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Physical Quality and Antibacterial Activity test of Ethanol Extract Gel Preparation of Cocoa Pod Husk (*Theobroma Cacao* L.) against *Staphylococcus aureus* and *Escherichia coli*

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Abstract

Indonesia becomes the third-ranking country over the world in producing and exporting cocoa. Cocoa production increment in Indonesia leads to the increase of cocoa pod husk waste. The heap of cocoa pod husk waste can cause environmental problems and foul odors. Cocoa pod husk contain potentially antibacterial compounds such as flavonoids, saponins, alkaloids, terpenoids and tannins. Surgical wound infections can be caused by bacteria, such as *Staphylococcus aureus* and *Escherichia coli*. These bacteria have been through a lot of resistance to antibiotics. This present study was conducted to determine the physical quality and antibacterial activity of ethanol extract gel of the cocoa pod husk (*Theobroma cacao L.*) against *S. aureus* and *E.coli*. This true experimental research used a post-test only control design. The stages of the research included extraction, testing the activity of antibacterial extract with various concentrations, gel formulations with variations in carbopol concentration, physical quality and antibacterial gel activity test. Data were analyzed using Kruskall-Wallis and Mann-Whitney. The results of this study were extracts with concentrations of 8%, 16% and 32% which had antibacterial activity against *S. aureus*, but did not have antibacterial activity against *E. coli*. The requirements for good physical gel quality only reached by the gel with carbopol concentration of 2% while gel with carbopol concentration of 1% and 3% did not meet the requirement of it. Gel with carbopol concentration of 1%, 2% and 3% have antibacterial activity against *S. aureus*. Cocoa pod husk extract gel with 8% extract concentration and 2% carbopol base concentration is the best formulation and has the antibacterial activity of *S. aureus*.

Key Words: Physical Quality, Antibacterial Activity, Gel, Cocoa pod husk.

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Introduction

Indonesia becomes the third-ranking country over the world in producing and exporting cocoa.The rate of cocoa production in Indonesia is around 16.65% of all cocoa production in the world (Central Statistics Agency, 2018). There are seven regions which become the centers of cocoa production, such as Southeast Sulawesi, West Sulawesi, Central Sulawesi, West Sumatra, South Sulawesi, Lampung and Aceh. Those seven provinces contribute 80.05% in the average of cocoa production in Indonesia (Ministry of Agriculture, 2017). Lampung Province in 2014 had a total area of 68,152 hectares of cocoa plantations (Central Statistics Agency of Lampung Province, 2014). The high production of cocoa in Indonesia resulted in an abundance of waste from the cocoa processing industry. Haryati and Mardjosuwito in 1984 in Listyati and Pakuwon (2015) reported that cacao fruit consisted of 3 parts, which are 21.74-24% of seed, about 74-75.67% of shelland 2-2.59% of placenta Purnamawati and Utami (2014) also stated that the largest component of cocoa waste comes from the skin of the fruit. If the cocoa pod husk are not managed intensively, they can cause problems such as environment pollution, foul odor and establishing an ideal place for the development of germs. Cocoa pod huskcontains the compounds that can be utilized in the field of medication. Research by Rachmawaty et al. (2017) revealed that cocoa pod husk extracts contains flavonoids, saponins, alkaloids and terpenoids, and tannins. These contents indicate that cocoa pod husk potentially becomes as an antibacterial. Nosocomial infection is an infection that occurs after hospitalization by germs from the hospital (Syahrurachman et al., 2010). According to Kasper et al. (2005) the frequent occurrence of nosocomial infections is Surgical Wound Infection, Urinary Tract Infection or UTI and pneumonia. The study that had been conducted in January - July in 2015 at Dr. Moewardi Hospital revealed that *Staphylococcus aureus*is the bacteria that mainly caused diagnosed patients with surgical wound infection (Sulistyaningrum, 2016). Other researchers reported that the *Escherichia coli*becomes the causative bacteria of surgical wound infections in the gynecology and fetus ward at Cipto Mangunkusumo Hospital (RSCM) during August - October 2011 (Wardoyo et al., 2014). Refdanita et al. (2004) reported that bacterial susceptibility patterns obtained from patients at Fatmawati Hospital from 2001 - 2002 showed that *Staphylococcus aureus* and *Escherichia coli* had experienced a lot of resistance to antibiotics. There is a need to conduct a research regarding the use of herbal medicines derived from natural ingredients as an antibacterial alternative to avoid the occurrence of antibiotic resistance.

