 60% sensitif. Persentase kategori inhibisi intermediet gentamisin terhadap E. coli 10% dan S. aureus 40%.

Kata kunci: *antibiotik, gentamisin, Escherichia coli, Staphylococcus aureus, Metode Cakram*

INTRODUCTION

Antibiotics are often used by the community to treat bacterial infections, but inappropriate usage can cause resistance to certain bacteria, thereby leading to a decrease in the effectiveness or ability of the drugs. Furthermore, this resistance needs to be avoided to ensure that the effectiveness of treatment in infected patients is optimal [1]. Apart from the increase in bacterial infections, attention must also be paid to the emergence of various types of antibiotics as well as the increase in their doses. Further studies are also needed to evaluate and investigate the ability of these drugs against bacteria.

Staphylococcus aureus is a gram-positive bacteria that is often found in the human body as normal flora, especially on the skin. After culturing in artificial media and observing using a microscope, it has a grape-like shape with clusters or spherical (coccus) [2]. *E. coli* also has a similar appearance with a round shape (coccus), but it is stained red during gram staining due to the absorption of safranin, hence, it is classified as a gram-negative bacteria [3]. Meanwhile, *S. aureus* is classified as a gram-positive bacteria due to its purple/violet appearance after the staining process [4]. One of the common diseases caused by *E. coli* is diarrhea, while *S. aureus* causes skin diseases, such as ulcers, infection due to wounds, as well as worsening inflammation and acne. In some cases, it can cause mastitis and infections of the human urinary tract [5].

Evaluation, susceptibility, and inhibition testing needs to be carried out on antibiotics that have been widely traded and commercialized to determine their effectiveness [6]. Several methods and techniques have been developed to test these drugs, and the WHO (World Health Organization) suggested that antibiotics developed in laboratories can be used by the public [3]. A similar recommendation was also given by the CLSI or clinical and laboratory standards institute that they need to be evaluated and routinely investigated for their effectiveness in inhibiting the growth and development of bacteria. One of the antibiotics that has been widely developed is gentamicin [7], which belongs to the aminoglycoside group and can be isolated from *Micromonospora pupurea*. It is also very active against gram-positive and gram-negative bacteria and has similar properties with other aminoglycosides [8].

In this research, a 10 µg doses of gentamicin were tested in order to determine its effectiveness and sensitivity in inhibiting the growth of bacteria, especially *E. coli* and *S. aureus*. Previous studies have shown that excessive use of gentamicin by chicken farmers to treat colibacillosis causes resistance to gentamicin antibiotics, with a decrease in gentamicin sensitivity from sensitive category to intermediate level category (3).

Based on this background, it is very important to monitor and test the effectiveness of gentamicin against *E. coli* and *S. aureus* in order to obtain empirical data related to the status and effectiveness of gentamicin antibiotics so that the public can better understand the importance of using antibiotics wisely to prevent antibiotic resistance from occurring. The test results can also provide information and be used as a reference for the use of gentamicin antibiotics to treat *E. coli* or *S. aureus* bacterial infections based on their sensitivity values.

The increase in disease-causing bacterial infections has led to the widespread development of antibiotics. However, inappropriate usage can cause resistance, which decreases their effectiveness and makes it easier for the bacteria to attack on the next exposure. Resistance is a problem that needs immediate attention to reduce the rate of infection in humans. Antibiotics, such as gentamicin, which are effective in suppressing gram-negative bacteria also need to be evaluated and investigated to determine their inhibitory power against *E. coli* and *S. aureus*.

