Original Research

In-vitro antibacterial activity of the seed extract of three-member Artocarpus towards Methicillin-Resistant Staphylococcus aureus (MRSA)

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HIGHLIGHTS

- The emergence of Methicillin-Resistant *Staphylococcus aureus* (MRSA) infections has become a serious health problem.
- Strategy to avoid this is by using alternative therapeutic agents from plants that are effective against MRSA
- Many plants are used as folk medicines to anti-MRSA, one of which is a seed of *Artocarpus*.

ARTICLE INFO

A B S T R A C T

Methicillin-Resistant *Staphylococcus aureus* (MRSA) infections have created a critical need for the development of natural antibacterials from a biological source. This research aimed to investigate the antibacterial activity of the seed extract of three-member *Artocarpus* (*Artocarpus heterophyllus*, *A. champeden*, and *A. camansi*) against MRSA which are the most prevalent causes of infections in patients. Crude seed extracts of three-member *Artocarpus* were evaluated for their antibacterial activity against MRSA. The antibacterial activity against MRSA of the three extracts was assayed in vitro by the agar well diffusion assay and agar microdilution method and minimum bactericidal concentration. The antibacterial activity, calculated as a zone of inhibition and MIC, MBC values. The Crude seed extracts of three-member *Artocarpus* showed antibacterial activity against the MRSA in the agar well diffusion assay (1.5-9 mm inhibition diameter). The MIC value of extract showed at 15.62 mg/mL and the MBC value of seed extract of *A. heterophyllus* at 62.5 mg/mL, *A. champeden* at 31.25 mg/mL, *A. camansi* at 250 mg/mL. All seed extracts have the potential to be developed as antibacterial agents, particularly against MRSA strain. Studies on the antibacterial activity against MRSA can provide new information about the benefits seed of members of *Artocarpus* as a source of natural antibacterial.

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1. INTRODUCTION

Staphylococcus aureus is one of the most common food-borne pathogenic bacteria. Toxins produced by it can cause food spoilage and food-borne diseases.[1] Due to the inappropriate use of antibiotics, methicillin-resistant *Staphylococcus aureus* (MRSA) became a new threat in the past decades. MRSA is a major cause of nosocomial infections worldwide including in Indonesia. The prevalence rates of MRSA in hospitals in some Asian countries such as Japan, South Korea, Taiwan, and China are 70–80%.[2] Although the prevalence varies considerably between regions or countries, MRSA has been detected in most countries worldwide.[3]

MRSA results in reduced efficacy of antibacterial drugs methicillin group, making the treatment of patients difficult. One strategy to avoid this is by using natural agents from plants,[4,5] honey,[6] Endophytes,[7] *Streptomyces,*[8,9] marine organisms,[10,11] mushrooms,[12] lichens,[13] lactic acid bacteria[14] and animal,[15] that are effective against antibiotic-resistant bacteria.

Many plants are used as folk medicines to antibacterial,[16] one of them is *Artocarpus*. These genera became one of the plants a great level of scientific interest as they contain important secondary metabolites possessing useful biological activities.[17] Several member *Artocarpus* species are used as food and for traditional folk medicines in South-East Asia, including Indonesia. Species of *Artocarpus* common cultivated in Indonesia are *Artocarpus heterophyllus* (jackfruit), *Artocarpus chempeden* (Chempedak), and *Artocarpus altilis* (breadfruit).

Research related to the antibacterial activity of *Artocarpus* has been reported. Extract from the bark of *Artocarpus rigida* Blume showed antimicrobial activity against *Escherichia coli* and *Bacillus subtilis*.[18] Research on anti-MRSA of seed extract of *Artocarpus* against MRSA has not been reported, so it is necessary to investigate the antibacterial potential of seed extract of *Artocarpus* against MRSA. The study on antibacterial activity against MRSA of seed extract of *Artocarpus* with Methanol solvent is expected to provide new information about the benefits of the seed of *Artocarpus*. Besides, it also can support the seed of *Artocarpus* as a source of natural antibacterial against MRSA. This research aimed to evaluate the antibacterial activity of the seed extract of three-member *Artocarpus* (*A. heterophyllus, A. camansi,* and *A. champeden*) against MRSA which are the most prevalent causes of infections in patients.

2. MATERIAL AND METHOD

Seeds

Fresh seeds (*A. heterophyllus, A. camansi,* and *A. champeden*) were collected from the Bandungan field in Semarang during the rainy season in January 2019 (Figure 1). The seeds were washed with water to remove all unwanted materials. They were then dried under sunlight for 7 days. The dried seeds were then milled into a fine powder using a milling machine and stored in a sterile air-tight container until further use.

Figure 1. Photographs seed of plants

Noted: (a) *Artocarpus heterophyllus* (jackfruit); (b) *Artocarpus chempeden* (Chempedak); and (c) *Artocarpus altilis* (breadfruit)

Preparations of extracts

Seeds of *Artocarpus* extracts were prepared for the method of maceration with Methanol. 100 g powdered seeds were soaked in 300 mL of solvent for 24 h at room temperature and protected from light with shaking. Solvent replacement is done every day. Replacement of solvent is done until the solution becomes clear with the assumption that there is no active compound contained in the dry powder. The supernatant was filtered through filter paper No.1 (Whatman). The maceration solution was concentrated under reduced pressure using a rotary evaporator at 50^0C. The crude extracts were collected and allowed to dry at room temperature.

Bacterial preparation

The MRSA for in vitro antibacterial screening in this study were isolated from patients of the hospital Dr. Kariadi, Semarang City, Indonesia. MRSA isolate was identified and susceptibility patterns were obtained using Vitek®MS (bioM´erieux). The bacteria were cultured for 24 h at 35±2 ^{0}C on 5% sheep blood agar (BAP). The bacteria colonies were homogenized and adjusted to 0.5 McFarland standards (5×10^8 CFU/mL) using spectrophotometry

Agar well diffusion assay

The antibacterial activity from seeds was evaluated using agar well diffusion assay.[19,20] In this method, 100 µL of MRSA which is equivalent to a 0.5 McFarland standard was inoculated on the MHA. Then it is spread onto the surface of the agar using a sterilized glass spreader. After 10 minutes of inoculation, the wells were prepared using a sterilized steel cork borer (1cm diameter). Wells were made in each plate, out of which five wells were loaded with the extract (100, 200, 500, 700, and 1000 mg/mL). Each test was done in triplicate. All the plates were then incubated aerobically at 35 ± 2 ^{0}C for 24 h. Dimethyl sulfoxide (DMSO) was used as a negative control. Vancomycin and oxacillin were applied as positive controls for MRSA. Antibacterial activities were evaluated by measuring the diameters of zones of inhibition (mm) against the test organism.

Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC).

The MIC and MBC of the extracts were determined using Mueller–Hinton broth microdilution,[21,22] MIC determination was performed by serial dilution technique using 12-well microwell plates. Extract amounts of 100 µL were placed into each well. Then, 100 µL of MRSA suspension (0.5 McFarland) was added to each. Each test was done in triplicate. The seeds extracts were serially diluted to produce final concentrations of 0.24; 0.48; 0.97; 1.95; 3.90; 7.81; 15.62; 31.25; 62.5; 125; 250; and 500 mg/mL. The microwell plates were then incubated for 24 hours at 35 ± 2^{0}C. Dimethyl sulfoxide was used as a control and Mueller–Hinton broth as a negative control. Oxacillin was used as a positive control. The MIC was determined as the lowest concentration of extract that completely inhibited the growth of the MRSA detected by the unaided eye.[23] The MBC was defined as the lowest concentration of the extract that did not any grow.[24] The wells were subcultured using a 10 µL inoculating loop on to a BAP at (35 ± 2)^{0}C for 16–20 hour incubation.

Phytochemical screening.

The seeds extract were screened for the presence of different classes of secondary metabolites, including alkaloids and flavonoids using previously described methods.[25]

3.   RESULTS AND DISCUSSION

Extraction is a process that aims to take active compounds from within cell bodies by dissolving active compounds that can then be extracted. The Methanolic extracts of the seed of three-member *Artocarpus* were calculated for the yield (Table 1), which showed that its

constituents were relatively polar. Methanol has a polarity index of 5.1 and is used for the extraction of polar compounds.[26]

Table 1. The extract yield

Plants	Part of Plants	Solvent	yield (%)
A. heterophyllus	seed	Methanol 96%	5.50
A. champeden	seed	Methanol 96%	6.68
A. communis	seed	Methanol 96%	6.70

The antibacterial activity against MRSA of the three extracts was assayed in vitro by the agar well diffusion assay and agar microdilution method.

The antibacterial activities against MRSA of the three seed extracts were assayed in vitro by agar diffusion method. The zones of inhibition are presented in Figure 2. All seeds demonstrated the zones of inhibition against MRSA. The diameters of the zones of inhibition with various concentrations *A. heterophyllus* and *A. champeden* (100-1000 mg/mL) and *A. communis* (500-1000 mg/mL) are presented in Figure 3. All extract showed antibacterial activity against MRSA in the agar well diffusion assay (1.5-9 mm inhibition diameter). This showed that all seed had the potential to be developed as antibacterial agents for MRSA strains. These results are similar to another previously obtained result, which indicated the efficacy of seed extracts with methanolic solvent.[27] Another study showed that the crude methanol extracts of the *Artocarpus heterophyllus* seed exhibited a minimum (<12 mm) antibacterial activity against MRSA.[28]
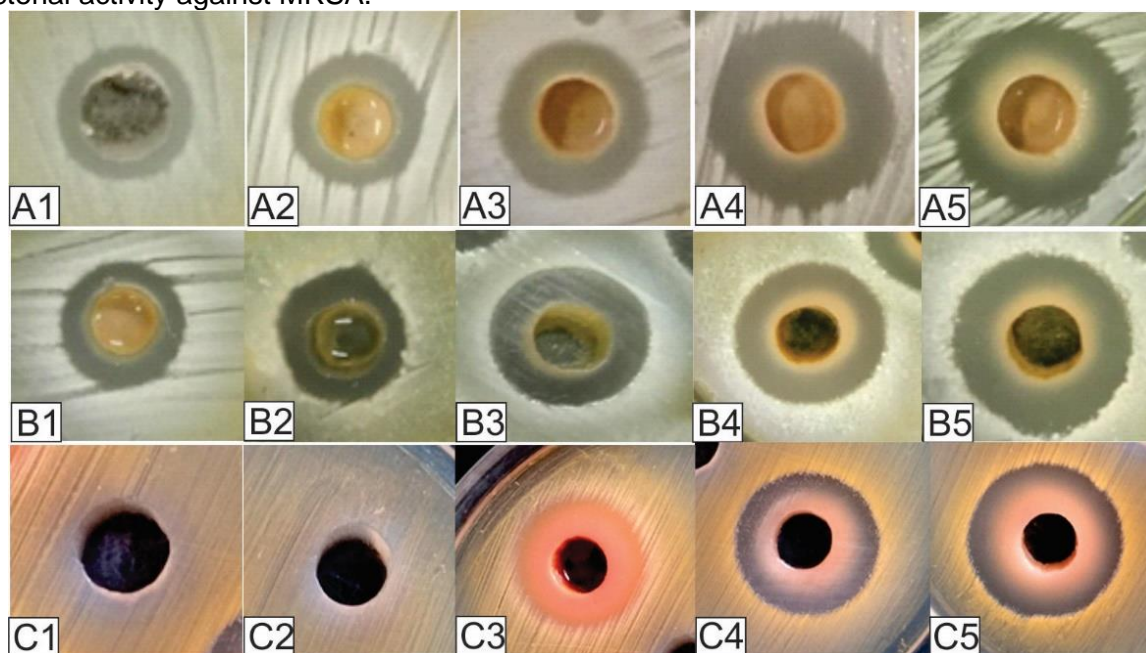

Figure 2. Zone of the inhibition of seeds extracts against MRSA

Note:
(A) *A. heterophyllus*
(B) *A. champeden*
(C) *A. communis*

(1) with concentration 100 mg/mL
(2) with concentration 200 mg/mL
(3) with concentration 500 mg/mL
(4) with concentration 700 mg/mL
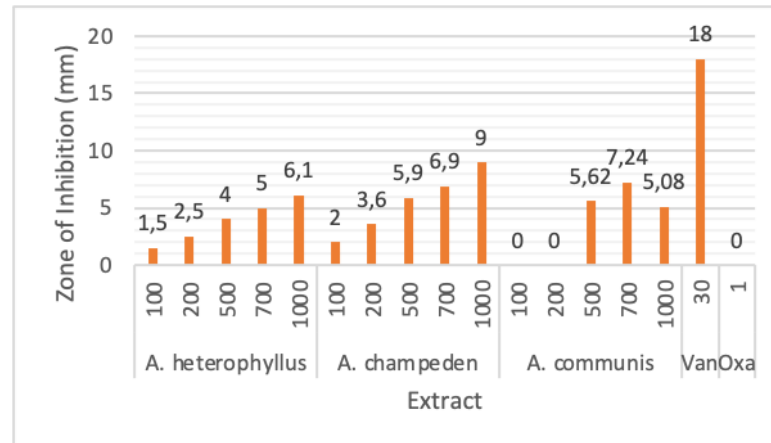(5) with concentration 1000 mg/mL

Figure 3. Graphical representations are indicated a zone of inhibition of seed extracts of *Artocarpus* against MRSA

Noted:
Van: Vancomycin
Oxa: Oxacillin

The antibacterial activity of the seed of *Artocarpus* was assayed in vitro by the agar microdilution method against MRSA. Table 2 shows all seeds exhibited the value of MIC against MRSA at 15.62 mg/mL. Figure 3, 4 and 5 shows value of MBC at seeds of *A. heterophyllus* (62.5 mg/mL), *A. champeden* (31.25 mg/mL) and *A. camansi* (250 mg/mL). This showed that seeds of *A. heterophyllus, A. champeden*, and *A. camansi* had the potential to be developed as antibacterial agents for MRSA strains. Plants extracted in methanol provide more consistent antibacterial activity compared to other solvent extracts of the same plants.[29] Most antimicrobial active compounds from plants that have been identified were soluble in methanol solvents.[30] Extraction techniques are also important to separate the active compounds, because some active compounds may be destroyed by heat. Seeds of *Artocarpus* extracts were prepared for the method of maceration with methanol this method does not use heat, so the active compound is not broken.

The methanolic extract of *Artocarpus* seed showed activity against MRSA. The antibacterial activity of the methanol, ethanol, acetone, chloroform, and petroleum ether extracts of the *A. heterophyllus* seed powder against MRSA strain by the disc diffusion method. It was observed that methanol extracts possessed good activity. The methanolic extracts were active against all the twelve isolates. The antibacterial effects of the methanolic extract were also better than that of methicillin (5 µg) used as the positive control.[28]

Table 2. MIC value of seed extract of *Artocarpus* against MRSA

Well	Concentrations(mg/mL)	*A. heterophyllus*	*A. champeden*	*A. camansi*
1	500	-	-	-
2	250	-	-	-
3	125	-	-	-
4	62.5	-	-	-
5	31.25	-	-	-
6	15.62	-	-	-
7	7.81	+	+	+
8	3.90	+	+	+
9	1.95	+	+	+
10	0.97	+	+	+
11	0.48	+	+	+
12	0.24	+	+	+

Noted: (+): Present; (−): Absent

Figure 4. MBC value of seed extract of *A. heterophyllus* against MRSA at 62.5 mg/mL (d), the arrow points to the growing bacterial colony
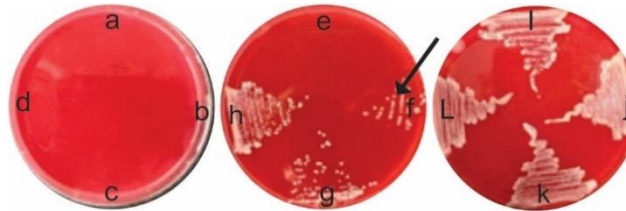

Figure 5. MBC value of seed extract of *A. champeden* against MRSA at 31.25 mg/mL (e), the arrow points to the growing bacterial colony
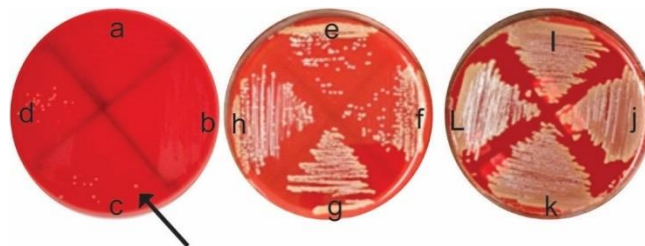

Figure 6. MBC value of seed extract of *A. camansi* against MRSA at 250 mg/mL (b), the arrow points to the growing bacterial colony

The screening of the phytochemical composition was conducted all for the seeds because they have the potential to be developed as antibacterial agents. The secondary metabolites are shown in Table 3 flavonoids were present in the all seed extract.

Table 3. The results of the phytochemical analysis of seeds extracts of the *Artocarpus*

Seed extracts	Secondary metabolites	
	Alkaloid	Flavonoid
A. heterophyllus	-	+
A. champeden	-	+
A. camansi	-	+

+: Present; -: Absent.

Flavonoids were present in all seed of *Artocarpus*. These bioactive compounds have been reported to be used by plants for protection against bacterial.[31] The mechanism of antibacterial activity flavonoids compound in the study still unknown, but many studies have shown that flavonoids have antibacterial activity. Flavonoid expressed a stronger antibacterial effect against *Escherichia coli*.[32] Antibacterial activities against *E. coli* (ATCC 25922), *Salmonella* (ATCC 51812), *Staphyloccocus aureus* (ATCC 25923), and *Streptococcus* (ATCC 49619), of flavonoids fraction, were also proved to be stronger.[33]

The seeds of *Artocarpus* were shown to be potentially developed as an antibacterial against MRSA strains. This study can provide new information about the benefits of *Artocarpus* seed as a source of natural antibacterial against MRSA. Further in vivo research

and discovery of action modes are needed to shed light on their antibacterial effects, so that potential antibacterial products could be developed.