The selection of gel preparations in this study is related to Elmitra (2017), the superiority of the gel is the cooling effect on the skin when it is being used, elegant and clear appearance, leaving a transparent film layer after drying, elastic, highly

adhesive, non-clogging pores, easy to wash , the release of the medicine and is able to spread well.

None of the research has been conducted that focused on the antibacterial activity of cocoa pod huskextracts in gel preparations against *Staphylococcus aureus* and *Escherichia coli.* A solution is needed to overcome the problems caused by an increase in the amount of cocoa pod huskwaste and the occurrence of antibiotic resistance, therefore this research conducted regarding "Physical quality and antibacterial activity test of ethanol extract gel of cocoa pod husk (*Theobroma cacao L.*) against *Staphylococcus aureus* and *Escherichia coli*".

Materials and Methods

This present research design used true experimental using a post test only control design. The study was conducted at the Botani Laboratory, Biology Department, Lampung University and Lampung Veterinary Center.

Plant Material

The sample in this study was the cocoa pod husk which is the waste from cocoa plantations in Negeri Sakti Village, Gedong Tataan District, Pesawaran Regency, Lampung Province. The plants have been identified and authenticated by Drs. M. Kanedi, M.Si, the chair of the Department of Biology, Lampung University and vouchers specimens from plant material have been stored at the Institute level.

Chemicals and Instruments

Staphylococcus aureus and *Escherichia coli* bacteria were obtained from Balai Veteriner Lampung, Indonesia.The tools used in this research were pH meter, round glass, stopwatch, whatman filter paper number 41, aluminum foil, object glass, adhesive testing equipment, scales, beaker (pyrex®), erlenmeyer (pyrex®), analytical balance, stirring rod, rotary evaporator, measuring cup (pyrex®), test tube (pyrex®), autoclave, laminar air flow, incubator, ose, micro pipette, calipers, millimeter block, vortex mixer, and blender.

The materials used in this study were erythromycin antibiotics disk, spiritus, ethanol 96%, carbopol, Neocenta® gel, Propylenglycol, *Triethanolamine* (TEA), aquadest, Mueller Hinton Agar (MHA), and physiological NaCl.

Sample Preparation and Extraction

Cocoa pod husk is thinly sliced followed by drying it at room temperature and not exposing it to direct sunlight. Dried cocoa pod huskwere blended with a blender until it turned out as a simplicia. Simplisia of 500 grams of cocoa pod huskwas put into an erlenmeyer glass, then soaked with 96% ethanol with a ratio of 1:1. Immersion was carried out for 3 days, with replacement of solvents every day. Filtering was done by whatman filter paper number 41, until the macerate was obtained. The solvent in the maserat was evaporated using a rotary evaporator at 45ºC by a speed of 115 rpm until a viscous extract was obtained.

Testing of Antibacterial Extract Activity

This test aims to get the Minimum Inhibitory ConcentrationConcentration (MIC). Testing of antibacterial extract activity was done by the Kirby Bauer method according to the Clinical and Laboratory Standards Institute (CLSI). The number of samples was calculated according to Hanafiah (1993) in Mardiansyah and Setiyanto (2015) using the Federer formula.

Bacterial culture was prepared with a turbidity standard of 0.5 McFarland. The bacterial suspension was applied to Muller Hinton Agar using a sterile cotton swab. The media was left at room temperature for 30 minutes. Paper discs that have been soaked for 15 minutes in a solution in accordance to the division of groups shown in Table 1.

Table 1: Extract grouping

Groups	Information
P1	Cacao pod husk extract with 8% of concentration
P2	Cacao pod husk extract with 16% of concentration
P3	Cacao pod husk extract with 32% of concentration
K-	Sterile aquadest
K+	Disk eritromisin

Petri dishes were incubated at 35ºC for 24 hours. The inhibition zone was measured by measuring the diameter of the clear zone in the cup using calipers (Sartini et al., 2009; Nurani, 2018). According to Warbung et al. (2013) the formula for calculating the inhibition zone is shown in Figure 1.

$$\frac{d1 + d2}{2} - X$$

Fig 1: Formula for calculating inhibition zones

Note:

d1 = The vertical diameter of media clear zone;

d2 = The horizontal diameter of media clear zone

X = wellhole (6 mm).