METHODS

Research design and materials preparations. This research was conducted at the Laboratory of Microbiology, Medical Laboratory of Technology Department, the Bina Cipta Husada College of Health Science. This research method used in this study is an experimental study using a completely randomized design (CRD), with a total of 25 experimental units that consist of 10 experimental units for *E. coli* treatment group, ten experimental units for *S. aureus* treatment group, and five experimental units for the negative control group. The subjects used were disc antibiotic (disc antibiotic) of gentamicin dose of 10 µg, isolates of *Escherichia coli* (EC) bacteria, and *Staphylococcus aureus* (SA), and negative control (DMSO 5%). DMSO 5% solution was prepared by adding 5 mL of pro-analysis DMSO to 95 mL of sterile distilled water. All materials and tools used were sterilized in an autoclave at 121ºC for 60 minutes. The bacterial isolates were obtained from the Bacterial Culture Center, Microbiology Laboratory, Jenderal Sudirman University (UNSOED), Purwokerto.

E. coli, S. aureus **isolates and nutrient agar medium preparations.** Bacterial inoculation was carried out in a laminar airflow (LAF) room. One ose of bacterial isolates were inoculated on lactose broth (LB) media, then incubated for 2-3 days. The culture results on LB media were then inoculated on 6 mL nutrient agar (NA) sloping media to be used as an isolates source for the treatment. Prepared 30 petri dishes containing 9 mL of nutrient agar medium for bacterial growth. Ten petri dishes contained NA were prepared for growing *E. coli* treatment, 10 petri dishes for *S. aureus*, and 10 plates for negative control (DMSO 5%) treatment.

Bacterial inoculation and gentamicin application. The bacterial inoculation was carried out by preparing a 10^{-3} suspension. Furthermore, 1 ose of pure isolate was diluted with 9 mL of sterile distilled water in the first tube, followed by homogenization using a vortex, after which 1 mL was taken. From the 3rd dilution, 100 µL of bacterial suspension was collected and poured into NA media using a pour plate and then leveled with drugalsky. Antibiotic discs were placed on the right and left sides in a petri dish using sterile tweezers. Incubation was then carried out for 2x24 hours, and the inhibition zone observed was measured with a ruler [10].

Observation. The method for evaluating the effectiveness of gentamicin was carried out by macromorphological observations, and measuring the inhibition zone formed around the disc (antibiotic disc) using a ruler (in millimeters), then taking a picture of the inhibition zone.

Data analysis. The data were then analyzed using ANOVA at a 5% confidence level ($p < 0.05$), using SPSS V.25.0 2021 software, and then continued with the Duncan's Multiple Range Test (DMRT). The results were then presented in a histogram and equipped with a notation to show the significance [9].

RESULTS AND DISCUSSION

The results showed that gentamicin could inhibit gram-negative and gram-positive bacteria, namely *Escherichia coli* and *Staphylococcus aureus*, respectively, as shown in Figure 1. The growth of *E. coli* colonies was only concentrated on the outer part of the disc. Furthermore, 100 µL of bacterial suspension with a concentration of 10^{-3}, which was poured on the NA medium showed very dense colony growth. The zone of inhibition was formed and concentrated 15-20 mm around the disc containing the antibiotic. There was no inhibition zone for the negative control group using sterile distilled water, and the disc was overgrown with bacteria.

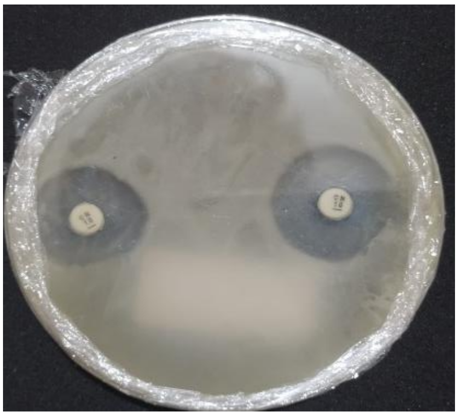

Figure 1. Inhibition activity of gentamicin against *E. coli* bacteria

Figure 2. Inhibition activity of gentamicin against *S. aureus* bacteria

Figure 3. Negative control of DMSO

The results showed that gentamicin could inhibit the activity of *E. coli* and *S. aureus* with a large inhibition zone (ZH), indicated by the clear zone in Figures 1 and 2. Previous studies also revealed that there was no colony growth around the disc, but they appeared and concentrated outside the area formed. [11]. The effectiveness of the ZH can be determined by observing the size of the clear zone formed around the disc containing the antibiotic. This indicated that the bacteria did not grow or die due to the bactericidal activity of the drug [12].