4.   CONCLUSION

All seed extracts have the potential to be developed as antibacterial agents, particularly against MRSA strain. Further in vivo research and discovery of mode action are needed to shed light on their antibacterial effects.

DISCLOSURE STATEMENT

The authors declare that they have no conflict of interest.

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SHORT BIOGRAPHY

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Original Research

Identification of Salmonella typhi contamination by amplification fliC gene in grass-jelly from traditional markets and minimarket in Semarang city

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HIGHLIGHTS

We found positive samples with *S. typhi* in the markets by amplification of *fliC* gene but amplification of only *fliC* gene cannot specific for *S. typhi* detection

ARTICLE INFO

ABSTRACT

Grass-jelly is one of the most popular plants consumed by people in various forms. Contamination can cause various diseases, one of those is typhoid fever by *Salmonella typhi*. The purpose of this study was to detect *S. typhi* in grass-jelly based on molecular detection by amplification of the *fliC* gene using PCR. Validation was done by culture methods on SSA media and biochemical testing. The *fliC* gene amplification results in grass-jelly samples (A1, A2, B1, B2, C1, and C3) showed the DNA fragments size of about 1500 bp. Colony and biochemical characters isolate Peterongan were lead to *S. typhi*, whereas another isolate was another *Salmonella* spp. Grass-jelly samples from the Peterongan market in Semarang were positively contaminated by *S. typhi* and isolate from Pedurungan and the minimarket was another *Salmonella* spp. Molecular-based food testing is fast enough and accurate for detecting types of bacterial contaminants but the amplification of only the *fliC* gene cannot specific for *S. typhi*.

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1. INTRODUCTION

Grass-jelly or commonly called cincau is a gel-like jelly that is processed from soaking certain plant leaves in water. The gel is formed because the carbohydrate content in the leaves is bind to water molecules.[1] In general, there are 2 types of grass-jelly on the market one of those is black grass-jelly, commonly called janggelan made from the leaves of *Mesona palustris* has fiber content that is useful for human digestion and green grass-jelly from the

leaves of *Premna oblongifolia* has several active compounds to relieve sore throat and keep blood pressure stable.[2] Both have a distinctive aroma and taste characters and both are popular drink mixes in the community.

The process of making grass-jelly recently is mostly done conventionally. It is food processing that was done traditionally with the usual procedures without good manufacturing practices such as the hygiene process. In order, the contamination of microorganisms can occur in grass-jelly due to several factors such as water for processing, pieces of equipment, and processing facilities, as well as people doing the processing.[3] This condition factors causing contamination by various pathogenic bacteria to black grass-jelly that sold in the market, one of which is *S. typhi* that causes typhoid fever.

A study on the identification of microbial contamination in grass-jelly at Wonodri Market, Semarang has been done.[4] The results of the study using culture methods and biochemical tests showed that there was contamination by *S. paratyphi A*, *Proteus mirabilis*, *Klebsiella oxytoca* and *Citrobacter diversus*.[5] stated that in Dr. Kariadi Hospital in Semarang city, population typhoid fever was associated with poor housing and inadequate food and personal hygiene. Based on the National Food and Drug Agency[6] Regulation No. 13 of 2019 concerning Maximum Limits of Microbial Contamination in Food should not contain *Salmonella sp.*, in the Fruit-Based Dessert Includes Fruit-Based Water-Based Dessert Food that is black and green grass-jelly included in it. Therefore a fast and accurate detection of the *Salmonella spp* group is needed to be done.

Salmonella is a broad spectrum of disease-causing bacteria. They can cause significant morbidity, and in some cases, death in humans and animals.[7] *S. enterica serovar typhi* or commonly called *S. typhi* is a rod-shaped, solitary, flagellar bacteria (peritric type or around on the entire cell surface), Gram-negative, intracellular bacteria, including in the family *Enterobacteriaceae*, causes typhoid fever and gastroenteritis in humans, with mild to severe clinical symptoms.[8,9] This bacterium has somatic antigen O composed of lipopolysaccharides, flagellate antigens or H antigens composed of flagellin proteins, Vi capsule antigens composed of carbohydrates and fimbriae antigens composed of fillins protein.[8,10] The gene that codes for the flagellin protein in *S. Typhi* is the *fliC* gene.[11]

The *fliC* gene in *S. typhi* has a unique sequence and size. The *S.typhi* H1 antigen consists of 2 serovars namely H1-d which is widespread in the world and H1-j which is only found in Indonesia. Both are encoded by the *fliC* gene that is on the bacterial chromosome, but the *fliC* gene that encodes the j antigen has deletion 251 bp which results in changes in shape in the antigen epitope of the flagel.[12] The study by amplification *fliC* gene with primer LPW 1857 Forward and LPW 1857 Reverse showed DNA about 1260 bp using *S. typhi* isolates from Salatiga and different from 9 other strains about 1500 bp.[13] Similar studies have also conducted the results showed that the flagellin *fliC* gene had a DNA band size of 1260 bp.[12,14] Previous research conducted by results showed the flagellin *fliC* gene had a DNA size of 1260 bp.[12,14] The purpose of this study was to detect contamination of *S. typhi* in grass-jelly from a traditional market in Semarang based on molecular detection by amplification of the *fliC* gene using PCR.

2.   MATERIAL AND METHOD

The samples in this study were black grass-jelly from two traditional markets in Semarang, namely Pedurungan Market, Peterongan Market, and one of the branded packaging products from a minimarket. The research was conducted at the Laboratory of Molecular Biology and Microbiology of Program of Study in Medical Laboratory Technology, Universitas Muhammadiyah Semarang.

DNA extraction

The sample came from 1 g of grass-jelly homogenized with 9 ml physiological NaCl then inoculated to the Brain Heart Infusion (BHI) slant agar medium and incubated 24 hours in 37°C. The growing colonies were then inoculated into 15 ml BHI broth media with 24-hour incubation in 37°C. After that, centrifuged 3000 RPM for 15 minutes to get pellets.

The DNA extraction protocol uses Phenol Chloroform Isoamyl Alcohol (PCIA) with composition 25:24:1. Pellets biomass added 750 µl lysis buffer (100 mM NaCl, 100 mM Tris-HCl pH 8, 50 mM EDTA, 2% SDS) and added 20 proteinase-K 10 mg/ml, then incubated at 55 °C in a water bath for 1 hour with shaking every 10 minutes. After incubation, the solution was centrifuged at 3000 RPM for 15 minutes. The supernatant was taken and added 700 µl PCIA (1:1) then centrifuged at 3000 RPM for 15 minutes. Furthermore, the aquous phase in the supernatant is transferred to new conicles and cold absolute ethanol (1:1) was added then thread-like DNA will form. The DNA was transferred to a new microtube tube. Then washed with 500 µl 70% ethanol 3 times. After that, let dry and added with 50 µl Tris-EDTA (TE).

Analyze quality of genomic DNA by 1% agarose electrophoresis

Agarose gel was made with a concentration of 1%. The DNA dye used SBYR safe. Buffer electrodes used Tris Acetic-EDTA (TAE). The sample was mixed with loading dye and then put in a gel pit. The electrophoresis was run with a voltage of 100 volts for 30 minutes.

DNA amplification

The Polymerase Chain Reaction (PCR) mix was made in a total volume of 25 µl containing 12.5 µl master mix (Promega) kits, Forward primers (5'-TTAACGCAGTAAAGAGAGGACGTT-3') and Reverse (5`-ATGGCACAAGTCATTAATACAA-3`)[14] each 2 µl, DNA template 1 µl and nuclease-free water 7.5 µl. The thermal cycler settings are predenaturation 95 °C for 4 minutes, denaturation 95 °C for 30 seconds, annealing 48 °C for 30 seconds, extension 72 °C for 2 minutes, with the PCR cycle repeated 35 times. Post extension 72 °C for 10 minutes, then temperature 4 °C for 10 minutes to maintain PCR product stability.

Analysis of PCR results with 2% agarose electrophoresis

Agarose gel was made with a concentration of 2%. The DNA dye used is SBYR safe. Buffer electrodes used TAE. The PCR sample was inserted into the wells and used a 100 bp (Vivantis) DNA marker. The electrophoresis was run with a voltage of 100 volts for 60 minutes

Inoculation and culture in *Salmonella Shigella* Agar (SSA) media

Grass-jelly samples are homogenized first. The homogenate sample was put into a test tube and added physiological NaCl in the ratio of 1:9 (sample 1 g, physiological NaCl 9 ml). After being homogeneous it is put into BHI broth media. Incubated for 24 hours at 37 °C. Then cultured on SSA agar, incubated for 24 hours at 37 ° C. Morphological characters of the growing colonies were observed, such as color, shape, size, edge, consistency, and elevation.

Biochemical testing

S. typhi suspected colonies with a black center in SSA were cultured on biochemical test media, those were Indol, Methyl Red (MR), Voges Proskauer (VP), Citrate, Motility, Urea and Triple Sugar Iron Agar (TSIA) incubated at 37 ° C for 24 hours.

Insilico PCR

In silico PCR analysis used website http://insilico.ehu.eus/ [15] then the sequences from the primer pair were added. These primers analyze in All strains of Genus *Salmonella*.

3. RESULTS AND DISCUSSION

Identification of *S. typhi* contamination in grass-jelly samples is needed to provide protection to consumers and prevent typhoid fever. In the two traditional markets of Semarang City (Pasar Pedurungan and Peterongan) that we observed, the process of selling black grass-jelly was placed in an open condition, exposed to dirty market air, dust, and flies attached to grass-jelly. This allows the contamination of microorganisms.

Grass-jelly samples were taken from Pedurungan Market (A1 and A2), Peterongan Market (B1 and B2), and one of the branded packaging products from minimarket (C1 and

C2) in Semarang City as a comparison of grass-jelly in factory packaging. This market location was chosen because it is the place most visited by the people to buy grass-jelly.

There were 2 steps carried out in this study, the molecular identification stage and the validation stage. The molecular identification stage was by testing the DNA of *S. Typhi* in grass-jelly samples using PCR based on the *fliC* gene. The validation stage was a bacterial culture method from grass-jelly samples using selective media and biochemical tests.

The results of genomic DNA extraction got genomic DNA (gDNA) isolates obtained from suspected *Salmonella* culture from grass-jelly samples visualized using agarose electrophoresis 1% in 100 volts for 30 minutes. The genomic DNA is closest to the good gel because of its large DNA size (Figure 1. A). DNA fragments under the genome bands were might be extrachromosomal DNA such as plasmids. DNA fragments that migrate to the bottom of the gel were suspected debris from cell proteins or protease enzymes while the smears formed are likely short pieces of DNA or RNA.
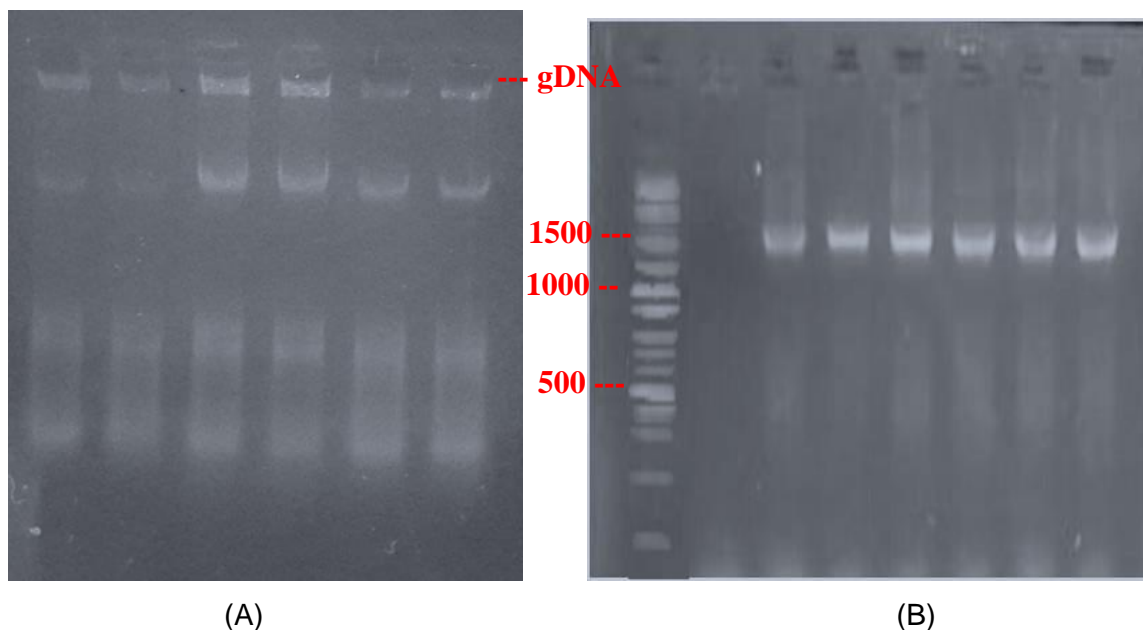

(A) (B)

Figure 1. Visualization of genomic DNA (A) and PCR products (B) from isolates culture

Primers that are used have specificity on the sequenced of *fliC* gene. The *fliC* and *fliB* genes play a role in the synthesis of flagellin proteins that compose bacterial cell flagella.[16] The results of amplification DNA isolates from grass-jelly based on *fliC* gene primer visualized using agarose electrophoresis 2% showed a single DNA fragment size around 1500 bp (Figure 1. B). This is in correspondence with another research.[12,13,14]

In silico PCR analysis from http://insilico.ehu.eus/ [15] using sequences from the primer pair, showed the results of amplified the *fliC* gene with a size of 1521 bp in *S. typhi* strain (Figure 2). The sequence database is derived from the Multiple Drug Resistant (MDS) *Salmonella enterica serovar typhi* CT18.[17] We used *in silico* PCR to matched the size of the PCR product that resulted in this research with the database reference.
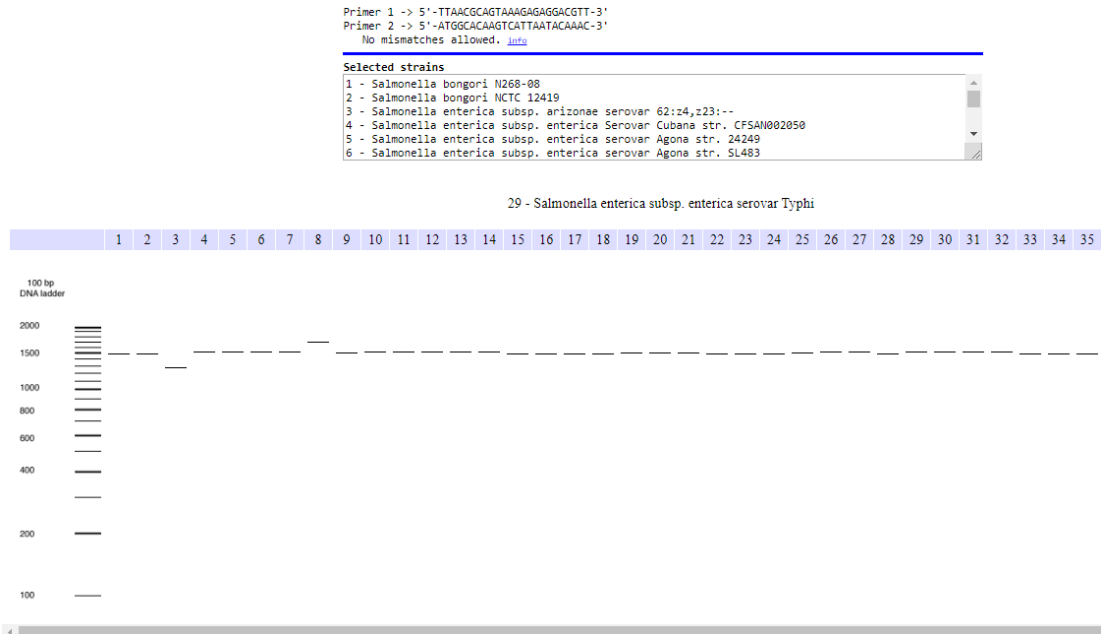
```
Primer 1 -> 5'-TTAACGCAGTAAAGAGAGGACGTT-3'
Primer 2 -> 5'-ATGGCACAAGTCATTAATACAAAC-3'
    No mismatches allowed. Info
Selected strains
1 - Salmonella bongori N268-08
2 - Salmonella bongori NCTC 12419
3 - Salmonella enterica subsp. arizonae serovar 62:z4,z23:--
4 - Salmonella enterica subsp. enterica Serovar Cubana str. CFSAN002050
5 - Salmonella enterica subsp. enterica serovar Agona str. 24249
6 - Salmonella enterica subsp. enterica serovar Agona str. SL483
```

29 - Salmonella enterica subsp. enterica serovar Typhi

Figure 2. *In silico* amplification of *FliC* gene using primer pairs LPW 1857 from all *Salmonella* genome in a database (http://insilico.ehu.eus/)

Molecular identification of *Salmonella* contamination in samples of juice drinks at the Wonodri traditional market, Semarang, was previously conducted it resulted in 3 of 16 fruit juice samples in Gunung Pati District of Semarang were Positive of *Salmonella*.[18] The test uses primers for *invA*1 and *invA*2 genes that produce 244 bp DNA amplification. Therefore, the study of development primers target for detecting specific genes in certain pathogenic bacteria needs to be carried out. Developing specific primers is important to detect specific bacteria such as the pathogenic strains, so the molecular detection results are precise on target.