Formulation of Gel Preparations

Gel base consisting of carbopol, *Triethanolamine* (TEA), *propyleneglycol* (PG), methyl paraben, and aquades. Gel base was made into three different formulations with variations in the concentration of carbopol. Methylparaben is dissolved in aquadest (distilled water) by heating to a temperature of 70°C, followed by adding a gel-forming (carbopol) and stirred until it unfurls and forms a gel, then adding other ingredients such as Propylenglycol and TEA. Carbopol was made with a concentration variation of 1%, 2% and 3%. The gel preparation base was made without active ingredients (Hidayanti et al., 2015). Gel base was added with cocoa pod husk extract with extract concentration according to Minimum Inhibitory Concentration (MIC). The gel then was stored at room temperature overnight at 10-15 °C (Sikawin et al., 2018).

Table 2 below shows the cocoa pod husk gel preparation formulation.

Table 2: Gel Preparation Formulations(Hidayanti et al, 2015)

Ingredients	Formula Basis (%b/b)		
	P1	P2	P3
Extract	MIC	MIC	MIC
Carbopol	1	2	3
TEA	1,5	1,5	1,5
PG	15	15	15
Aquadest	Ad 100	Ad 100	Ad 100

Physical Quality Evaluation of Gel Preparations

Evaluation of preparations was carried out on cocoa pod husk extract gel with carbopol concentration of 1%, 2% and 3%. Evaluation of the physical quality of cocoa pod huskextract gel preparations includes organoleptic testing, homogeneity, pH, spreadability, and adhesion (Sikawin *et al.*, 2018).

The test results will be compared with a good standard physical gel quality based on literature.

Testing of Antibacterial Gel Activity

Antibacterial activity test of cocoa pod husk extract gel against *Staphylococcus aureus* and *Escherichia coli* was carried out using the well-diffusion method. The wells method was chosen because it allows the gel preparation test material to be directly in contact with the wall media, thus it visually will be easier for inhibitory with the measurement of the presence of a clear zone, clear zone is an area around the well where bacteria are inhibited by antibacterial (Brooks *et al.*, 2007). Five wells were made on the Mueller Hinton Agar (MHA) media according to the group division shown in Table 3.

Table 3: Gel grouping

Group	Information
P1	Cacao shell extract gel with carbopol concentration of 1%
P2	Cacao shell extract gel with carbopol concentration of 2%
P3	Cacao shell extract gel with carbopol concentration of 3%
K-	Gel base
K+	Neocenta®Gel (*neomycin sulfate* 0,5% and *bovine placenta extract*),

Dip a sterile cotton swab with a bacterial suspension according to the turbidity of 0.5 Mc Farland, then apply by rubbing the cotton on the entire surface of Mueller Hinton Agar (NCCLS, 2002). Make a well in the media agar. Each gel was taken as much as 50 µL using a micro pipette and dropped on each well. Incubate Mueller Hinton's media at 35 °C for 16-18 hours. Clear zones were measured in diameter by calipers (Sikawin et al., 2018; Mahon and Lehman, 2019).

Data analysis

The results of the physical quality evaluation of the gel preparations were compared with the

physical quality of the good gels in the literature to find out whether the preparations that had been made had fulfilled the requirements as the good gel preparations.

In testing antibacterial activity, data analysis was performed using a non-parametric statistical test, namely Kruskall-Wallis, because the data were not homogeneous and there were zero data variations.

Further analysis with Mann-Whitney further tests was conducted to determine the differences in the diameter of the inhibitory zone that were significant at each concentration. Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) (Mulyatni et al., 2012; Dahlan, 2014).

Results and Discussion

Preparation of sample and Extraction

The results of sample preparation and extraction of cocoa pod huskwere viscouscocoa pod huskextracts with a concentration of 100%. The extract was 100% liquid, smells like cocoa pod husk and has a yellowish brown in color.

The 100% extract was diluted to a concentration of 8%, 16% and 32%. The extracts of 8%, 16% and 32% have the same shape and odor as the 100% extract which was liquid and smells like cocoa pod husk extract. Cocoa pod huskextract of 8% and 16% have a lighter color than the concentration of 32%.