The average size of the inhibition zone formed after gentamicin administration on media containing *E. coli* and *S. aureus* bacteria is presented in Figure 4. The results showed that the ZH formed for each replicate treatment group for *E. coli*. and *S. aureus* varied. The highest value of 22.5 mm was recorded in K4 for *E. coli*, while the lowest of 19 mm was obtained in K1 and K2. Meanwhile, for *S. aureus*, the highest ZH value was 18.5 mm in groups K2 and K4, and the lowest was 15.5 mm in K3.

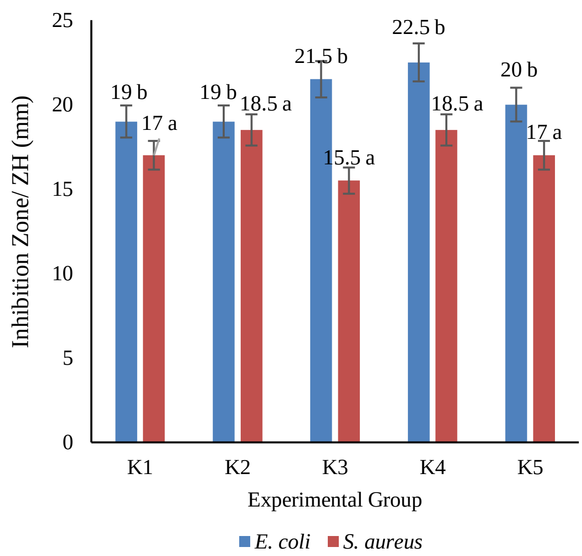

Figure 4. Average zone of gentamicin inhibition against *E. coli* and *S. aureus*

Figure 4 showed that after the analysis, each experimental group and replicates of gentamicin against *E. coli* and *S. aureus* gave significant results ($p < 0.05$). In the experimental groups, both bacteria observed were significantly different. The inhibition zone obtained in this study ranged from 15–23 mm. The greater the ZH value of gentamicin for each experimental group, the higher the effectiveness and inhibitory activity caused by the antibiotic.

Previous studies also showed that gentamicin has a 19% inhibitory power on gram-negative bacteria, namely *E. coli*, while a value of 7% was obtained for gram-positive bacteria, namely *Staphylococcus epidermidis* [13]. Another study revealed that the minimum inhibitory concentration (MIC) was greater against *E. coli* compared to *S. aureus*, namely 128 g/mL and 64 128 µg/mL, respectively [1].

The observation results of the ZH values in each experimental group were then compared with the inhibition categories presented in Table 1.

Table 1. Category of inhibitory ability (inhibition) of gentamicin antibiotics

No	Standard Value	Category
1	≤ 12 mm	Resistant (RT)
2	13 – 16 mm	Intermediate/ Medium (IM)
3	≥ 16 mm	Sensitive (SF)

Table 1 showed the standard inhibition values for gentamicin. It had standard values for the resistant (RT), intermediate (IM), and sensitive (SF) categories with an inhibition zone of < 12 mm, 13-16 mm, and 16 mm, respectively [14]. The ability of gentamicin as an antibiotic has a broad-spectrum effect because it can inhibit gram-positive and gram-negative bacteria. Furthermore, it inhibits bacterial growth by suppressing the growth rate in the log or exponential phase, which involves destroying the peptidoglycan wall. However, its bactericidal activity is often found in gram-negative bacteria [2].

These findings are consistent with previous studies, where gentamicin had high effectiveness in inhibiting the growth and development of *S. aureus*. Its MIC100 value against *S. aureus* was 0.156, which was categorized in a high level. The antibiotic can also inhibit microbial growth in culture placed in petri dishes at a dose of 10 g/mL per disc with an inhibition zone of > 20 mm [15].