Figure 3. Morphology of culture colonies resulted on SSA media

SSA is a bacterial medium that uses lactose as a source of carbohydrates, contains a color indicator of pH changes is Neutral Red, and contains ferric citrate as an indicator of H_2S gas formation. Interpretation of the results of bacterial culture samples on SSA media that were colorless or transparent colony because does not ferment lactose and was able to produce black sediment due to producing H_2S (Figure 3). A colony that had the character of *Salmonella typhi* which is the black center was used for biochemical testing.[9,19]

Interpretation of the biochemical results of three samples with double repetitions showed differences (Table 1). The indole test was negative because a pink ring did not form when the Kovach reagent was added. Kovac or Erlich reagents containing hydrochloric acid and p-dimethylaminobenzaldehyde in amyl alcohol will react with indole derived from tryptophan hydrolysis by showing pink color.[9] MR test result in isolates from Pedurungan and

minimarket were negative because there was no red color when Methyl Red reagent was added but in Peterongan isolates were positive. Methyl red is an indicator of organic acids at pH 4.5 and below to indicate the fermentation of glucose. All *S. typhi* isolate was negative in the indole test result and *S. typhi* isolates from Peterongan Market had positive Methyl Red result while others were negative.

The results of the VP test were negative in isolates from Peterongan and minimarket because no red color was formed when the α-Napthol reagent and 40% KOH were added but in Pedurungan isolates were positive. VP test to see the presence of acetyl-methyl carbinol (acetoin) production from glucose fermentation. Acetoin will be reacted with 40 % KOH to form diacetyl then diacetyl and the guanidine group in the media will be reacted with α -naphthol which will produce condensation showing pink color. Citrate test results were negative because all isolate could not turn media to blue due it uses a color indicator for changing the pH of Bromothymol Blue (BTB). The citrate test is to determine the ability of microorganisms to use citrate for carbon compounds as an energy source. When a bacterium uses citrate, the ammonium salt will be converted to alkaline ammonia so that the pH in the media increases, and the BTB indicator will show blue.[9] *S. typhi* isolates from Pedurungan Market had a positive result in VP test while others were negative. All *S. typhi* isolates had a negative result in the citrate test.

Motility test using semisolid media with 0.4% agar and Triphenyltetrazolium Chloride (TTC), the results were positive because the media turned turbid and red color formed on the puncture marks from the TTC reaction that showed cell movement. Urea test results were negative because all parts of the media did not turn pink. TSIA results were Alkalis/Acid and H_2S (+), which means red on the slope because it does not ferment sucrose and lactose, while a yellowish black color at the bottom of the media due to glucose fermentation and H_2S formation. Whereas gas formation in Pedurungan and minimarket isolates were positive but in Peterongan isolates were negative.

Table 1. Result of biochemical testing from colony cultures such Indol, MR, VP Citrate, motility, urea, and TSIA test

Biochemical test	Pedurungan Market		Peterongan Market		Minimarket		*S. typhi* (Hall and Woods, 2017)
	A1	A2	B1	B2	C1	C2	
Indol	-	-	-	-	-	-	-
MR	-	-	+	+	-	-	+
VP	+	+	-	-	-	-	-
Citrate	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+
Urea	-	-	-	-	-	-	-
TSIA	Alkaline/Acid and H_2S (+), gas (+)	Alkaline/Acid and H_2S (+), gas (+)	Alkaline /Acid and H_2S (+), gas (-)	Alkaline/Acid and H_2S (+), gas (-)	Alkaline /Acid and H_2S (+), gas (+)	Alkaline/Acid and H_2S (+), gas (+)	Alkaline/Acid and H_2S (+), gas (-)

Biochemical test results can represent the physiological characteristics of certain bacteria. Based on the results of biochemical tests in this study, isolates from the Peterongan market matched with the characteristics of *S. typhi*[9,19] supported by the *fliC* gene amplification result. Biochemical identification test results and colony culture on SSA media were used as a comparison of the results of the amplification *fliC* gene in DNA isolated from grass-jelly samples. Identification of *S. typhi* using the *fliC* gene only without biochemical test is less specific because all other *Salmonella* isolates can also amplify the fliC gene by PCR.

Molecular-based food testing is fast enough and accurate to detect types of pathogenic bacteria contaminants qualitatively in food compared to culture methods and biochemical tests but the accuration has to increase. As a result of Table 1, we can see that isolates from Pedurungan and the minimarket have different biochemical characteristics with reference. For

increases the specificity we can use multiplex PCR with more that one primer such for detecting S. Typhi we can use genes such *rfbE, fliB, fliC* and *invA1*, and *invA2*.[20,21] However, the use of conventional PCR methods has limitations that can only be used to detect types of bacteria but are unable to count the number of bacterial cells. The method for detecting and calculating the predicted number of bacteria can be done by Quantitative PCR (qPCR).[22,23,24,25]

Based on the results of this study grass-jelly samples from two traditional markets and one branded packaging product, both were positive for *S. typhi*. In traditional markets it was observed that the process of selling black grass-jelly was placed in an open condition, exposed to air, dust, and the presence of flies attached to grass-jelly so that it was suspected to be a source of contamination beside from its conventional processing. Whereas the branded grass-jelly sample from a minimarket should not contain *S. typhi* because it is more controlled by the regulation of the Good Manufacturing Practice (GMP) system of industrial companies.[26] Suspected contamination in branded food samples was predicted comes from suppliers, storage processes, mixing, filling, or packaging processes in the company.[27] From the findings of *S. typhi* in the sample, manufacturing companies must improve and pay attention to the hygiene in the manufacturing process to maintain product safety.

4. CONCLUSION

In this study it can be concluded that the three grass-jelly samples from the Peterongan Market were contained *S. typhi* contamination and isolate from Pedurungan and minimarket were *Salmonella* spp. The method of identification pathogenic bacteria of S. typhi on food used amplification of *fliC* gene cannot be specific in *S. typhi* because isolates that have different biochemical characters from the *S. thypi* reference remain amplified so reducing the accuracy of the results.

DISCLOSURE STATEMENT
The authors declare that they have no conflict of interest.

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SHORT BIOGRAPHY

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Original Research

OPEN ACCESS

The potential of ethanol extract of white pomegranate leaves (Punica granatum L) as anti-bacterial

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HIGHLIGHTS

We were found that the extract of white pomegranate leaves was able to inhibit the growth of positive Gram bacteria strains MRSA and the extract was unable to inhibit the growth of Escherichia coli bacteria strain ESBL.

ARTICLE INFO

A B S T R A C T

Treatment of infections using penicillin-derived antibiotics such as methicillin has been found to cause antibiotic-resistant bacteria. This bacteria could produce a beta-lactamase enzyme to form a resistant strain. Research on antibacterial activity continues to develop. Pomegranate (Punica granatum L.) was one of the herbal plants whose fruit has long been used for the treatment and prevention of various diseases. This study aimed to determine the potential inhibition of white pomegranate leaf extracts (Punica granatum L.) on the growth of Gram-negative bacteria including Escherichia coli Extended-Spectrum Beta-Lactamase (ESBL) strain and Gram-positive bacteria Staphylococcus aureus Methicillin-Resistant Staphylococcus aureus (MRSA) strain. White pomegranate leaf extract macerated with ethanol 96%, evaporated to obtain pure extracts made with a concentration of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and with 100% tested with invitro diffusion method. It was found that the extract of white pomegranate leaves with 30% (10.00 ± 0.0) concentration was able to inhibit the growth of positive Gram bacteria strains MRSA and the extract was unable to inhibit the growth of Escherichia coli bacteria strain ESBL.

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1. INTRODUCTION

Compounds that work by inhibiting bacterial growth or causing bacterial death called antimicrobials. The use of excess antimicrobials, incorrect dosages[1] can lend to resistance. This bacterium has the defense power of antibiotics by mutating the active and binding sites, forming trans membrane proteins known as efflux and plasmid proteins that encode the antibiotic-resistant gene.[2,3] Gram-negative bacteria in the beta-lactam class were one of the bacteria that can perform that ability. Bacteria produce beta-lactamase enzymes that can break the beta-lactam ring so that the antimicrobial becomes inactive.[1] The resistance of beta-lactamase producers is formed mainly by inappropriate use of antibiotics.[1,4] Bacteria that produce the beta-lactam enzyme called Extended-Spectrum Beta-Lactamase (ESBL) such as *Escherichia coli*,[1] *Staphylococcus aureus* strain Methicillin-resistant Staphylococcus aureus (MRSA).[3,5] Bacteria have been resistant to penicillin-derived antibiotics such as meticillin, so it is necessary to choose the right drug.[1,6]

Bacteria that are resistant to antibiotics need proper treatment.[6,7] Treatment of infectious diseases using herbal plants that have been used because it has active compounds that act as antibacterial compounds such as pomegranate leaves.[8] Pomegranate (Punica granatum L.) herbal plants that have long been used as a fruit treatment and prevention of various diseases, including cancer-preventing diseases,[9,8,10] cardiovascular disease,[11,3] inflammation,[12] and protection against ultraviolet radiation.[13,9]

Pomegranate has various types, namely red, white, black pomegranate. Red pomegranate leaf extract has a natural antibacterial power against *Escherichia coli*[12] There is no research on white pomegranate leaves against Gram-negative bacteria that produce the beta-lactam enzyme. This study aimed to determine the potential of white pomegranate leaf extract (Punica granatum L.) on inhibiting the growth of Gram-negative bacteria including the extended Spectrum Beta-Lactamase (ESBL) and Gram-positive strains of Methicillin-Resistant Staphylococcus aureus (MRSA) strains.

2. MATERIAL AND METHOD

This research is experimental. White pomegranate leaves were taken in Kayu Putih Village, Oebobo District, Kupang, East Nusa Tenggara. White pomegranate leaves are taken, washed thoroughly with water, and air-dried for 5 days, then mashed until leaf powder is found. White pomegranate leaf powder weighed 300 grams, then macerated first with 70% ethanol in a ratio of 1:10 and the second 1:5. Ethanol extract was thickened with a rotary evaporator, dissolved with 5% dimethyl sulfoxide (DMSO) made in series of concentrations of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100%.

Test bacteria Staphylococcus aureus strain Methicillin-Resistance Staphylococcus aureus (MRSA) was compared with Mc Farland 0.5. A total of 100 µl of test bacteria was leveled on Muller Hinton Agar (MHA) medium and then made into wells with a diameter of 8 mm using the well diffusion method. Extracts of white pomegranate leaves that have made various concentrations are put into the well as much as 50 ul, then incubated at 37^0 C for ± 24 hours. Amoxicillin 1 mg/ml is used as a control. The observed parameter was the diameter of the inhibition zone of each treatmen.[14] Each concentration of white pomegranate leaf extract was repeated three times and then the average was calculated.

3. RESULTS AND DISCUSSION

The measurement results of the inhibition zone formed for the extract of white pomegranate leaves with concentrations ranging from 10%, 20%, 30%, 40%, 50% against *Staphylococcus aureus* bacteria strain Methicillin-Resistance Staphylococcus aureus (MRSA) can be seen in table 1.

Tabel 1.The inhibition zone formed of extract white pomegranate

Concentration (mg / ml)	The mean diameter of the zone of inhibition against S. Aureus Bacteria ± SD (mm)			The average diameter of the zone of inhibition against E. Coli bacteria ± SD (mm)		
100	14.50	±	0.50	0	±	0
90	13.83	±	0.29	0	±	0
80	13.67	±	0.58	0	±	0
70	11.50	±	0.50	0	±	0
60	10.83	±	0.29	0	±	0
50	9.83	±	0.29	0	±	0
40	10.33	±	0.58	0	±	0
30	10.00	±	0.00	0	±	0
20	0.00	±	0.00	0	±	0
10	0.00	±	0.00	0	±	0

DP 10 50 ESBL
DP 60 100 EC

DP 10 50 MRSA
DP 60 100 MRSA
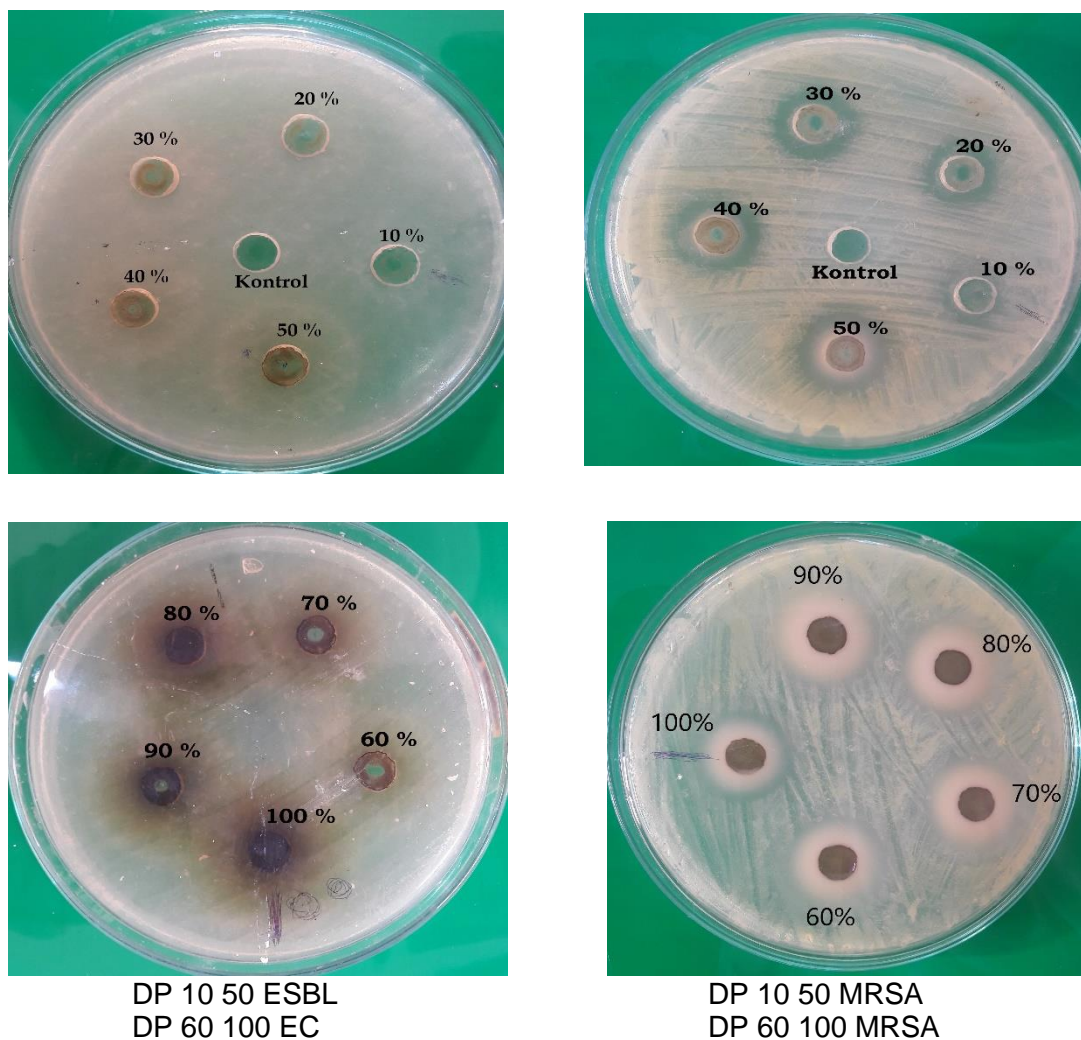
Figure 1. The inhibition zone formed of extract white pomegranate to ESBL and MRSA

White pomegranate leaf extract was tested with bacteria and measured by the clear zone formed around the extract. This shows (Figure 1) the inhibitory white pomegranate leaf extract has antibiotic or antimicrobial properties when tested with *Staphylococcus aureus* strain of Methicillin-Resistant Staphylococcus aureus (MRSA), although it gives different sensitivity results.

The difference in anti-bacterial sensitivity is influenced by the structure of the bacterial cell wall.[1,15,16] This corresponds to the morphology of the *Staphylococcus aureus* bacteria that are included in Gram-positive bacteria, which tends to be more sensitive because of the structure of the simple cell wall compared to Gram-negative, so it makes it easier for anti-bacterial compounds to enter into cell.[17] *Staphylococcus aureus* cell wall structure consists of peptidoglycan with little lipid and cell walls containing polysaccharides (teicoat acid).[3] Teicoat acid is a water-soluble polymer, which functions as a positive ion to exit or enter. This water-soluble indicated that the cell wall of Gram-positive bacteria is more polar.[15] Anti-microbial compounds found in white pomegranate leaves are part that is polar so that makes it easier penetrates the polar layer of peptidoglycan.[3]

White pomegranate leaf extract has the smallest inhibitory zone at a concentration of 30% which is 10 mm, then increasing to a concentration of 100% 14.50 mm. The concentration of white pomegranate extract which is effective as an antibacterial against *Staphylococcus aureus* bacteria was analyzed with a P-value 0.000 so that there is an effect of white pomegranate extract on the growth of *Staphylococcus aureus* (MRSA) bacteria.

The difference in inhibition of each concentration is influenced by the levels of the extract. According to small inhibitory zones indicate lower antibacterial activity while large inhibitory zones indicate greater antibacterial activity.[2] This is because secondary metabolites in white pomegranate leaves contain saponins, phenols, and tannins that are antibacterial[1,16,14,12]. Antibiotic compounds contained in white pomegranate leaf extract can inhibit the growth of MRSA.