This results were consistent with a research conducted by Pappa *et al*., (2019) which also produces cocoa pod husk extract with a yellowish brown color.

Testing of Antibacterial Extract Activity

The results of antibacterial extract activity testing of cocoa pod huskagainst*Staphylococcus aureus* and *Escherichia coli* can be seen in Table 4.

Table 4 shows that all extract concentrations formed inhibitory zones, thus it possessed antibacterial activity against*Staphylococcus aureus*. The Kruskal Wallis test result was 0,000 (<0.05), thus $H_0$was rejected, which means that there was a significant antibacterial activity of cocoa pod husk extract against *Staphylococcus aureus* bacteria. There were significant differences in Mann Whitney test results, in all groups both treatment and control.Minimum Inhibitory Concentration (MIC) of cocoa pod husk extract against *Staphylococcus aureus* in this study was a group with a concentration of 8%, thus the concentration of 8% will be used in the cocoa pod husk extract gel formulation. This was in line with the research of Mulyatni et al (2012) who also received a MIC of 8%. So that in the manufacture of cocoa pod husk extract gel will use an extract concentration of 8%.

The testing results of antibacterial activity of cocoa pod husk extract against *Escherichia coli* showed that all extract concentrations did not form.

Table 4. Zone of Extract Inhibition

Group	Average of inhibition diameter zone (mm)			
	Staphylococcus aureus		*Escherichia coli*	
P1	5,19	± 0,44[a]	0	± 0
P2	7,73	± 2,08[b]	0	± 0
P3	10,61	± 1,39[c]	0	± 0
K-	0	± 0[d]	0	± 0
K+	29,42	± 1,63[e]	9,11	± 1,03

*The average number followed by different superscript letters shows a significant difference with the Kruskal Wallis statistical test followed by the Mann Whitney test at a 95% confidence level.

inhibitory zones, hence, they did not have antibacterial activity against*Escherichia coli*.

According to Davis and Stout in 1971 quoted by Rastina et al. (2015) that concerning the criteria for the strength of antibacterial in which 5 mm diameter inhibition zone or less is categorized as weak, 5- 10 mm inhibition zone is categorized as medium, 10-20 mm inhibition zone is categorized strong and 20 mm inhibition zone or more is categorized very strong. Cocoa pod husk extract with concentration of 8% has an inhibition zone of 5.19 mm with the inhibitory power produced of medium category. Cocoa pod huskextract with concentration of 16% has an inhibition zone of 7.73 mm with the inhibitory power produced of medium category. Cocoa pod huskextract with concentration of 32% has an inhibition zone of 10.61 mm with the resulting inhibition of strong category.

Inhibition of bacterial growth against*Staphylococcus aureus* by cocoa pod husk extract is thought to originate from the activity of dissolved bioactive compounds. Rachmawaty et al (2017) explained that the extract of cocoa pod husk in each treatment contained alkaloids, flavonoids, tannins and saponins, and terpenoids. The results of antibacterial activity testing showed that the extract of cocoa pod husk did not have antibacterial activity against*Escherichia coli*. E. coli is a Gram-negative bacterium that tends to be more resistant to active compounds, because it has a thin wall structure of cell which is about 10-15 µm which consists of three layers, namely the outer membrane, inner membrane and thin peptidoglycan layer on the inside with high lipid content (11-21%). The outer layer consists of two layers, such as lipopolysaccharide and lipoprotein (Hawley, 2003). This is likely to cause the bioactive compounds presents in the extract of cocoa pod huskconcentrate to not be able to penetrate the cell membrane, therefore the inhibition of the growth of Escherichia coli bacteria did not occur.

Physical Quality Evaluation of Gel Preparations

The making of this cocoa pod huskextract gel used carbopol as a gelling agent and propylene glycol as a humectant which will maintain the water content in the preparation so that the

physical properties and stability of the preparation during storage can be maintained. Carbopol is often used as a gelling agent because it can provide good and stable gel characteristics. Propylenglycol is chosen as a humectant because of its good solubility with water and suitable as an ingredient of phenolic groups which are the secondary metabolites of cocoa pod husk. Another material used is TEA, which is a base agent, neutralyzing agent which helps to form the gel character (Rowe et al., 2009).