Compared with the results in Figure 1, the average ZH value was in the range of 15.5 – 22.5 mm, indicating that it can be included in the intermediate or sensitive category. The category for each experimental unit of gram-negative and positive bacteria using *E. coli* and *S. aureus* was presented in Tables 2 and 3.

Table 2. Value of gentamicin inhibition zone against *E. coli* bacteria

No	Value of n (Experimental Group) (mm)	Standard Value	Category
1	17	≥ 16 mm	SF
2	21	≥ 16 mm	SF
3	22	≥ 16 mm	SF
4	16	13 – 16 mm	IM
5	26	≥ 16 mm	SF
6	17	≥ 16 mm	SF
7	23	≥ 16 mm	SF
8	22	≥ 16 mm	SF
9	21	≥ 16 mm	SF
10	19	≥ 16 mm	SF

Table 2 showed that ZH value for gentamicin in the ten experimental units against *E. coli* ranged from 16–26 mm. Based on the classification in Table 1, where the standard is 13-16 mm, 90% or nine units were in the sensitive category with a ZH value of 16 mm, while 1 unit or 10% were in the intermediate category/medium (IM).

Evaluation and investigation of the effectiveness of antibiotics can provide a better understanding for the community on the right use of the drugs. Antibiotic resistance can cause financial loss as well as death. For example, *S. aureus* resistance to methicillin caused the loss of thousands of dollars [16].

Table 3. Value of gentamicin inhibition zone against *S. aureus* bacteria

No	Value of n (Experimental Group) (mm)	Standard Value	Category
1	20	≥ 16 mm	SF
2	14	13 – 16 mm	IM
3	16	13 – 16 mm	IM
4	21	≥ 16 mm	SF
5	14	13 – 16 mm	IM
6	17	≥ 16 mm	SF
7	20	≥ 16 mm	SF
8	17	≥ 16 mm	SF
9	15	13 – 16 mm	IM
10	19	≥ 16 mm	SF

The categorization results of ZH values in each experimental unit using *S. aureus* with the antibiotic treatment of gentamicin showed more diverse values, as shown in Table 3. The inhibition zone value ranged from 14– 21 mm, and the highest value of 21 mm was obtained in unit 4. Meanwhile, when viewed from the standard, it was in the range of 13-16 mm and then categorized in the intermediate or sensitive group. The categories for

ZH in *S. aureus* were more diverse with a total of 4 and 6 units in the IM and SF groups, respectively. When compared with the percentage data presented in Figure 5, gentamicin was very influential on *E. coli*.

The results also showed that gentamicin has very good effectiveness, which was indicated by the ZH value and the percentage of the inhibition category, namely sensitive to *E. coli* and *S. aureus*. The mechanism of action of the antibiotic is intrinsic, and it involves damage to the peptidoglycan cell wall as well as genetic material. This condition then suppresses the growth and kills the bacteria optimally [17].

Gentamicin is effective in causing autolysis in bacterial cells, which helps to reduce their growth and bacterial infection effects [18]. Previous studies on its sensitivity to *S. aureus* and *E. coli* also showed an inhibition percentage of >40% [16].

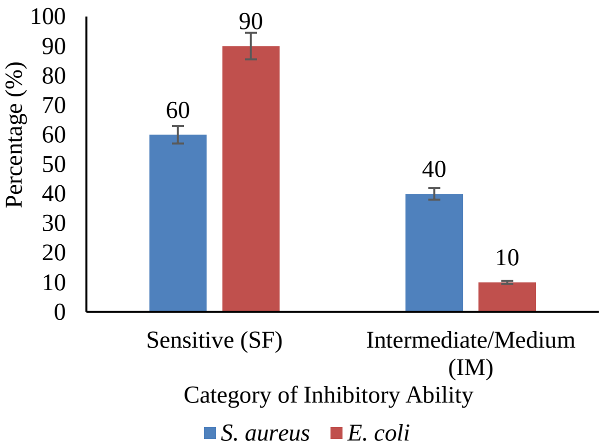

Figure 5. Percentage of gentamicin inhibition ability category against *E. coli* and *S. aureus*

Based on the classification of ZH value in Table 3, gentamycin treatment against *E. coli* was more effective compared to *S. aureus*. Its sensitive (SF) category against *E. coli* was 30% higher than that of *S. aureus*, which was only 40%. The comparison for the intermediate category between *E. coli* and *S. aureus* had a ratio of 1: 4. These findings indicate that the highest effectiveness of gentamicin was against gram-negative bacteria, namely *E. coli*.