Suggested three categories of extract antibacterial activity,[15] antibacterial activity of white pomegranate leaf extract against MRSA bacteria is included in the medium category. The increase in concentration is directly proportional to the diameter of the formed inhibition zone. Hardana explained that the factors influencing the diameter of inhibitory zones are the diffusion rate of antibacterial substances, the degree of bacterial sensitivity, and the speed of bacterial growth.[5] The diffusion of solutes from higher concentrations is faster than lower concentrations. Extracts with higher concentrations spread faster and more widely in agar media.[15] This results in the growth of bacteria that are around so that is passed by the extract are inhibited.[3]

Metabolite compounds found in pomegranate leaves such as alkaloids and tannins are considered antibacterial.[2,8,10,18,19] According to Saaed[9] the alkaloid compounds found in pomegranate leaves are 2-(2-propenyl) piperidine. Alkaline groups in alkaloids react with amino acid groups in cells that cause changes in the structure and composition of amino acids so that the cell is damaged.[9,20,21]

Flavonoids in pomegranates can inhibit bacterial growth by inhibiting the process of DNA gyrase in bacteria based on antibacterial activity by damaging cell membranes that cause intracellular leakage.[8,21] Queercetin (a flavonoid in pomegranate) can kill bacteria by increasing the permeability of membranes and damaging bacteria inside membrane potential, causing bacterial ATP production to be disrupted, disrupting membrane transport and movement of bacteria.[12] The content of polyphenols in pomegranates can kill bacteria by denaturing enzymes, but it can also be attached to substrates such as minerals, vitamins, carbohydrates so that bacteria cannot be used for their metabolism.[13] Polyphenols can also be absorbed in cell walls disrupting the structure and function of cell membranes.[17,10,21]

White pomegranate leaf extract after being tested with *Escherichia coli* strain Extended-Spectrum Beta-Lactamase (ESBL) showed no inhibitory or low level of antibacterial sensitivity. ESBL bacteria are Gram-negative bacteria tend to be more resistant to antibacterial, because the cell wall of Gram-negative bacteria is more complex where it consists of 3 components located outside the peptidoglycan layer, namely lipoprotein, outer membrane, and lipopolysaccharide.[12,15] The lipoprotein layer is useful for stabilizing the outer membrane and attaching it to the peptidoglycan layer. The outer membrane has a double-layered structure in which larger antibiotic

molecules are relatively slow when penetrating the outer membrane so that Gram-negative bacteria are relatively more resistant to antibiotics.[12,16]

4.   CONCLUSION

White pomegranate leaf extract can inhibit the growth of the MRSA strain (Methicillin-Resistant Staphylococcus aureus) *Staphylococcus aureus* bacteria with the smallest concentration of 30% (10.00 ± 0.0). White pomegranate leaf extract cannot inhibit the growth of *Escherichia coli*, with Extended-Spectrum Beta-Lactamase (ESBL).

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FUNDING INFORMATION

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Original Research

Erythrocyte index of residents exposed to lead in Tambaklorok, Semarang, Indonesia

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HIGHLIGHTS

The erythrocyte index value was mostly in the normal category

ARTICLE INFO

A B S T R A C T

Erythrocyte index can form erythrocyte morphology in the peripheral blood smear preparation. Lead exposure in Tambaklorok exceeds the threshold that affects anemia. The purpose of this study was to determine the erythrocyte index confirmation with erythrocyte morphology in the peripheral blood smear preparation. A cross-sectional study was conducted in 2month in Tambaklorok Semarang residents and 104 samples were taken using the purposive technique. Erythrocyte index was measured using the hematological analyzer and erythrocyte morphology in the peripheral blood smear preparation using Giemsa painting. Erythrocyte index confirmation with the peripheral blood smear preparation was analyzed using the Gamma statistical relationship test. The results showed that the erythrocyte index value was mostly in the normal category, i.e., MCH 68 (64.4%), MCHC 61 (58.6%) and MCH 58 (56%) and below normal category were MCH 45 (42.95%), MCHC 41 (39.4%), and MCV 36 (34.3%). Erythrocyte morphology was mostly hypochromic, namely 46 (44.23%), normochrome 40 (38.46%), and hyperchrome 18 (17.3%). Relationship of MCH with erythrocyte color p-value 0.037 with size p-value 0.038. Conclusion of erythrocyte index confirmation, especially MCH with the peripheral blood smear preparation, there was a match on the color and size of erythrocytes, while the MCV and MCHC values had no significant relationship.

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1. INTRODUCTION

Erythrocyte index was determined by calculating three parameters, namely hemoglobin (Hb), hematocrit (Ht) levels, and the number of Erythrocytes. Erythrocyte index calculation results show the average volume of erythrocytes and the average hemoglobin per erythrocyte, the average concentration of hemoglobin per erythrocyte. Clinically examination of the erythrocyte index is used as a filter for diagnosing anemia and its classification based on erythrocyte morphology.[1] Measurement of erythrocyte index needs to be confirmed using erythrocyte morphology examination on the peripheral blood smear preparation.[2]

Erythrocyte index consists of mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC). MCV is measured by comparing Ht levels with the number of erythrocytes in million / ul multiplied by 10 in femtoliter units (fl), MCH is measured by comparing Hb levels with the number of erythrocytes in million / ul multiplied by 10 by picogram units (pg), MCHC is measured by comparing Hb levels with Ht multiplied by 100%. MCV reference values 82-92 fl, MCH 27-31 pg, and MCHC 30-35 gr / dl.[3]

Peripheral blood smear preparation is a strategy to see the morphology of blood cells including erythrocytes, leukocytes, and thrombocytes.[4] Erythrocyte morphology is examined based on size, color, shape, and inclusion. High MCV and MCH will give an overview of the size of erythrocytes in peripheral blood smear preparation enlarge (macrocytes) and otherwise if the MCV and MCH are low, then the size of erythrocytes in peripheral blood smear preparation becomes smaller (microcytes).[3] It is important to know how the erythrocyte morphology in peripheral blood smear preparation is useful for confirming the erythrocyte index.

Increased MCV is found in aplastic anemia, hemolytic anemia, pernicious anemia, folic acid deficiency anemia, while decreased MCV is found in microcytic anemia, iron deficiency anemia, thalassemia, sickle cell anemia, lead/ plumbum poisoning.[5,3]

Lead exposure can cause anemia through the Delta-aminolevulinic acid dehydratase (ALAD) enzyme inhibition pathway in the heme biosynthesis process and has been proven,[6] but the erythrocyte index is not yet known with confirmation of peripheral blood smear preparation so that anemia is known. The benefits of anemia classification are very useful in the process of managing patients with anemia.

Various causes of anemia include lead exposure either through inhalation, digestion, or skin.[7] Lead that enters the body will accumulate and cause detoxification in both liver and kidney detoxification organs.[8,9] Biochemically erythropoietin hormone which functions for erythropoiesis is disrupted so that the formation of erythrocytes is inhibited. Lead can also inhibit the ALAD delta enzyme in the process of heme synthesis, so that heme is not formed which results in decreased levels of hemoglobin in the blood and the volume of erythrocytes becomes abnormal as indicated by a decrease in hematocrit levels. These parameters are used to determine the erythrocyte index.

Tambaklorok is part of the city of Semarang which is located in the northern part close to the north coast. Currently, the Tambaklorok region is a densely populated and industrious area. Various industries that have the potential as sources of lead contamination are the textile, pharmaceutical, power plant, ship repair, paint industry, cosmetics, batteries, pipes, ceramic coatings, children's toys, and others[10] located in Tambaklorok. Industrial waste management is not all good, a lot of industrial waste before going through the treatment process is discharged through the flow of water up to the edge of the sea resulting in polluted marine commodities such as green shells, seaweed, and fish consumed by many Tambaklorok residents. According to research conducted by Supriyantini and Soenardjo 2015, waters around Tanjung Emas including Tambaklorok area found to contain a lead level of 0.06 ppm, while the maximum standard quality was 0.008 ppm.[11] Other research conducted by Tm NK 2011, showed Pb levels that polluted the air in Tambaklorok region was 8.41 µg / m3 above the threshold value of quality 2 µg / m3 per 24 hours, the highest compared to other regions in the city of Semarang.[12] Results of research conducted by Marianti A, Prasetya AT, 2013, lead content in hair of North Semarang residents showed that 56 residents had the highest lead content of 17,028 ppm and an average of 8,304 ppm. The cause is thought to

originate from drinking water consumed by the residents containing an average lead level of 6 ppm.[13] Based on this description, it is probable that lead exposure in Tambaklorok can cause anemia. Therefore the classification of anemia based on the erythrocyte index becomes important to know so that the handling of anemia due to lead exposure can be overcome.

2. MATERIAL AND METHOD

This type of analytic research is a cross-sectional approach, study from January to March 2020. The study population is the residents of Tambaklorok Semarang and the number of samples is 104 taken based on the Lemeshow formula.[14] Sampling uses a purposive technique by not limiting age groups to residents who have lived for at least five years. Lead content data from Tambaklorok residents in the form of secondary data were obtained from the study of Marianti A, Prasetya AT, 2013. The erythrocyte index was obtained from measurements of venous blood samples using a hematology analyzer with the impedance principle. Erythrocyte morphology in the form of erythrocyte size and color was identified using peripheral blood smear preparation with Giemsa staining. To confirm the erythrocyte index with erythrocyte morphology in the peripheral blood smear preparation, a relationship test was performed using the Gommer test.

The study was conducted after obtaining ethical clearance from the Medical Faculty of UNISULA Semarang *No.064/III/2020/Komisi/Bioetik*. The Head of the Clinical Pathology Laboratory of the University of Muhammadiyah Semarang agreed to research after receiving notification of the results of ethical clearance. Erythrocyte index examination and erythrocyte morphology was carried out in the clinical pathology laboratory of Unimus.

3. RESULTS AND DISCUSSION

Erythrocyte index examination in the form of MCV, MCH, MCHC as well as a morphological description of erythrocytes based on color and size was carried out on Tambaklorok residents of Semarang who were suspected of being exposed to lead. MCV, MCH, MCHC values can be seen in figure 1. The graphs of increase and decrease in MCV, MCH, MCHC in each sample are almost the same. If there is an increase in MCV will be followed by an increase in MCH and MCHC and vice versa, although the increase or decrease can not be measured with the same number. It can be explained that each increased erythrocyte volume will be followed by a decrease in the mean and the percentage of hemoglobin levels. Likewise, a decrease in erythrocyte volume will be followed by a decrease in the mean and percentage of hemoglobin levels.

The mean value of MCV is still in the normal category even though it is at the lowest limit of its normal value (83.1), so is the mean value of MCHC that is also at the lowest limit of its normal value of 32. Whereas the mean value of MCH is below normal (26.8) (Table 1). The erythrocyte index value is mostly normal (Table 2), the highest normal category is MCH 68 (64.4%), followed by MCHC 61 (58.6%) and MCH 58 (56%). The highest erythrocyte index values below normal were MCH 45 (42.95%), MCHC 41 (39.4%), and MCV 36 (34.3%).

Table 1. Average MCV, MCH, MCHC among Tambaklorok residents in Semarang

Variable	n	Average	SD	Highest	Lowest
MCV	104	83.2	4.99	84.20	82.30
MCH	104	26.8	1.99	27.20	26.40
MCHC	104	32.0	3.40	32.30	31.30

Table 2. Values of MCV, MCH, and MCHC based on normal values

Variable	N	Results		
		< Normal (%)	Normal (%)	>Normal (%)
MCV	104	36 (34.30)	68 (64.40)	1 (1.00)
MCH	104	45 (42.90)	58 (56.00)	1 (1.00)
MCHC	104	41 (39.40)	61 (58.60)	2 (1.90)

In the peripheral blood smear preparation that has been stained with Giemsa staining, erythrocyte morphology can be grouped into hypochromic, that is, pale color with a diameter exceeding 1/3 central polar, normochromic that is normal with a pale color 1/3 the diameter of the central polar, and hyperchromic that is pale the entire surface of the erythrocyte does not appear to have a central poll. Besides being grouped by color, the morphology of erythrocytes is also seen based on their size, which is normal (normocyte), which is 6.9 μ and 9.6 μ., Small (microcytes), and large (macrocytes). The following in figure 2 an example of hypochromic normocytic erythrocyte morphology.
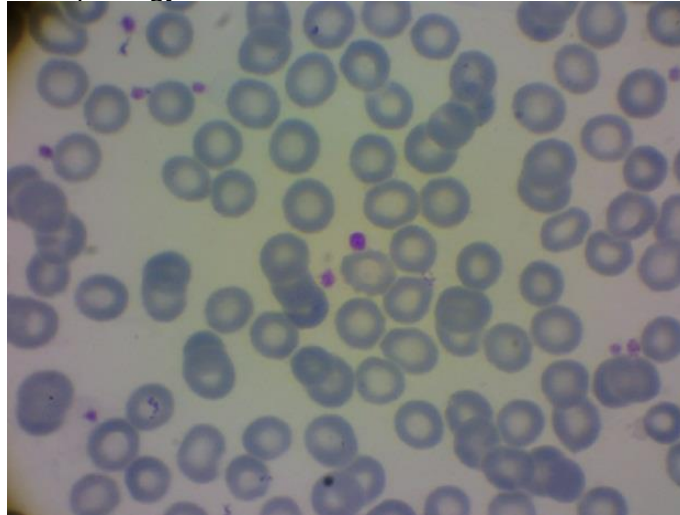

Figure 2. Hypochromic Normocytic
In Figure 2, it comes from sample no. 50 whose MCV value is 87.7 fl and MCH is 26.6 pg

The morphology of erythrocytes (Table 3) based on color abnormalities was mostly found hypochromic, namely 46 samples (44.23%), while normochrome 40 samples (38.46%), and the least hyper chrome was 18 (17.3%). Erythrocyte morphology (Table 4) based on size abnormalities, found 72 (69.23 samples of normal size (normocytic), only 14 (13.46%) were microcytic, and 18 (17.3%) were macrocytic.

Table 3. Erythrocyte morphology based on color abnormalities

Color	Frequency	Percentage (%)
Hypochromic	46	44.23
Normochromic	40	38.46
Hyperchromic	18	17.30
Total	104	100

Table 4. Erythrocyte morphology based on the size of the measurement

Size	Frequency	Percentage (%)
Microcytic	14	13.46
Normocytic	72	69.23
Macrocytic	18	17.30
Total	104	100

Tables 5 and 6 are a recap of the results of statistical tests of the relationship between the erythrocyte index and the morphology of erythrocytes based on the color and size of the erythrocytes. Based on the results of the gamma statistical test, only MCH with color and MCH with a size that has a relationship, respectively, the p-value is 0.038 and 0.037. MCV and MCHC both in color and size are statistically not significant.

Table 5. Erythrocyte index based on the color of erythrocytes in the peripheral blood smear preparation

Erythrocyte index	Category	Color			P-value
		Hypochromic	Normochrome	Hyperchrome	
MCV	< N	17	12	7	
	N	28	28	11	0.783
	>N	0	1	0	
MCH	< N	25	14	6	
	N	20	26	12	0.038
	>N	0	1	0	
MCHC	< N	19	10	12	
	N	26	29	6	0.587
	>N	0	2	0	

Table 6. Erythrocyte index based on the size of erythrocytes in the Peripheral Blood Smear Preparation

Erythrocyte index	Category	Size			P-value
		Microcytic	Normocytic	Macrocytic	
MCV	< N	6	24	6	
	N	7	48	12	0.632
	>N	0	1	0	
MCH	< N	9	31	5	
	N	4	41	13	0.037
	>N	0	1	0	
MCHC	< N	3	35	3	
	N	10	36	15	0.516
	>N	14	73	18	

Based on the results of the study it was found that the average value of MCH was in the lowest normal limit and most were less than normal when compared with MCV and MCHC. The statistical test results obtained a p-value of 0.038 between MCH and erythrocyte color and size, meaning that there is a significant relationship between MCH and erythrocyte color and erythrocyte size. MCH is the average level of hemoglobin contained in erythrocytes. The higher the level of hemoglobin in the blood, the higher the hemoglobin content in erythrocytes will be and vice versa so that it can be used as an indicator of anemia.[3,15] Such a condition, when viewed in terms of erythrocyte morphology in the preparation of peripheral blood smear, will give a picture of pale erythrocytes. The pale color is determined by the width of the central polar diameter of the erythrocytes. The pale coloration of erythrocytes in peripheral blood smear preparations is increasingly apparent with Giemsa staining.[16]

In this study, MCH with erythrocyte size was also found to have a significant relationship. Some studies of erythrocyte size are related to erythrocyte volume.[15] In this study showed that the hemoglobin content was also related to the volume of erythrocytes. If the hemoglobin content in erythrocytes decreases, the size of erythrocytes also decreases.[17] Several studies have shown that the color and size of erythrocytes were associated with the type of anemia. A decrease in MCH occurs in patients with microcytic anemia and hypochromic anemia, whereas an increase in MCH occurs in patients with iron deficiency anemia.[18,19,20]

Hypochromic anemia based on the results of this study occurred due to low hemoglobin levels in the samples/ respondents. The low level of hemoglobin of Tambaklorok Semarang residents was inseparable from the previous history of lead exposure that was above the threshold. Hemoglobin is composed of heme and globin, the presence of lead can inhibit heme synthesis.[21,22,23,24] The mechanism of lead heavy metal poisoning in the heme synthesis process occurs as a result of suppressing enzyme activity at the beginning, middle, and end of heme biosynthesis. Enzyme δ ALAD is a starting enzyme that is inhibited by the presence of lead. Due to the inhibition of lead in the δ ALAD enzyme, it will cause the cessation of δ ALA to become *porphobilinogen* (PBG). This can result in increased levels of ALA in the

blood and urine. The intermediate enzyme that is inhibited by lead exposure is *coproporphyrinogen* oxidase which can cause an increase in *coproporphyrinogen* levels. The last enzyme that is inhibited by lead in the heme biosynthesis process is *ferrochelatase*. Obstacles that occur in ferrochelatase will cause elevated levels of protoporphyrin in red blood cells / free erythrocyte protoporphyrin (EPP) so that the porphyrin ring is not formed and results in the failure of heme formation.[25,26]

The results of statistical tests for MCV and MCHC values obtained an average p-value above 0.05, which means that statistically there is no significant relationship with the size and color of erythrocytes, although descriptively there is a downward trend in the mean value. This could be possible through hematocrit levels and the number of erythrocytes was decreasing, some were still within normal limits. The main finding of this study is that erythrocyte morphology in peripheral blood smear preparation can be used as confirmation of erythrocyte index results, although the strength of this study is only significant in the relationship of MCH to erythrocyte color and size. The limitation of this study is that in a population with mild anemia, further research is needed to confirm the erythrocyte index based on erythrocyte morphology in peripheral blood smear preparation preparations in a severe anemia population.