The results of the physical quality evaluation of the cocoa pod huskextract gel and its comparison with the literature can be seen in Table 5 below.

Table 5: Comparison of Physical Quality of Gel with Literature

Testing	Literature	Group		
		P1	P2	P3
Organoleptic	Distinctive colors and aromas such as active substances and viscous shapes	Brown, extract smell and runny	Brown, extract smell and viscous*	Brown, extract smell and very viscous
Homogenity	Homogen	Homogeneous*	Homogeneous*	Non Homogeneous
pH	4,5-6,5	6,4 ± 0.1*	6,4 ± 0.1*	6,2 ± 0.12*
Spread ability (cm)	3-5	4,5 ± 0,5*	3,25 ± 0,61*	2,15 ± 0,47
Adhesion (seconds)	> 4	3 ± 3	120 ± 70,36*	300 ± 67,08*

*: showed the testing results in accordance with the parameter criteria.

Source: Priawanto & Hadning (2017), Sayuti (2015), Draelos & Thaman (2006), Garg *et al.*, (2002) dan Ulaen *et al.* , (2012).

Organoleptic Testing

Organoleptically good gels must meet the requirements of having distinctive colors and aromas same as active substances and their viscous shape (Priawanto and Hadning, 2017). Gel with 2% carbopol concentration meets the requirements because its brown color was similar to thecocoa pod huskextract, it has a distinctive smell like cocoa pod husk extract and has a viscous shape. Ulfa et al (2016) also reported that the preparation of cocoa pod husk extract gel masks had a clear brown color, with a consistency or viscous form.

Homogeneity Testing

Homogeneity testing aims to determine whether all substances have been mixed evenly so that when it is being applied to the skin in need, all skin areas have the same opportunity to get the properties of substances contained in a preparation (Soba, 2018). The gel is declared homogeneous if there are no coarse grains, there are evenly colored similarities and no different particles are found (Sayuti, 2015; Sikawin et al., 2018). Gel with 1% and 2% carbopol concentration did not show any coarse grain and color difference, so it was declared

homogeneous. Gels with 3% carbopol concentration were found to have different colors, so they were declared as not homogeneous. The gel was not homogeneous due to the concentration of gelling agent which was too high thereby it increased the viscosity of the gel preparation and formed clots that were difficult to be removed when applied to a piece of glass (Supomo et al., 2015).

PH testing

A good degree of gel acidity should be in accordance with the pH of human skin. PH values that are too acidic can cause skin irritation, on the other hand, pH with high value of alkaline can cause scaly skin (Saraung et al., 2018). The normal pH value of human skin is 4.5-6.5 (Draelos and Thaman, 2006). The cocoa pod husk extract gel with 1% and 2% carbopol concentration had the same pH value of 6.4, while the 3% concentration had a pH value of 6.2. The pH values of all groups of ethanol extract gel of cocoa pod huskwere in accordance with the pH of the skin, thus it was safe to be applied to the skin.

Spreadability Testing

The spreadability of the gel shows the gel ability to spread at the location of application when applied to the skin (Afianti and Murrukmihadi, 2015). The difference in the spread ability greatly influences the speed of diffusion of the active substance across the membrane. The wider the preparation membrane is spread, the greater the diffusion coefficient resulting in the improvement of drug diffusion, thus, the greater spread of a preparation is better (Hasyim et al., 2011). According to Garg et al (2002) the spreadability for semisolid preparation is divided into two parts, namely semi-stiff and semi-fluid. In semi-stiff, the specified distribution strength is 3-5 cm, while for semi-fluid is 5-7 cm.

The spreadability of cocoa pod husk extract with carbopol concentration of 1% was 4.5 cm, while the spreadability of cocoa pod husk extract with carbopol concentration of 2% was 3.25 cm, thus, both concentrations include semi-stiff gel preparations. The spreadability of cocoa pod husk extract gel with carbopol concentration of 3% was 2.15 cm, thus, the spread ability did not meet the requirements for good gel preparation.