Testing for antibiotics based on WHO recommendations are very important and must be carried out intensively. The increasing rate of resistance to these drugs is a problem that must be addressed [19]. The effectiveness of antibiotics in inhibiting the growth of bacteria, especially pathogenic bacteria, can be a good evaluation.

The sensitivity of gentamicin to *E. coli* and *S. aureus* are also in line with previous studies. Furthermore, several studies showed that it has a low resistance value against *E. coli* [16]. It also has excellent bactericidal activity against *S. aureus* and *Pseudomonas aeruginosa*, and its mechanism of action involves degrading and damaging the peptidoglycan cell walls, thereby killing the bacterial cells [18].

CONCLUSION

The activity of gentamicin in inhibiting two types of bacteria, namely gram-negative *E. coli* and gram-positive *S. aureus* is very effective and can be categorized into effects that have a broad spectrum. Furthermore, it inhibited the growth of *E. coli* bacteria with the smallest inhibition zone of 17 mm and the highest of 22.5 mm. The lowest was 15.5 mm for *S. aureus* and the highest was 18.5 mm. The percentage of inhibition ability in the sensitive category for *E. coli* was 90% and 60% for *S. aureus*. In the intermediate category, 10% and 40% values were obtained for *E. coli* and *S. aureus*, respectively. These findings indicate that gentamicin is very effective in inhibiting and suppressing the growth of *E. coli*. Further studies can also be carried out by comparing its effectiveness with that antibiotic.

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REFERENCES

[1] Kusriani RH, Nawawi A, Turahman T (20150 Uji aktivitas antibakteri ekstrak dan fraksi kulit batang dan daun sungkai (*Peronema canescens* Jack) terhadap *Staphylococcus aureus* ATCC 25923 dan *Escherichia coli* ATCC 25922. J. Farm.Galen. 2 (1): 8–14.

[2] Mates SM, Eisenberg ES, Mandel LJ, Patel L, Kaback HR, Miller MH (1982) Membrane potential and gentamicin uptake in *Staphylococcus aureus*. Proc. Natl. Acad. Sci. USA 79 (21): 6693–6697. doi: 10.1073/pnas.79.21.6693.

[3] Mukti A, Harris A, Masyitha D (2017) Resistensi *Escherichia coli* terhadap antibiotik dari daging ayam Broiler di Pasar Rukoh. Jimvet 01 (3): 492–498.

[4] Ramadheni P, Mukhtar H, Prahmono D (2018) Uji aktivitas antibakteri ekstrak etanol daun katuk (*Sauropus androgynus* (L.) Merr) terhadap bakteri *Staphylococcus aureus* dan *Escherichia coli* dengan metode difusi agar. Indones. Nat. Res. Pharm. J. 3 (2): 34–45.

[5] Rachman NO, Prenggono MD, Budiarti LY (2016) Uji sensitivitas bakteri penyebab

infeksi saluran kemih pada pasien diabetes melitus terhadap seftriakson, levofloksasin, dan gentamisin. Berk. Kedokt. 12 (2): 205. doi: 10.20527/jbk.v12i2.1871.

[6] A'lana L, Sari R, Apridamayanti P (2017) Penentuan nilai FICI kombinasi ekstrak etanol kulit daun lidah buaya (*Aloe vera* (L) Burm.f) dan gentamisin sulfat terhadap bakteri *Escherichia coli*. Pharm Sci Res. 4 (3): 132–142.