CONCLUSION

Based on the results of research and discussion it can be concluded that the erythrocyte index confirmation, especially MCH with the morphology of erythrocytes in the form of color and size is appropriate as evidenced by a significant relationship, while the values of MCV and MCHC have no significant relationship.

DISCLOSURE STATEMENT

The authors declare that they have no conflict of interest.

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Figure 1. Distribution of MCV, MCH, MCHC values in Respondents
(Y=MCV, MCH,MCHC. X=no sample)

Original Research

Peripheral stem cell mobilization strategies in patients with autologous hematopoietic cell transplantation: A single center's experience

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HIGHLIGHTS
The white blood cell count was inversely correlated with the success of mobilization

ARTICLE INFO

A B S T R A C T

This research is to investigate the parameters which may affect the mobilization of stem cells in patients receiving autologous hematopoietic peripheral blood stem cell transplantation (PBSCT). A retrospective study was carried out using the data derived from the medical files of 242 patients who received PBSCT. Descriptive, clinical, and laboratory parameters were compared between patients with successful and unsuccessful stem cell mobilization. Successful stem cell mobilization ratio was 4.463 times higher when preemptive plerixafor was administrated; 1.032 times higher when CD34+ cell count increased 1 unit at the beginning of mobilization. The white blood cell count was inversely correlated with the success of mobilization. An increase of 1 unit in WBC count was associated with a 1.027 times decrease in the success rate. The data indicated that the administration of preemptive plerixafor and CD34+ cell count at the beginning of mobilization were directly related to the success of mobilization after PBSCT. On contrary, WBC count was inversely associated with the success rate.

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1. INTRODUCTION
High dose chemotherapy and bone marrow transplantation are common therapeutic modalities used in the management of hematological malignancies. An adequate number and

quality of stem cells must be infused to achieve a favorable autologous bone marrow transplantation. Relevant studies indicated that infusion of minimally $2x10^6$ cells/kg of CD34+ stem cells is enough for a satisfactory neutrophil and platelet engraftment on the 14th day following the transplantation.[1] Mobilization is defined as the removal of the hematopoietic progenitor cells (HPCs) from the bone marrow to the peripheral blood.[2]

Autologous transplantations and most allogeneic transplantations are currently performed primarily with peripheral blood stem cells (PBSCs) rather than stem cells from the BM. Because it is associated with high rates of cell collection, quicker engraftment diminished possibility of complications, simpler accessibility, lesser rates of tumor contagion, and quicker hematopoietic and immune restructuring.[3] Since successful hematopoietic peripheral blood stem cell transplantation (PBSCT) is associated with the quality and number of infused stem cells, the factors that may influence stem cell mobilization have been primarily studied.[3] It has been reported that the diagnosis of the patient, chemotherapy protocols, frequency of relapses, growth factors, and brand of apheresis devices as well as leukocyte and CD34+ cell counts on the 1st day of apheresis may influence the success of mobilization. The discrepancies in the outcomes of studies may be due to the small sample size, the inclusion of various mobilization regimens in the analysis, and the vagueness of the well-established benchmarks for successful mobilization.

The mobilized PBSCs constitute the primary source for hematopoietic PBSCT after myeloablative therapy. The conventional protocols for PBSCs mobilization involve the employment of granulocyte colony-stimulating factor (G-CSF) only or in combination with other myelosuppressive chemotherapeutics.[4] The recognition and elucidation of factors that influence stem cell mobilization are critical in optimizing therapeutic outcomes. Our purpose was to investigate the success of peripheral stem cell mobilization strategies in patients with autologous hematopoietic cell transplantation in our center and variables that may affect the mobilization of stem cells.

2. MATERIAL AND METHOD

Study design

This retrospective study was performed using medical records of 242 patients (158 males, 84 females) treated in the bone marrow transplantation unit of the hematology department of our tertiary care center between 2014 and 2018. The approval of the local Institutional Review Board was obtained before the study. The study has been implemented in adherence to the principles of the Helsinki Declaration.

According to our institutional policy, the initial mobilization attempt was performed with 10 µg/ kg/d G-CSF alone in patients with low tumor burden. Patients who needed salvage chemotherapy received G-CSF (10 µg/kg/d) in combination with chemotherapy. For poor mobilizers in G-CSF alone group, chemotherapy followed by G-CSF was preferred as a second line mobilization regimen. Chemotherapy was either high dose cyclophosphamide or salvage chemotherapies according to the primary disease of the patients.

On the other hand in patients who were mobilized with G-CSF plus chemotherapy enumeration of CD34 + cells in the peripheral blood was assessed when blood leukocyte count exceeds $1000/mm^3$ and apheresis was performed when the peripheral CD34+ cell count was $>20/mm^3$. Total nucleated and CD34+ cell count of the apheresis product was measured with flow cytometry.

Stem cell mobilization was assigned as unsuccessful in 65 (26.9%) patients, and successful in 177 (73.1%) patients according to the criteria defined by Gertz et al.[5] The success of mobilization is classified into three groups with respect to the CD34+ cell count collected after mobilization and leukapheresis: 1) Failure: CD34+ cell count $< 1x10^6$/kg; 2) Poor: $1x10^6$/kg $\leq$ CD34+, cell count $< 5x10^6$/kg; 3) Successful: CD34+ cell count $\geq 5x10^6$/kg.[5] In this study, failure and poor mobilization were accepted as unsuccessful, and their descriptive, clinical, and hematological parameters were compared with patients with successful outcomes.

The most common diagnoses were multiple myeloma (n=111; 45.9%), non-Hodgkin lymphoma (n=70; 28.9%), and Hodgkin's disease (n=43; 17.8%). For stem cell mobilization,

Filgrastim was utilized in many patients (n=231; 95.5%), while lenograstim was used in 11 (4.5%) patients. Accompanying comorbidity was detected in 61 patients (25.2%). Radiotherapy was administered in 24 patients (9.9%). The disease was stage 3 (n=107; 44.2%), stage 4 (n=62; 25.6%), stage 2 (n=23; 9.5%) and stage 1 (n=8; 3.3%).

A single dose of preemptive plerixafor was administered for stem cell mobilization in 28 patients (11.6%). There was refractory thrombocytopenia in 11 cases (4.5%). Leukapheresis has performed in case the CD34+ cell count in circulation was greater than 10/μL, for patients who were treated by chemotherapy and granulocyte colony-stimulating factor (G-CSF). The leukapheresis was performed on the 5th day in patients treated by G-CSF alone.

Statistical analysis

Data were analyzed via the Statistical Package for Social Sciences program version 21.0 (*SPSS Inc., Chicago, IL, USA*). Data are expressed as mean±SD or median (interquartile range), as appropriate. All differences associated with a chance probability of .05 or less were considered statistically significant. The initial evaluation of variables that may influence the success of mobilization was performed with univariate logistic regression analysis. The variables that yielded a p-value <0.020 were determined and involved in multiple logistic regression analysis.

3.   RESULTS AND DISCUSSION

In Table 1, the descriptive statistics and univariate logistic regression analysis results for categorical variables are presented. The comparison of groups with successful and unsuccessful stem cell mobilization indicated that there was no statistically significant difference between 2 groups as for sex distribution (p=0.864), disease stage (p=0.946), G-CSF type (filgrastim or lenograstim) (p=0.511), presence of comorbidities (p=0.464), refractory thrombocytopenia (p=0.476) and administration of radiotherapy (p=0.478). A significant relationship between the administration of preemptive plerixafor and success rate was observed.

The administration of preemptive plerixafor increased the success rate by 4.463 times. The comparative analysis of the impacts of various chemotherapy protocols indicated that doxorubicin, bleomycin, vinblastine and dacarbazine (ABVD) and gemcitabine, dexamethasone and cisplatin (GDP) (p=0.554); high dose dexamethasone (HIDEX) and Velcade®, cyclophosphamide and dexamethasone (VCD) (p=0.497); and Velcade®, cyclophosphamide and dexamethasone (VCD) alone had no significant effect on the success of mobilization (Table 2).

Table 3 demonstrates the results of the univariate logistic regression analysis for quantitative variables. There was a noteworthy correlation between CD34+ cell count at the beginning of mobilization and the success rate. Every increase in CD34+ cell count was associated with a 1.032 times amplification of the success rate of mobilization.

Multivariate logistic regression analysis was performed on variables with a p-value < 0.020 in univariate logistic regression analysis. The results of multivariate regression analysis are demonstrated in Table 4, and the mobilization success rate is affected by white blood cell (WBC) count and CD34+ cell count at the onset of mobilization. Every increase in WBC count was associated with 1.027 times decrease in the success rate of mobilization. On the other hand, each increase in CD34+ cell count was associated with a 1.037 times increase in the mobilization success rate.

The determination of risk factors linked with the accumulation of peripheral blood stem cells in various malignancies is essential in taking appropriate therapeutic decisions. Recently, some of the confounding and contributory factors have been identified, and efforts have been spent on the development of new mobilization strategies.

These results supported the publication by Jansen et al., which suggested that the CD34+ cell count may have a predictive role in the myeloablative therapy.[6] In contrast to studies supporting that prior radiotherapy may adversely affect mobilization, we did not observe any unwanted impact of radiotherapy.[7,8] In this study, the most unfavorable predictive

factor for progenitor cell output was the intensity of previous chemotherapy.[9,10] Chemotherapy regimens under investigation did not yield any obvious effect on the success rate of stem cell mobilization; however, we had a wide spectrum of chemotherapy protocols, and only 3 of them could be analyzed due to the small number of participants in each subgroup. Pre-emptive plerixafor administration has a remarkable and favorable effect on the success rate of mobilization. Further studies of new mobilization agents and their combination regimens are warranted to evaluate the outcomes of stem cell mobilization and to overcome the failure of mobilization.

The stem cells collected after a sequence of chemotherapy contained notably fewer plasma cells compared to those collected after a single high-dose drug administration. Different strategies were studied in MM patients for HSC harvesting and tandem transplantation.[11,12,13] There was an inverse correlation between CD34+ stem cell counts on the 9th day and neutrophil engraftment. This inverse correlation is noteworthy since it reminded that higher CD34+ stem cell levels by the 9th day, resulted in earlier neutrophil engraftment. Thus, the quantification of CD34+ stem cell levels on the 9th day may yield valuable information for autologous hematopoietic PBSCT.[14,15] Further trials must be implemented to evaluate the validity and significance of this finding.

Factors likely to affect total harvested cells involve >3 cycles of apheresis for an apheresis period, mobilization with lenograstim, harvesting with Fresenius device, and ≥ 35000 WBC counts on the 1st day of apheresis.[16,17,18] Moreover, factors that affect total harvested CD34+ cell count were reported as diagnosis, peripheral blood WBC counts on the 1st day of apheresis, harvesting with Fresenius device, and mobilization with filgrastim. The patient's diagnosis is an important parameter for the achievement of the highest total CD34+ cell counts. The success of the collection of CD34+ cells was highest in Hodgkin's disease and multiple myeloma. On the other hand, the lowest rate was detected in acute leukemias. This difference may arise from the high rate of stem cell damage due to the salvage treatments used in acute leukemia. This negative effect on the CD34+ cell mobilization is consistent with previous studies.[16,17,18] Many studies reported the impact of peripheral blood WBC and CD34+ cell counts on the success of mobilization at first-day apheresis.[16,19,20,21,22]

Our data yielded that gender distribution, disease stage, type of G-CSF type (filgrastim or lenograstim), the presence of comorbidities, refractory thrombocytopenia, and administration of radiotherapy did not influence the success of mobilization. However, the administration of preemptive plerixafor increased the success rate in univariate analysis. In multiple clinical studies, the combination of plerixafor with G-CSF resulted in more significant mobilization in CD34+ cells than G-CSF alone and more successful retrieval of hematopoietic stem cells from donors and better engraftment in recipients.[23] Fergadis et al. stated that plerixafor was useful to mobilize enough numbers of peripheral blood stem cells in relapsed malignancies after previous single or tandem high-dose chemotherapy and PBSCT.[24] Tolomelli et al., evaluated 37 multiple myeloma patients and stated that the timing of plerixafor administration influences immunological recovery.[25] Yang et al., analyzed the effectivity of plerixafor use for successful stem cell mobilization in non-Hodgkin lymphoma and multiple myeloma. Their findings indicated that the additional use of plerixafor to G-CSF provides an increased HSC collection in a shorter duration without any increase in adverse events.[26] Gutiérrez-Aguirre et al. demonstrated that the use of reduced doses of plerixafor might suffice to gather at least 2×10^6 /kg CD34.[27] Yoshifuji et al., stated that the use of plerixafor with an enough washout period may contribute to the successful mobilization after the use of pomalidomide.[28] Danner et al., have addressed the impact of serum albumin on homeostatic hematopoiesis and pharmacological mobilization of hematopoietic stem and progenitor cells.[29] Multivariate analysis indicated that higher counts of CD34+ cell count were associated with better success rates for mobilization, while increased WBC count was associated with the diminished success of mobilization. Although some publications supported that lenograstim was more potent than filgrastim for improvement of the success rate, our data did not confirm this postulate.[30,31] However, there was a remarkable equilibrium between patients receiving filgrastim and lenograstim in our series. Thus, understanding whether filgrastim is more

effective in the mobilization of progenitor cells and the recruitment of mature cells to the peripheral blood cells necessitates further prospective trials on larger series.

These findings yielded that gender, radiotherapy, and comorbidities did not alter the number of harvested CD34+ cells. This finding is controversial to the reports stating that radiotherapy exerted a negative effect on stem cell mobilization.[7,8] The other factors that may affect the success rate of mobilization involved qualitative and quantitative variability of the hematopoietic stem cells in the bone marrow, differences in the migration capacity of hematopoietic stem cells, and decreased response to G-CSF. Our results are consistent with previous reports stating that leukapheresis success was associated with CD34+ cell count in peripheral blood before the intervention.[32]

The factors reported herein may be also important for other cell therapies such as autologous bone marrow-derived mononuclear cells (BMDMC). Morales et al., reported that the instruction of BMDMCs through bronchoscope seems to be a feasible and safe method in accelerated and chronic silicosis.[33] Assmus et al., stated that repeated intra-coronary administration of BMDMCs seems to be linked with improvement of clinical outcomes compared with single treatment at 2 years in patients with heart failure after myocardial infarction.[34]

The main restrictions of this study are retrospective design and data confined to the experience of a single center. Although the patient number analyzed in this study was not few for a single-center study, still those are heterogeneous in many clinical backgrounds and the sample size of each patient group with a similar background is relatively small.

To sum up, these factors must be remembered before stem cell apheresis and more convenient decisions can be made in terms of the preparation procedures, the selection of technical measures, and apheresis devices. These parameters may all contribute to the improvement of the success of mobilization. A better understanding of mechanisms of mobilization will aid in the determination of the optimal time and using synergistic agents to have enough CD34+ cells. This approach will help the accomplishment of a cost-effective modality for hematopoietic stem cell transplantation.

CONCLUSION

To conclude, this research results indicated that the administration of preemptive plerixafor and CD34+ cell count at the beginning of mobilization were directly related to the success of mobilization in hematopoietic stem cell mobilization. On the contrary, WBC count was inversely associated with the success rate. Consideration of these points during the selection of patients and the establishment of the treatment plan may be useful to achieve better treatment outcomes.