Adhesion testing

Adhesion is the ability of the gel to coat the surface of the skin in a waterproof, does not clog the pores and the physiological functions of the skin. The longer the gel is attached to the skin, the more active substances that diffuse into the skin, resulted in making it more effective in its use (Voigt, 1984). The adhesiveness of cocoa pod husk extract gel with carbopol concentration of 1%, 2% and 3% respectively were 3 seconds, 120 seconds and 300 seconds. The time requirement for good adhesion is not less than 4 seconds (Ulaen et al., 2012). Hence, the cocoa pod huskextract

gel with carbopol concentration of 2% and 3% qualified as gel with good adhesion, while the cocoa pod huskextract gel with carbopol concentration of 1% did not meet the requirements.

From the findings of the study, it was known that differences in terms of the concentration of carbopol as a gelling agent affected the consistency, pH, adhesion and spread ability power of cocoa pod husk gel preparations, where the increase in carbopol concentration affected to the increase of consistency, the longer proceeds of adhesion, the decrease of pH, and the smaller of spreadability. This is because the greater the level of gelling agent, it will increase the viscosity of a preparation (Chandra, 2019). Octavia (2016) states that the inherent power of a preparation is directly proportional to its viscosity. The higher the viscosity of a preparation, the higher its stickiness. Unlike the inherent power, the spreadability of a preparation is inversely proportional to its viscosity and adhesion. The higher the viscosity and adhesion of a preparation, the lower its spreadability (Octavia, 2016). Cocoa pod husk extract gel with a carbopol concentration of 2% fulfilled the requirements of good gel quality based on organoleptic tests, homogeneity, adhesion, spread ability and pH determination.

Testing of Antibacterial Gel Activity

The testing results the antibacterial activity of cocoa pod husk extract gel can be seen in Table 6 below.

Table 6: Zone of Gel Inhibition

Group	Diameter of gel inhibition			
	Staphylococcus aureus		*Escherichia coli*	
P1	8,37	± 1,52[a]	0	± 0
P2	6,55	± 1,01[a]	0	± 0
P3	4,18	±1,47[b]	0	± 0
K-	0	± 0[c]	0	± 0
K+	25,95	± 2,01[d]	21,66	± 0.48

*The average number followed by different superscript letters shows a significant difference with the Kruskal Wallis statistical test followed by the Mann Whitney test at a 95% confidence level.

From the measurements results that have been made and showed in Table 6, it revealed that the cocoa pod huskextract gel had antibacterial activity against*Staphylococcus aureus* bacteria forming the largest inhibitory zone at carbopol concentration of 1% at 8.37 mm with the inhibitory power produced included in themedium group, followed by cocoa pod huskextract gel with carbopol concentration of 2% at 6.55 mm with the inhibition produced included in the medium category and the smallest inhibitory zone at the concentration of 3% carbopol base at 4.18 mm with the resulting inhibition included in the weak group . The Kruskal Wallis test result was 0,000 (<0.05), hence H_0 was rejected, which means that there was a significant antibacterial activity of cocoa pod husk extract gel

against*Staphylococcus aureus* bacteria. The Mann Whitney test results showed that there were significant differences in all groups, except for the cocoa pod huskextract gel group with a 1% carbopol concentration and 2%carbopol concentration

The testing results of the antibacterial activity of cocoa pod huskextract gel against*Staphylococcus aureus* showed that all carbopol concentrations formed inhibitory zones. The antibacterial activity of the cocoa pod huskextract gel was due to the presence of secondary metabolite compounds contained in the extract. The presence of secondary metabolites becomes an important factor through its mechanism against bacteria. Cocoa pod huskextract gel did not have antibacterial activity against*Escherichia coli* because the cocoa pod husk extract used also did not have antibacterial activity against*Escherichia coli*.

The higher the concentration of carbopol, the lower the antibacterial inhibition. This is because the greater level of gelling agent will increase the viscosity of a preparation, and the greater the viscosity of a preparation, the greater its resistance (Chandra, 2019), thus it blocked the release of the active substance and resulted in a decrease in the inhibition of gel formulation against*S. bacteria. Aureus* (Afianti and Murrukmihadi, 2015).

Conclusion

The preparation of cocoa pod huskgel with 8% extract concentration and 2% carbopol base concentration was the best formulation and had the antibacterial activity of *Staphylococcus aureus* with inhibition zone of 6.55 mm, thus the inhibitory power produced is included in the medium group.

Conflict of Interest

The authors declare no conflicts of interest.

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