[7] Basit A, Sari R, Luliana S (2019) Optimasi aktivitas antibakteri rutin daun singkong (*Manihot esculenta* Crantz.)-gentamisin sulfat terhadap bakteri *Staphylococcus aureus*. J. Mhs. Farm. Fak. Kedokt. UNTAN, vol. 4, no. 1, pp. 1–10, 2019.

[8] Tiwari A, Sharma P, Vishwamitra B, Singh G (2021) Review on surface treatment for implant infection via gentamicin and antibiotic releasing coatings. Coatings 11 (8): 1–21. doi: 10.3390/coatings11081006.

[9] Husen F, Ratnaningtyas NI, Samiyarsih S, Muljowati JS, Fitrianto N (2022) Soybean selection against *Cercospora* leaf blight disease caused by *Cercospora kikuchii* based on anatomical resistance. Biosaintifika J. Biol. Biol. Educ. 14 (1): 90–102. doi: 10.15294/biosaintifika.v14i1.34865

[10] Yalinca Z, Yilmaz E, Taneri B, Bullici FT (2013) A comparative study on antibacterial activities of chitosan based products and their combinations with gentamicin against *S. epidermidis* and *E. coli*. Polym. Bull. 70 (12): 3407–3423. doi: 10.1007/s00289-013-1030-0.

[11] Simoes M, Rocha S, Coimbra M, Vieira M (2008) Enhancement of *Escherichia coli* and *Staphylococcus aureus* antibiotic susceptibility using sesquiterpenoids. Med. Chem. 4 (6): 616–623. doi: 10.2174/157340608786242016.

[12] Astrini D, Singgih W, Nugrahani II (2014) Aktivitas antibakteri madu pahit terhadap bakteri gram negatif dan gram positif serta potensinya dibandingkan terhadap antibiotik kloramfenikol, oksitetrasiklin dan gentamisin. Acta Pharm. Indones. 39 (3 & 4): 75–83.

[13] Hamid S, Widjajanto PH, Laksono IS (2016) Evaluasi sensitivitas antibiotik dengan demam neutropenia. Sari Pediatr. 15 (4): 220. doi: 10.14238/sp15.4.2013.220-4.

[14] Barus DO, Gelgel KTP, Suarjana IGK (2013) Uji kepekaan bakteri *Escherichia coli* asal ayam pedaging terhadap antibiotik doksisiklin, gentamisin, dan tiamfenikol Indones. Med. Vaterinus 2 (5): 538–545.

[15] Mazlan NA, Azman S, Ghazali NF, Yusri PZS, Idi HM, Ismail M, Sekar M (2019) Synergistic antibacterial activity of mangiferin with antibiotics against *Staphylococcus aureus*. Drug Invent.Today 12: 14–17.

[16] Chudlori B, Kuswandi M, Indrayudha P (2012) Pola kuman dan resistensinya terhadap antibiotika dari spesimen pus di RSUD Dr. Moerwadi tahun 2012. Pharmacon Pharm. J. Indones. 13 (2): 70–76.

[17] Bakhsheshi-Rad HR, Hadisi Z, Ismail AF, Aziz M, Akbari M, Berto F, Chen XB (2020) In vitro and in vivo evaluation of chitosan-alginate/gentamicin wound dressing nanofibrous with high antibacterial performance. Polym.Test. 82: 106298. doi: 10.1016/j.polymertesting.2019.106298.

[18] Kadurugamuwa JL, Beveridge TJ (1996) Bacteriolytic effect of membrane vesicles from *Pseudomonas aeruginosa* on other bacteria including pathogens: Conceptually new antibiotics. J. Bacteriol. 178 (10): 2767–2774. doi: 10.1128/jb.178.10.2767-2774.1996.

[19] Ummah ZK, Sari N, Fortuna J, Boy E (2017) Perbandingan efektifitas chitosan sisik ikan bandeng dengan gentamisin terhadap perkembangan *Escherichia coli*. Yars. Med. J. 25 (2): 108. doi: 10.33476/jky.v25i2.358.