DISCLOSURE STATEMENT

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Table 1. The comparison of groups with successful and unsuccessful stem cell mobilization for clinical variables (univariate logistic regression analysis)

Variable		Unsuccessful (n=65)	Successful (n=177)	Odds Ratio	Sig.*
Sex	Female	22 (26.2)	62 (73.8)	1.054	0.864
	Male	43 (27.2)	115 (72.8)		
Diagnosis	AML	0 (0.0)	1 (100.0)	1.235	0.160
	HL	11 (25.6)	32 (74.4)		
	MM	26 (23.4)	85 (76.6)		
	NHL	25 (33.7)	49 (66.3)		
	Solid tumor	3 (23.1)	10 (76.9)		
Stage	1	3 (37.5)	5 (62.5)	1.014	0.946
	2	4 (17.4)	19 (82.6)		
	3	34 (31.8)	73 (68.2)		
	4	17 (27.4)	45 (72.6)		
Remission before mobilization	CR	39 (31.0)	87 (69.0)	N/A	N/A
	Chemosensitive	0 (0.0)	2 (100.0)		
	PR	23 (28)	59 (72)		
	Progressive	0 (0.0)	2 (100.0)		
	Refractory	3 (18.8)	13 (81.3)		
	Stable	0 (0.0)	3 (100.0)		
	VGPR	0 (0.0)	10 (100.0)		
Mobilization regimen	G-CSF	36 (28.3)	91 (71.7)	4.285	*0.03*
	G-CSF + CT	13 (14.8)	75 (85.2)	6.923	*0.004*
	G-CSF+ Plerixafor	10 (66.7)	5 (33.3)	3.033	0.81
	CT + G-CSF + Plerixafor	6 (54.5)	5 (45.5)	0.6	0.532
G-CSF type	Filgrastim	63 (27.3)	168 (72.7)	1.687	0.511
	Lenograstim	2 (18.2)	9 (81.8)		
Filgrastam type	Granocyte®	2 (18.2)	9 (81.8)	1.125	0.895
	Leucostim®	30 (32.6)	62 (67.4)	0.517	0.167
	Neuopogen®	26 (25.0)	78 (75.0)	0.75	0.548
	Tevagrastim®	7 (20.0)	28 (80.0)	-	-
Preemptive plerixafor	Yes	16 (57.1)	12 (42.9)	4.463	*<0.001*
	No	49 (23.0)	164 (77.0)		
Co-morbidity	No	45 (24.9)	173 (75.1)	1.295	0.464
	Yes	15 (30.0)	46 (70.0)		
Refractory thrombocytopenia	No	61 (26.5)	169 (73.5)	1.582	0.476
	Yes	4 (36.4)	7 (63.6)		
Radiotherapy	No	57 (26.5)	158 (73.5)	1.385	0.478
	Yes	8 (33.3)	16 (66.7)		

* p value obtained after univariate logistic regression analysis.
CT: chemotherapy; AML: acute myeloid leukemia; HL: Hodgkin lymphoma; NHL: non-Hodgkin lymphoma; MM: multiple myeloma; DLBCL: diffuse large B-cell lymhoma; N/A: not applicable; G-CSF: granulocyte colony stimulating factor; CR: complete remission; PR: partial remission; VGPR: very good partial remission

Table 2. The comparison of groups with successful and unsuccessful stem cell mobilization for the impact of chemoterapy regimens (univariate logistic regression analysis)

Variable	Total number	Unsuccessful	Successful	Odds Ratio	Sig.*
VCD	4	1 (25.0)	3 (75.0)	N/A	N/A
ABVD + GDP	19	4 (21.1)	15 (78.9)	1.412	0.554
ABVD + ICE	6	2 (33.3)	4 (66.7)	N/A	N/A
BEP + TIP	4	0 (0.0)	4 (100.0)	N/A	N/A
CHOP	7	2 (28.6)	5 (71.4)	N/A	N/A
HIDEX + VCD	40	9 (22.5)	31 (77.5)	1.321	0.497
HIDEX + VTD	8	3 (37.5)	5 (62.5)	N/A	N/A
R- CHOP	8	6 (75.0)	2 (25.0)	N/A	N/A
RCHOP + GDP	9	4 (44.4)	5 (55.6)	N/A	N/A
VAD + VCD	13	5 (38.5)	8 (61.5)	N/A	N/A
VTD	27	4 (14.8)	23 (85.2)	2.278	0.143

| VIDE | 5 | 2 (40.0) | 3 (60.0) | N/A | N/A |

VCD: Velcade®, cyclophosphamide and dexamethasone; ABVD: Doxorubicin, bleomycin, vinblastine and dacarbazine; GDP: gemcitabine, dexamthasone and csiplatin; ICE: ifosfamide, carboplatin and etoposide; BEP: bleomycin, etopopsid and platinum; TIP: paclitaxeli ifosfamide and cisplatin; CHOP: cyclophosphamide, doxorubicin, vincristine and prednisolone; HIDEX: high dose dexamethasone; VTD: Velcade®, thalidomide and dexamethasone; VAD: vincristine, doxorubicin and dexamethasone; N/A: not applicable

Table 3. The comparison of groups with successful and unsuccessful stem cell mobilization for hematologic parameters (univariate logistic regression analysis)

Variable	Unsuccessful (n=65)	Successful (n=177)	Odds Ratio	Sig.*
Age	52.25± 14.95 55 [17 - 75]	49.58± 14.051 53 [14 - 75]	1.013	0.199
No. of cures before mobilization	7.51 ± 3.37 6 [3 - 19]	6.80 ± 3.37 6 [2 - 34]	1.059	0.162
No. of sequences before mobilization	1.91 ± 0.99 2 [1 - 6]	1.86 ± 0.70 2 [1 - 4]	1.072	0.701
No. of mobilization procedures	2.12 ± 0.72 2 [1 – 4]	1.92 ± 0.85 2 [1 – 4]	1.351	0.089
White blood cell count before mobilization	32.60± 20.64 35.41 [2.14 – 85.8]	27.05± 20.08 22.8 [1.52 – 69.67]	1.013	0.061
Haemoglobin level before mobilization	11.86 ± 2.03 12.1 [6.96 – 16.1]	11.49 ± 1.99 11.7 [0 – 15.5]	1.102	0.202
Platelet count before mobilization	158.63 ± 87.77 155 [12 - 366]	149.57± 95.60 139 [6 - 418]	1.00	0.503
Peripheral CD34+ count in the beginning of mobilization	30.57± 28.34 23.13 [0 – 208.82]	73.43± 80.20 40.28 [0478.86]	1.032	*<0.001*
The duration of mobilization	7.34 ± 5.85 4 [3 - 33]	8.65 ± 5.89 5 [1 - 39]	1.042	0.131

* p value obtained after univariate logistic regression analysis.

Table 4. Multivariate logistic regression analysis of the variables with statistical difference in univariate logistic regression analysis

	B-value	SE	Wald	Odds Ratio	95% CI	Significance
Preemptive plerixafor administration	-0.561	0.474	1.401	0.1752	0.692 – 4.434	0.237
No. of cure(s)	-0.020	0.047	0.182	0.980	0.893 – 1.075	0.670
Sequences of mobilization	0.296	0.231	1.646	1.344	0.855 – 2.112	0.199
White blood cell count	-0.026	0.009	8.235	0.974	0.957 – 0.992	*0.012*
CD34+ cell count in the initiation of mobilization	-0.737	0.008	18.624	1.037	1.020 – 1.054	*<0.001*

Original Research

Detection of B1 gene as Toxoplasmosis marker in women of childbearing age in West Bandung Regency, Indonesia

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HIGHLIGHTS
- B1 gene was detected in 7 of 50 women of childbearing age in West Bandung Regency
- Transmission of *Toxoplasma gondii* in the area is influenced by frequent contact with pets

ARTICLE INFO

A B S T R A C T

Congenital toxoplasmosis can cause damage and death to the fetus, to prevent this case, toxoplasmosis testing is important for the woman of childbearing age. One of the methods to screening the presence of *T. gondii* in the blood is Polymerase Chain Reaction (PCR). One of the *T. gondii* genes which can be used as a marker is the B1 gene. There are many toxoplasmosis cases in Indonesia, but the data is still difficult to find in West Bandung Regency. This study aimed to determine the number of toxoplasmosis cases in a woman of childbearing age in West Bandung Regency using the B1 gene as a marker and to determine the factors that influence these cases by conducting statistical analysis on the results of the questionnaire. The sample used in this study was 50 women of childbearing age (got married and domiciled in West Bandung). All samples have met the inclusion criteria and signed the informed consent. DNA from blood specimens was isolated using the Wizard Genomic DNA Purification Kit. The concentration and purity of isolated DNA were measured using a nanodrop device. Besides, the B1 gene from *T. gondii* was amplified using a pair of specific primers and visualized by the agarose electrophoresis method. Data were analyzed using the logistic regression method. The results showed that 7 women of childbearing age women (14%) in West Bandung Regency had toxoplasmosis. Frequent contact with pets, especially cats, was a significant factor (p <0.005) in this disease transmission.

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1. INTRODUCTION

Toxoplasmosis is the most common parasitic infection in the world. Until now, 1/3 of the world population is estimated to have this infection.[1] Moreover, Sakikawa et al[2]., showed the prevalence of toxoplasmosis in the world, ranging from 6.1 to 74.5%. Toxoplasmosis is a

zoonotic disease caused by obligate intracellular protozoa, *Toxoplasma gondii*. There are 3 types of *T. gondii*, such as tachyzoite (the proliferative form), cyst (containing bradyzoite), and oocyst (containing sporozoites).[3] The main host of *T. gondii* is a cat[4] but humans and the other warm-blooded vertebrates (goats, pigs, dogs, chickens, or birds) can be intermediaries.[5,6,7] Humans can become infected with *T. gondii* by consuming food or water contaminated with cat feces,[8,9] raw meat or vegetables,[7] and non-pasteurized milk.[10]

Toxoplasmosis is divided into five categories, such as toxoplasmosis in immunocompetent, immunocompromised, pregnant, ocular, and congenital patients.[3] Congenital Toxoplasmosis is a disease occurred in a fetus due to *T. gondii* from the mother. In unexposed pregnant women, *T. gondii* can transfer to the fetus through the placenta.[2] Hide et al.,[11] stated that the transmission rate of congenital toxoplasmosis can reach 19.8%. Moreover, congenital toxoplasmosis is the most dangerous type because it can cause miscarriage,[5,9] visual damage,[1,3] and nerve damage in the infant, like hydrocephalus, mental retardation, cerebral calcification, and chorioretinitis.[2,10] Gargate[7] also stated that congenital toxoplasmosis can affect neurological disorders, particularly schizophrenia, and bipolar disorder. To prevent the adverse effects on the fetus, some studies suggested that toxoplasmosis testing is performed on childbearing age women before being fertilized.[18]

In 2014, 56,737-176,882 children per year in India are estimated to have a risk of congenital toxoplasmosis infection.[9] Furthermore, in Ethiopia, infant mortality due to toxoplasmosis is around 28 cases of 1000 births.[10] Besides, congenital toxoplasmosis also occurs in Indonesia, with different prevalence in each region, as 58% in Surabaya, 70% in Jakarta, and 80.2% in Bandung.[12] West Bandung is one of the regencies in West Java having 804,219 female population and 1-11% couples of childbearing age in each district.[13] Most of the population live together with pets or livestock and have a less hygienic lifestyle. It is possible that women in West Bandung infected by *T. gondii* but until now the data of toxoplasmosis in this area are difficult to find.

T. gondii presence in the body can be detected by the serological test, as an Indirect Fluorescent Antibody Test (IFAT) or Enzyme-Linked Immunosorbent Assay (ELISA).[4] The component detected in the serological test is the antibody. So, this test often shows the false-negative result in the initial infection and immunocompromised patient.[5,14] Therefore, some previous studies suggested that toxoplasmosis can be checked by PCR.[15,16,17] Moreover, Rahumatullah et al.,[14] and Mousavi et al.,[4] stated that PCR was used to detect and identify parasites from various specimens, as blood amniotic fluid. Bourdin et al.,[16] also showed that PCR successfully detected the presence of *T. gondii* in immunocompromised patients.

The B1 gene is one of the genes which can be used as a marker to detect toxoplasmosis. This gene has been widely recommended by several researchers because of consisting of 35 copies in one *T. gondii* cell.[5,15,17] A high number of gene copies can increase the detection sensitivity. Based on observations, research on toxoplasmosis in women of childbearing age in West Bandung Regency using PCR detection techniques and the B1 gene as a marker has never been done before. Therefore, this study aimed to determine the number of toxoplasmosis cases in a woman of childbearing age in West Bandung Regency using the B1 gene as a marker and to determine the factors that influence these cases by conducting statistical analysis on the results of the questionnaire.

2. MATERIAL AND METHOD

Population and samples

The population of this study was the childbearing age women domiciled in West Bandung. There were several inclusion criteria used in the selection of the samples, such as willing to be involved in the research, getting married, having age between 20 and 45 years, and having pets (cat, dog, goat, chicken, or bird). Then, 50 samples were collected in this study. All samples had known the background, objective, benefit, and research procedure also signed the informed consent.

Materials and equipment

The materials used in this study was agarose (Thermo Scientific), Buffer TAE (Thermo Scientific), Buffer MgCl$_2$ (Thermo Scientific), ddH$_2$O (Thermo Scientific), dNTPs (Thermo Scientific), DNA Taq Polymerase (Thermo Scientific), Genomic DNA Isolation Kit (Promega Wizard Genomic DNA Purification Kit), Ladder (Thermo Scientific Gene Ruler 100 bp), Loading dye (BioRad), and Primer (Thermo Scientific) (Table 1).

Table 1. Primer Specifications

Primer	DNA Sequence	Amplicon size (bp)
B1 Forward Primer	5'-GGAACTGCATCCGTTCATGAG-3'	200
B1 Reverse Primer	5'-TCTTTAAAGCGTTCGTGGTC-3'	

Furthermore, the tools used in this study were the vacutainer needle (BD Vacutainer), micropipette (BioRad), microtube (Biologix), nanodrop (Thermo Scientific, Nanodrop 2000 Spectrophotometer), a set of electrophoresis (Mupid-EXU), a vacutainer tube (One Med), thermocycler (Thermo Fisher Scientific), UV transilluminator (Avenge).

Research steps

The research was started by filling out the questionnaire by all participants. The questionnaire used consisted of several questions related to identity, lifestyle (including the transmission risk factor of *T. gondii*), types of pets or livestock, and the disease history. Then, the specimen of venous blood from the participant was taken after this stage.

The genomic DNA was isolated by DNA isolation Kit. Then, the concentration and purity of isolated DNA were measured by nanodrop. Moreover, the B1 gene was amplified by the same primer as the study of Bin Dajem and Almushait, but modifying some of the reaction composition and amplification conditions. The total volume of the amplification reaction was 25μl, consisting of 16μl ddH$_2$O; 3μl buffer MgCl$_2$ 10X; 1μl dNTPs 10mM; 1μl B1 Forward primer 10mM; 1μl B1 Reverse primer 10mM; 1μl Taq DNA Polymerase 5U/μl; and 2μl sample with DNA concentrations of at least 50ng/μl.

The amplification process started with the pre-denaturation of DNA for 5 minutes at 95°C. Then, the amplification was carried out as 40 cycles, consisting of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for 30 seconds. The final elongation was carried out for 1 minute at 72°C. The amplification results were visualized by the electrophoresis, with the agarose concentration of 1%. All stages from sample collection to analysis results followed the applicable code of ethics and received approval from the Health Research Ethics Committee of School of Health Sciences number 09/KEPK/V/2019.

Data analysis

To find out the significantly influential factors with the *T. gondii* transmission in the women of childbearing age in West Bandung, the questionnaire data and the B1 gene amplification were analyzed by logistic regression method, using statistical software.

3. RESULTS AND DISCUSSION

Based on the B1 gene amplification result in 50 blood specimens from the women of childbearing age in West Bandung, 7 women (14%) were positively infected by *T. gondii*. There was a 200 bp DNA band in 7 positive samples. The size of the DNA band was the same as the positive control and the pre-designed amplicon size (Table 1). The positive control used in this study was the genomic DNA from the blood of patients positive to toxoplasmosis. The patients had proven positively infected in the anti- *T. gondii* IgM testing. The amplicon B1 gene process in this study was specifically, where there was no DNA band in the negative control. The negative control used was ddH$_2$O.
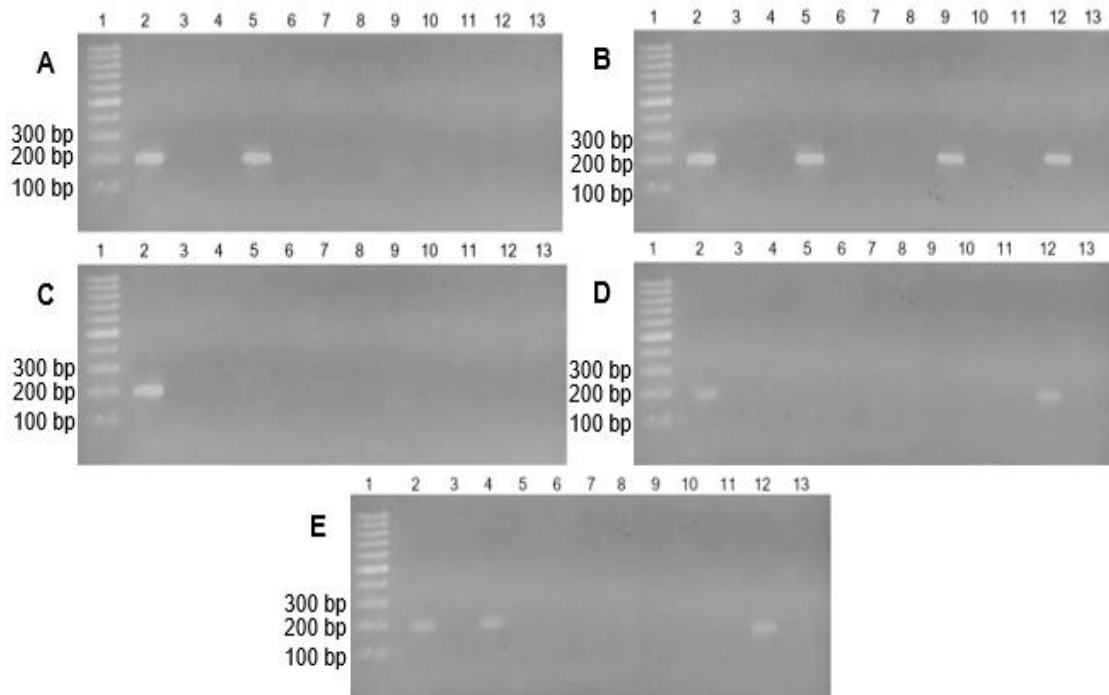
Figure 1. Agasore Electrophoresis Result of the B1 Gene Amplification in Childbearing Age Women in West Bandung

Lane 1: Ladder Thermo Scientific 100 bp; Lane 2: Positive Control; Lane 3: Negative Control.
Lane 4-13: (A) sample number 1-10; (B) sample number 11-20; (C) sample number 21-30; (D) sample number 31-40; (E) sample number 41-50.
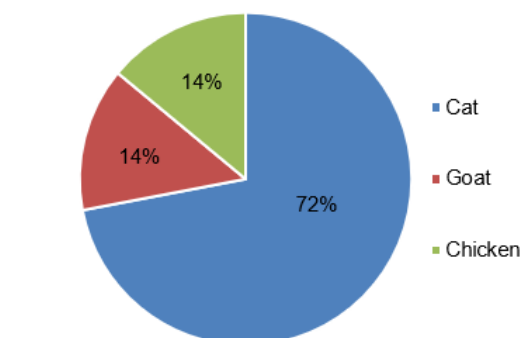

Figure 2. The pet types of the women of childbearing age infected by *Toxoplasma gondii* in West Bandung Regency

Based on the data, it was known that all woman samples had pets or livestock. All samples infected with *T. gondii* admitted that they raised cats, chickens, and goats, in which the three animals were the most raised by the toxoplasmosis women in West Bandung Regency (Figure 2).

All women also admitted that they often contacted the animals. The animals they had were rarely bathed, did not have the litter box, and could go in and out of the house. Besides, the samples also carried out other activities, like consuming raw meat and vegetables and contacting soil (Table 2). The statistical analysis showed that frequent contact with the animal, especially cat was a factor that significantly related to the *T. gondii* transmission (P < 0.005).

Table 2. Characteristics of Samples

Variable	Total				*Toxoplasma gondii* infection	
	n	%	N	%	Regression coefficient	p-value
The tenure of pets or livestock					0.1053	0.1337
< 1 year	17	34	1	5.8		
> 1 year	33	66	6	18.2		
In contact with pets or livestock					0.3239	0.0117
Frequently	18	36	7	38.8		
Infrequently	11	22	0	0		
Never	21	42	0	0		
Bathing pets or livestock					0.3313	0.1129
Frequently	8	16	0	0		
Infrequently	17	34	2	11.7		
Never	25	50	5	20		
The abode of pets or livestock					0.0019	0.4228
Always inside the house	2	4	0	0		
In and out of the house	14	28	5	35.7		
Always outside the house	34	68	2	5.8		
Providing the litter box					0.1187	0.0914
Yes	23	46	0	0		
No	27	54	7	28		
Consuming raw vegetables					0.4412	0.0954
Yes	36	72	3	8.3		
No	14	28	4	28.5		
Consuming raw meat					0.4451	0.7719
Yes	3	6	3	100		
No	47	94	4	8.5		
Contacting with soil					0.0448	0.7323
Yes	10	20	5	50		
No	40	80	2	5		

Toxoplasmosis can be detected by many methods. Furthermore, the earliest detection method developed is cell inoculation in culture and microscopy, but these methods are time-consuming, expensive, and less sensitive. There are also Giemsa and Haematoxylin & Eosin Staining. Both methods are simple and cost-effective, but the samples used only feces, water, and other environmental materials.[18] Therefore, the researchers use the serological test to detect *T. gondii* antibody circulating. The serological test performance is quite good but has several drawbacks. The serological tests often show false-negative results at the first infection, because the human body takes 1-2 weeks to form the anti- *T. gondii* IgM (window period).[18] The false-negative result is often found in people with immune systems disorder because their bodies are slow or failed to produce the IgM or IgG. Testing of anti-*T. gondii* IgM cannot show the active phase of the parasite because the IgM can survive for several months until more than 1 year.[19]

This study proved that the molecular examination method, like PCR, can be used as an alternative method for detecting toxoplasmosis. Moreover, PCR is more sensitive, specific, and no time-consuming. PCR has also been shown to detect *T. gondii* DNA in the blood of pregnant or unpregnant women.[5] Now, many PCR types were developed to detect the toxoplasmosis, like nested PCR and quantitative PCR. Both methods are more sensitive than conventional PCR, but conventional PCR is more desirable because of simpler, faster, and relatively cheaper. Therefore, Rahumatullah et al.,[14] stated that conventional PCR is suitable for routine detection of *T. gondii*, especially in developing countries such as Malaysia and Indonesia. The success of the PCR is determined by several factors, like the number of marker gene copies.[15]

The B1 gene used as a marker in this study has been widely recommended by researchers. Mousavi et al.,[4] reported that the B1 gene is a specific gene for detecting *T. gondii*, and it is conserved in all strains. Some studies also report that the detection of the B1

gene is more sensitive than the rDNA,[14] P30,[5] and 529 RE.[4,15] PCR method using B1 as a target gene can detect 10 parasites in 100,000 human leukocyte cells.[14] On the other hand, some studies further suggested using the 529 RE gene to increase the sensitivity due to the greater number of copies (200-300 copies of genes in one *T. gondii* cell).[17,18] Moreover, Rahumatullah et al.,[14] reported that the difference in sensitivity and specificity level of PCR in every study can be influenced by several factors (like the annealing temperature and the solution composition of PCR). Also, Priyowidodo et al.,[19] showed that the detection performance of PCR in blood specimens can be influenced by inhibitors (like immunoglobulins, polypeptides, and lactoferrin which can reduce the sensitivity of detection).

The number of women found positive to toxoplasmosis of childbearing age in West Bandung is lower than the other regions, such as Venezuela,[8] Kosovo,[20] Ethiopia,[6] India,[9] and Saudi Arabia.[21] The difference in the prevalence rate of every study can be caused by several factors, including method, number, and characteristics of the samples used. Some studies used more than 100 samples, with a wider age range of 11 – 45 years.

In this study, the significant factor influencing the *T.gondii* transmission was the frequent contact with pets, especially cats. This result was in line with Subasinghe et al.,[22] in Sri Lanka. Furthermore, the majority of the seropositive respondent of toxoplasmosis had contacted cats at home and outside. Diaz-Suarez and Estevez[8] also stated that *T.gondii* inspection is strongly influenced by oocysts in cat feces. The rarely bathed cat can increase the risk of *T. gondii* transmission because the feces can stick to the fur, nails, and body of the cat. Besides, the misplaced cat feces can contaminate soil and water in the surrounding environment, so increasing *T. gondii* infection.[9] Agustin and Mukono[23] reported that cat feces left in the environment can produce infective oocysts. Oocyte can survive for years in soil, sand, and water. The maturation and transmission of oocyte will be faster in humid and wet conditions.[7,21] Covering the cat feces with soil or sand cannot prevent the sporulation of oocyst. This only delays the oocyst to sporulate.[24]

Besides in contact with the cat or other pets, consuming raw meat and vegetables can increase the toxoplasmosis risk. In this study, these activities did not show a significant effect on the *T. gondii* transmission. However, Sakikawa et al.,[2] reported that consuming raw meat and vegetables was the major factor in the transmission of *T. gondii* in women in Japan. Therefore, it is suggested that the women of childbearing age limit their physical contact with pets which can be the *T. gondii* host, and also do not consume raw meat and vegetables.

CONCLUSION

Seven of 50 women of childbearing age (14%) in West Bandung were infected by *Toxoplasma gondii*. Frequent contact with pets (especially cats) was a significant factor in toxoplasmosis transmission ($p < 0.005$). The results of this study are useful for the Health Office and the public, especially the people in West Bandung Regency, to know the number of cases and the factors that influence toxoplasmosis in the area. The community, especially women of childbearing age, can prevent toxoplasmosis. Besides, this study provides information about alternative methods that can be used to detect *T. gondii*.

DISCLOSURE STATEMENT

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FUNDING INFORMATION

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SHORT BIOGRAPHY

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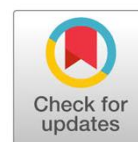
Original Research.

Anti-inflammatory activities of squalene compound of methanol extract of Abroma augusta L

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HIGHLIGHTS

- Characterized isolate from methanol extract of *Abroma augusta* then we obtain squalene compound which belongs to triterpene that intermediate in the biosynthesis of steroids.
- The anti-inflammatory activity of isolate F211 has good results with edema values approaching positive control results with a reduction in edema of 28-30%.

ARTICLE INFO

A B S T R A C T

Abroma augusta L plant traditionally was used to treat swellings, cuts, sores, and bruises. In the province of Jambi, *A. augusta* is used in folk medicine to treat wounds. This study aims to isolate the steroid compound from the root of *A. augusta* L and determine its anti-inflammatory activities. Extraction and fractionation have been done with graded maceration using solvents with different polarities, which are n-hexane, ethyl acetate, and methanol. The separation was performed by column chromatography, followed by preparative thin-layer chromatography. The characterization of the isolate was carried out using UV-Vis spectrophotometry and infrared spectrophotometry, GC-MS. The anti-inflammatory activities of methanol extract and isolate of *A. augusta* was performed in this study was designed to evaluate the dose-response relationship of the anti-inflammatory activity in rat models of chronic inflammation chromatography to obtain isolate 2.1.1 that characterize and showed maximum absorbance at 265. The result of IR showed the presence of functional groups, -C=C-H, -C=H, -CH, CH_3, CH_2, and –CO belongs to the steroid compound. The results of the GC-MS shows that isolates contain squalene compounds with a value of m/z 410, Isolate and crude extract showed an anti-inflammatory activity that almost approached the positive control of sodium 4-chlorophenolate. It could be concluded that isolate and extract provide good anti-inflammatory activity, that promise for new drug candidate squalene-based *A. augusta*.

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1. INTRODUCTION

Indonesia is a country that has mega biodiversity, which is rich in plants that need to be explored in its potential. In Indonesia's tropical forests it is estimated that there are 30,000 species of plants, and it is estimated that about 9,600 species are known to have medicinal properties and 200 of them are important medicinal plants for the traditional medicine industry as raw materials.[1] The content of medicinal compounds in plants needs to be isolated, to separate active compounds that are mixed to get pure compounds.[2] The process of isolating compounds from natural materials is targeted at the isolation of secondary metabolites because secondary metabolite compounds can provide benefits for human life. The utilization of secondary metabolites is very much among them as antioxidants, antibiotics, anticancer, anti-inflammatory, as well as environmentally friendly pest control antigens. Some secondary metabolite compounds are alkaloids, terpenoids, flavonoids, steroids, and others. The content of secondary metabolites found in many leaves.[3]

Traditional medicinal plants are natural ingredients used for treatment based on the experience and diversity of medicinal plants. One of them is Abroma augusta L. which is widely used as a fracture drug or inflammatory drug. Inflation is a response from the body to injury or infection. When had an injury occurs, the body will try to neutralize and eliminate harmful agents from the body and make preparations for tissue repair.[4] The protective response due to damage to the tissue that serves to destroy, reduce, or localize both the injured agent and the injured tissue. The main signs of acute inflammation include swelling or edema, redness, heat, pain, and changes in function.[5] Inflammation can occur acute and chronic which causes pathological abnormalities. Other than that, the inflammatory process underlies the pathogenesis of several diseases such as cancer, rheumatoid arthritis, Chronic, Obstructive Pulmonary Disease (COPD), atherosclerosis, and cardiovascular.[6]

Abroma augusta Linn (Family-Malvaceae) is commonly known as the Caterpillar of the genus of large evergreen plants, growing rapidly on shrubs, small trees with velvety branches. The *Abroma augusta* L plant is found in tropical Asia, South and East Africa, and Australia.[7] Based on the ethnobotany survey conducted by the author through an interview with a resident in Muaro Jambi District, Mendalo Darat Village, it is known that the broken steering plant (*Abroma augusta* L.) is used as traditional medicine. The community uses mucus from the bark to treat inflammation of the joints and for fractures. The previous study was done by Pastel and Dhanabal[8] found that *Abroma augusta* Linn contained glycosides, alkaloids, carbohydrates, and steroids. Inflammation is usually treated using both steroid class anti-inflammatory drugs (AIS) and a nonsteroidal class of anti-inflammatory drugs (NSAIDs).[5,9] These phytoconstituents had been reported to have many biological effects such as antibacterial, antidiabetic, and anti-inflammatory, based on our previous studies known that all parts of the plant *Abroma augusta* have anti-inflammatory activity. This study aims to analyze secondary metabolite compounds in *Abroma augusta* and characterize the potential of steroid compounds as anti-inflammatory using phytochemical screening, chromatography, GC-MS, IR spectrophotometer (FTIR), UV-Vis, and anti-inflammatory assay.

2. MATERIAL AND METHOD

Material and research tools

Fresh leaves of *Abroma augusta* L. were collected from the District of Muaro Jambi, Jambi 2019 (5 kg). The Chemical Solvents such as Methanol, n-hexane, ethyl acetate, acetone, aquadest, silica gel 40 (70-230) mesh were purchased from Merck (Germany). ASTM for column chromatography, glassware (Pyrex®), FeCl3 5%, NaOH 10 %, Mg-HCl, H2SO4 (p), 6% HCl, chloroform, TLC silica gel 60 F254.

Extraction, isolation, and fractionation

The leaves of *Abroma augusta* were dried for a week at room temperature in the laboratory, mashed and macerated in 8 L of n-hexane then for 24 hours, then filtered and the filtrate is collected. The filtrate obtained was combined and concentrated with a rotary evaporator until a thick extract was obtained. The n-hexane residue was then macerated successively with 8 liters of ethyl acetate and methanol for 24 hours, each filtrate was collected

and concentrated with a rotary evaporator. The methanol fraction was isolated and purified by column chromatography, then analyzed by thin-layer chromatography (TLC) to select the appropriate eluent, then separated by open column chromatography using silica gel 60 silent phases. Purification of the main components contained in the active fraction was carried out by column chromatography and recrystallization until a pure compound with a single stain was obtained at TLC.[10,11]

Characterization of compound

We did a qualitative analysis of secondary metabolites of both extract and isolate by phytochemical screening.[12] Furthermore, the isolated compounds were characterized using a UV-Vis Spectrophotometer (Biochrom Libra S70), IR Spectrophotometer (FTIR) (Perkin Elmer), and GC-MS (Shimadzu GCMS-QP2010 Ultra, Australia). The analysis was carried out using a UV-Vis spectrophotometer, the crystalline fraction obtained was dissolved using methanol as a solvent. In the UV-Vis spectrophotometer and determined the wavelength to be 200-400 nm.[10] For analysis using an IR spectrophotometer, crystals from the obtained fraction were used as much as 0.5 mg then mixed with 50 mg KBr and crushed homogeneously. The FTIR spectrophotometer is first performed with a blank baseline in the form of air. Samples were put into KBr cells and put into a device with a hole leading to the radiation source then analysis was carried out ranging from 2.5-micron wavelengths (υ 4000 cm-1) to 25 microns (υ 400 cm-1). In the GCMS tool, 1 μl of the pure isolate was injected into the GC-MS tool, then the column used was the capillary model number 19091S-433 HP-5MS 5% Methyl Siloxane with a length of 30 m, a diameter of 250 μm and a thickness of 0.25 μm. Oven temperatures were used between 100-220 ^{0}C. The rate of temperature increase is 15 ^{0}C / minute and the flow velocity is 1.0 ml/minute. The carrier gas is pressurized helium 10.5 psi and a total rate of 140 ml/min and a split ratio of 1:50.[10,13]

Anti-inflammatory assays

Anti-inflammatory effectiveness test of leaves of *Abroma augusta* using eight male white mice aged around two months with an average weight of 20-30 g. Mice were randomly divided into 4 groups, each group containing 3 mice (Table 1). Mice are acclimatized and fasted for 24 hours by being given a drink. The inaction of injection from the beginning of the treatment, half an hour after we treatment, the rats were given an injection both of extract and isolates on the sole of the left rear foot. The volume of Edema was measurement using calipers for 90 min at 30 min intervals.

Table 1. Grouping of Animal test for anti-inflammatory assays

No	Group Name	Total a mice host
1	Positive control	1
2	Negative control	1
3	The isolate of *Abroma augusta*	3
4	Extract of *Abroma augusta*	3

Calculation of determination of several test animals
Calculated according to the Federer formula: $(n-1)(t-1) \geq 15$
Where n: Number of experimental animals per group; and t: Number of groups

Diclofenac sodium dose calculation

$$VAO = \frac{Body\ Weight\ (kg) \times Dose\ (mg/kgWeight)}{Concentration\ (mg/mL)}$$

Determination of anti-inflammatory activity
Calculated percent edema and percent inhibition of edema by following the previous study.[4]

$$\% \text{ udem} = \frac{V_t - V_o}{V_o} \times 100\%$$

Determination of phytochemical analysis

This research to find out the content of compounds from the roots of the *Abroma augusta* L, was carried out through phytochemical tests by looking at the color changes or reactions that occur in the sample caused by the reaction between the reagent and the sample.

3. RESULTS AND DISCUSSION

Percentage of yield

The percentage yield for the extract was obtained and tabulated in table 2, by weighing the weight of each fraction and then calculated. The following results are obtained:

Tabel 2. The percent yield of Abroma augusta L extract with different solvent

Fraction	Fraction weight (gr)	% Yield
n-hexane	1.4016	0.14016
Ethyl- acetate	2.014	0.2014
Methanol	16.6159	1.66159

Among the three extracts, the methanol fraction gave the highest percentage yield of 1.66159%, then ethyl acetate 0.2014% and n-hexane 0.14016% gave the lowest percentage yield. That seems due to the ability of each solvent to extract compounds in the roots is different. Besides, also this is influenced by the root extracted of the plant, where the concentration of the chemical compound is not too high. Methanol extract shows the content of secondary metabolites alkaloids, tannins, steroids, and triterpenoids which are non-polar compounds (Table 3). Moreover, we found that the steroid group is the major compound of the secondary metabolites group in the isolate.

Table 3. Phytochemical screening of *A. augusta* L

Secondary Metabolites	Method	Result	
		Total Extract	Isolate
Alkaloids	Meyer reagent	+	-
	Dragendorff reagents	-	-
Flavonoids	HCl 2N + Mg powder	-	-
Tannins	$FeCl_3$	+	-
Steroids	Lieberman Burchard	+	+
Triterpenoids	Lieberman Burchard	+	-
Saponins	Water (Boiled) shake until foam appears	-	-

(+) Present/ (-) Absent

Isolation of Abroma augusta L.

The isolation of bioactive compounds was carried out by column chromatography which begins with vacuum column chromatography to separate and simplify the total compounds in the extract, then continued for the isolation process using gravity column chromatography to obtain four sub-fractions, and the best fraction we continued for the second of the KKG process to obtain a pure of isolates. Finally, we found that fraction 2.1.1 (Figure 1) has a staining pattern. Somehow, we wonder what the compound in that isolate. We did the phytochemical screening of the fraction to determine the content of chemical compounds in the isolates. The results of the phytochemical test (Table 3) showed that the isolate contained a single compound is steroids. This data showed that the isolate is a natural polymer, which was supported by the results of three eluent tests that showed an elongated TLC pattern that indicated that the compound obtained was a polymer.

Characterization of isolate

To identify the groups of secondary metabolites found in the isolated methanol extract of *Abroma augusta*, the characterization was performed using a spectrophotometer.

Characterization using a UV-Vis spectrophotometer

The number of absorbance of the compound the isolates were obtained from the leaves of *Abroma augusta* L. against UV light which has absorption at a maximum wavelength of 265 nm (1.1487 A) (Figure 2). This data shows the characteristics of steroid compounds that indicate conjugated double bonds which may also be aromatic.[14]
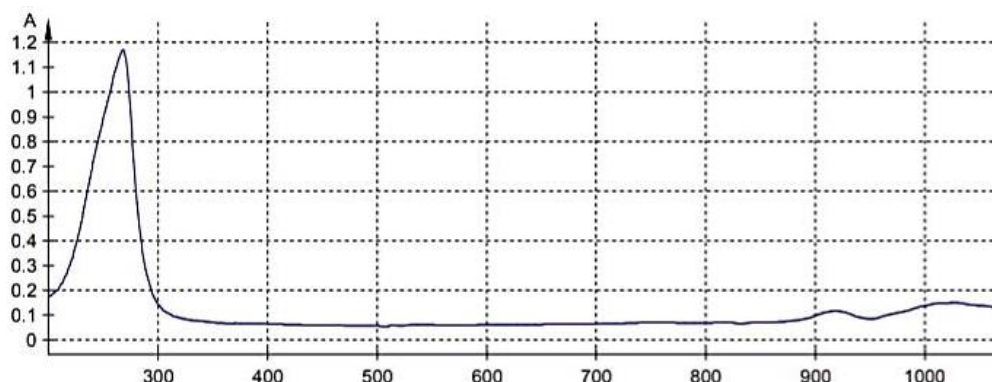
Figure 1. UV-Vis Spectrum of Isolate 2.1.1

Characterization using spectrophotometer of FT-IR

The FTIR analysis of compound F211 is used to find out functional groups of compounds, where the results of the FTIR spectrum of compound F.2.1.1 and comparison can be seen in Figures 3 and 4.
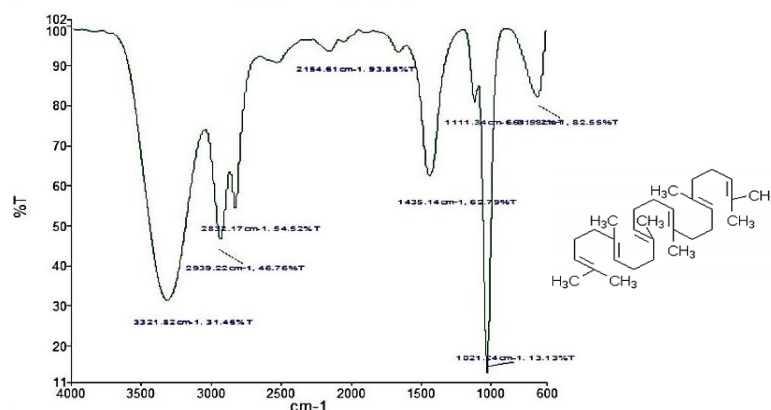
Figure 2. The FT-IR spectrum of Isolate 2.1.1 and Squalene structure interpretation

The results of the isolated spectrum (figure 3) showed that the existence of functional groups of a compound based on the electromagnetic spectrum in the IR region. Wavenumber 3321.82 cm^{-1} showed there is the existence of stretching absorption of O-H hydroxyl groups, and 3030,2 is a C=C-H group. This assumption is strengthened by the existence of wave number at 2939 cm^{-1} which is belongs to an aliphatic CH group (2850-2970 cm^{-1}). The absorbance at wavenumber 668 cm-1 is caused by the C = C group which is an aromatic CH group. There is also a peak at 2924.09 cm^{-1} means the C-H strain of CH_3. Moreover, the peaks in both 1643 cm^{-1} and 1512.19 cm^{-1} indicate the presence of aromatic C=C and wave numbers 1273.02 and 1226.73 cm^{-1} which indicate C-O uptake, this indicates the presence of terpenoid compounds or modified of squale may occur even due to impure of the isolate.[14] The FTIR spectrum of isolate F2.1.1 has high similarity with the previous results which is confirmed a similar compound.[15]

Tabel 4. A functional cluster of FTIR spectrophotometer

No	This Study (F211)	The previous Study*		Wavelength (cm⁻¹)	Functional Group
		Squalene	Squalene (Modified)		
1	3321,82	3030,2	3031,2	3650-3500	C=C-H
2	-	3051,18		3100-3000	C- H
3	2939,22	2920,2	2920,2	3000-2850	C – H
	2832,17	2885,4	2885		
4	1435,14	1667,3	1737,7	1650-1450	C = C
			1667,3		-CO–O
5	1021,24	-	-	1300-1000	C – O alkohol

*Erizal, (2005)

According to previous research, the results of our study are suspected to have CO-O type bonds; C – H; C = C and C = C – H, it can be seen in Table 4. Based on the results of the comparative spectrum in the table above with the isolated compound F2.1.1 have similarities with the results of Erizal[15] study which stated positively the steroid group compounds. To find out specifically needed further characterization using GC-MS for the determination of compounds.

Characterization of molecules compound by GC-MS

To confirm the results of our FT-IR analysis, the characterization was carried out using the GC-MS spectrophotometer, to know the molecular weight of the compounds was obtained. The isolate that was characterized showed the pattern of fragmentation of MS data with molecular ions valued at m/z 410.0 which had similarities with MS data of squalene compounds obtained from *Saussurea obvallata* extract.[16] The pattern of fragmentation between isolates and literature can be seen in Figure 3.
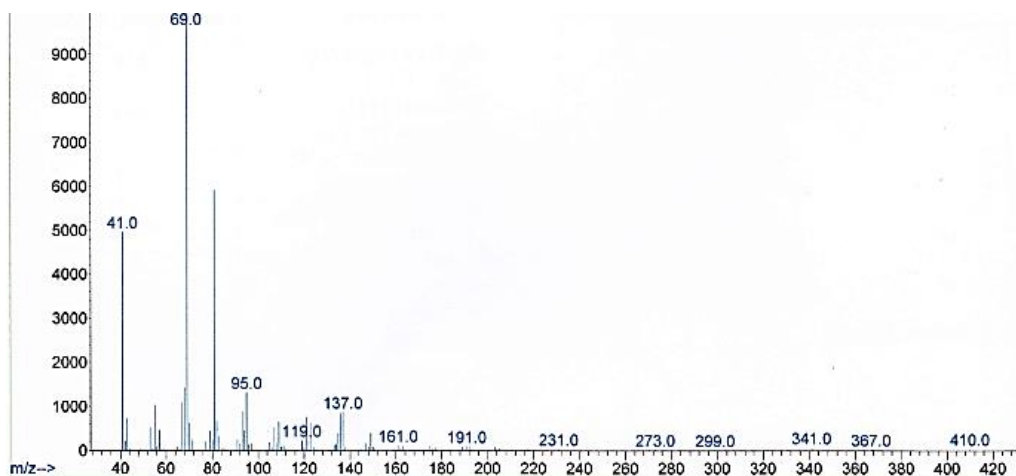

Figure 3. Spectrum GC-MS of isolate F211

Upon comparing with previous studies shows that indeed, the FT-IR spectrum of isolate F.2.1.1 corresponds to Squalene and GC-MS spectrum results also show that isolate F.2.1 has belonged to squalene which is a steroid group.
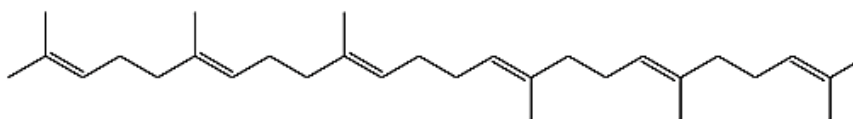

Figure 4. Structure of squalene: (*E*)-2,6,10,15,19,23-Hexamethyl-2,6,10,14,18,22-tetracosa-hexane[16,17]

Table 5. Chemical and physical properties of squalene

Properties	Value
Molecular weight	410.7 g mol^{-1}
Melting point	-75^{0}C
Refractive index	1.499
Viscosity at 25^{0}C	12 cp
Density	0.858 g.ml
Boilling point at 25^{0}C	285^{0}C
Flash point	110^{0}C
Iodine number	381 g/100 g
Infrared peaks	2728, 1668, 1446, 1380, 1150, 1180, 964, 835 cm^{-1}
Surface tension	~32 mN/m

Anti-inflammatory assays

Anti-inflammatory testing was done by injecting test samples on experimental animals that have been injected with carrageenan. The results of measuring the diameter of the test animal's legs can be seen in Table 6.

Table 6. Anti-inflammatory activity of both extract and isolate F2.1.1

Sample	The feet diameter after injection (mm)				% Decreased Edema
	0 min	30 min	60 min	90 min	
Positive control (0.14 mg)	0.46	0.42	0.39	0.38	39.67
Negative control	nd	nd	nd	nd	nd
Extract (5 mg)	0.36	0.33	0.31	0.31	31.66
Extract (10mg)	0.38	0.36	0.32	0.31	33
Extract (15mg)	0.33	0.30	0.29	0.27	28.66
Isolate F211 (5mg)	0.31	0.31	0.32	0.27	30
Isolate F211 (10mg)	0.28	0.29	0.28	0.27	28
Isolate F211 (15mg)	0.35	0.32	0.30	0.28	30

Based on the data in Table 6 it can see a decrease in swelling of the legs of the test animals that have been given test samples, due to the activity of the isolate as an anti-inflammatory agent. The ability of anti-inflammatory activity can be seen from the % of edema of test animal feet. The formation of inflammation due to the induction of carrageenan consists of two phases. The first phase (early phase) is 1-2 hours after carrageenan injection which causes trauma. In the second phase (late phase) prostaglandin release occurs and is mediated by bradykinin, leukotrienes, polymorphonuclear cells, and prostaglandin production by macrophages.[6]

Inflammatory and drug response

Inflammation is a response to inflammation of tissue damage due to various adverse stimuli, both chemical and mechanical stimuli, infections, and foreign objects such as bacteria and viruses.[18] Anti-inflammatory drugs that are generally used are divided into two major groups, namely anti-inflammatory steroids and non-steroidal anti-inflammatory groups. However, both classes of drugs have side effects that are quite serious in their use. Anti-inflammatory steroids can cause peptic ulcers, decrease immunity to infections, osteoporosis, muscle atrophy, and fat tissue, increase intraocular pressure, and are diabetes.[3] Some compounds that have anti-inflammatory activity from plants are classified as flavonoids. The development of natural-based compounds as an alternative to anti-inflammatory steroids and non-steroid which are known to have side effects on health.[19] In our study we were able to obtain isolates from the *Abroma augusta* plant which were thought to be squalene compounds. The results of our isolate characterization using UV-Vis spectrophotometry and phytochemical tests confirm that isolate 2.11 is a steroid class. Further characterization using FT-IR and GC-

MS strengthens our hypothesis that the compound isolate 2.11 is squale, by comparing with some previous studies.[13,20] Squalene is a triterpene that intermediate in the biosynthesis of sterols in eukaryotes and a few bacteria,[21] with the formula $C_{30}H_{50}$, an intermediate for the biosynthesis of phytosterol or cholesterol in animal, plants, and even humans, widespread in animal and vegetal kingdom.[22] In previous research have reported that squalene (SQ) from virgin olive oil (VOO) possesses preventive effects against skin damage and anti-inflammatory properties.[17] Hence, Squalene could be a useful natural product that might cane manage would healing by its immunomodulation of macrophages, the main innate cells involve in wound healing.[17] Squalene play role in reducing free radical oxidative stress and reduced intracellular levels of ROS, nitrites, pro-inflammatory enzymes (iNOS, COX-2, and MPO), and cytokines (TNF-α, IL-1β, IL-6, and IFN-γ). Besides, squalene enhanced expression levels of anti-inflammatory enzymes (HO-1) and transcription factors (Nrf2 and PPARγ).[23]

CONCLUSION

Based on the results of this research it can be concluded that the results of UV-Vis and FTIR characterization show that the compound contained in isolate F211 is a steroid group and confirmed by GC-MS it is concluded that the steroid compounds obtained are squalene, it strengthened by the results of phytochemical test produce a brown positive steroid with a white needle-shaped crystal. Moreover, the anti-inflammatory activity of the extract and isolate F211 has the same potency as an anti-inflammatory with edema values approaching positive control results with a reduction in edema of 28-30%.

DISCLOSURE STATEMENT
The authors declare that they have no conflict of interest.

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SHORT BIOGRAPHY

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Original Research

Identification of molecular markers for type 2 Diabetes mellitus in Sidoarjo, Indonesia

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HIGHLIGHTS
Based on our study we found molecular markers to determine the likelihood of a person suffering from type 2 diabetes using the PCR-RAPD method

ARTICLE INFO

A B S T R A C T

T2DM can be triggered by two collaborating factors, namely genetics and the environment. This study aimed to identify genetic markers that can be used to detect the possibility of a person having T2D using the random amplified polymorphism DNA (RAPD) method. The study was carried out cross-sectional and involved 60 samples consisting of 30 positive T2D samples and 30 negative samples T2D. The primer used for PCR-RAPD was D20 (5'-ACCCGGTCAC-3'). The PCR-RAPD results were then analyzed using the scoring method and analyzed using the non-parametric Chi-Square test (cl: 95%). Among T2D, 576 bp band were confirmed to be markers in the patients.

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1.   INTRODUCTION

Diabetes mellitus (DM) sufferers continue to increase. 463 million people suffer diabetes mellitus and it is estimated to increase to 578 million in 2030 and 700 million in 2045. Indonesia is the seventh most DM sufferer in the world.[1] These conditions are very worrying that DM will worsen the incidence of other diseases,[2] especially in the current COVID pandemic.[3] DM can be grouped into four categories, type 1 diabetes mellitus (T1D) which is a failure of insulin production and is usually congenital, type 2 diabetes mellitus (T2D) is an event that is more likely to be experienced by adults and is caused by genetic disorders and environmental factors. Gestational diabetes (GD) is usually owned by women who are pregnant, and the last is diabetes with other symptoms that may be caused by infectious disease syndrome of the pancreas or the use of drug substances in the case of HIV AIDS therapy or after organ transplantation.[4,5]

T2D is characterized by increased levels of glucose in the blood due to the malfunctioning of cell receptors to enter insulin, several studies show that it followed by decreasing insulin production.[6] T2D incidence can change the stability of body fluids.[7,8] The complexity of T2D makes it difficult to control.[9] T2D is caused by genetic factors and environmental factors. Genetic factors are related to factors that cannot be changed and are inherited from both parents, while environmental factors are the factors that sufferers can manipulate, including changing social habits, especially due to lack of physical activity and excessive food consumption.[5,10]

Analysis of the relationship between the effects of genetic mutations with T2D is widely carried out. More than 120 genes are involved and some are closely related.[11,12,13,6,14,15] However, type 2 DM is still difficult to control due to other factors that have a tendency such as obesity,[16] ethnicity,[17] and lifestyle changes.[18]

Previous analyzes regarding the identification of polymorphisms showed different polymorphisms between type 2 DM and non-DM patients.[19] PCR-RAPD has advantages such as simple, easy, inexpensive, does not require target gene analysis, and can analyze the whole genome. The purpose of this study was to determine specifically as a marker of the possibility of a person suffering from type 2 diabetes or not using primers that produce specific polymorphisms.

2. MATERIAL AND METHOD

This research method was cross-sectional with an exploratory descriptive. Sampling had approved by the ethical committee of dentistry, Faculty of Medicine, Airlangga University, Surabaya with number: 091 / HRECC.FODM / III / 2020. A total of 60 people consisting of 30 positive T2D and 30 negative T2D were used in this study. Sampling through macro sampling as much as 3 cc is stored in the EDTA tube. Sampling was done by purposive sampling. The inclusion criterion for a positive sample was had diagnosed by a doctor or glucose test showed more than 200 mg/dl (random blood test). A negative sample is a subject that has less than 180 mg/dl in the glucose test. An interview was also given whether he had a family history of DM or not. If the sample had a family history of DM, the sample was canceled.

DNA isolation was carried out using the standard procedure of the GeneAid DNA Isolation kit for blood. The pure DNA was then analyzed quantitatively using a Thermo Evolution 201 spectrophotometer. PCR-RAPD was done using Bio-rad T100 thermocycler with 15 µL total reaction (DNA Template 2 µL, Primer A10 2 µL (5'-ACCCGGTCAC-3 '), PCR Mix 5 µL, ddH2O 6 µL). The reaction was Pre-denaturation 94˚C at 5 minutes, Denaturation 94˚C at 1 minute, Annealing 36˚C at 1 minute, Extension 72˚C at 2 minutes, Cycle 45 cycles, Final Extension 72 ˚C at 10 minutes. The band PCR-RAPD results were visualized with 2% gel agarose and were analyzed using Chi-square (cl: 95%) were done with SPSS ver.16.

3. RESULTS AND DISCUSSION

A total of 60 people from different individuals (30 positives for T2D & 30 as control) were used to look for molecular markers on T2D. The primer used is D20 (5'-ACCCGGTCAC-3'). This study strengthens previous research that has shown polymorphism in T2D samples.[19] Multiplication of samples and statistical analysis were carried out as well as looking for polymorphism characteristics from Sidoarjo, Indonesia.

PCR-RAPD produced 16 bands ranges from 174 bp to 3677 bp in length. Three bands were monomorphic (478 bp, 944 bp, & 2109 bp) and the others were polymorphic (Table 1). It was found that the band at 576 bp was significantly different between controls and T2D (p-value: 0.001). The number of bands found shows not the same number as the previous study.[19] However, the lengths were found to be different. The differences in polymorphisms previously were found around 1500 bp.

Confirmation using statistical tests is needed for this study because Zahid[19] studies used a relatively small sample. With the use of more waste, the reliability and accuracy of the research can be accepted. However, RAPD requires a higher reliability test than other methods. The reason for the band appearance is one of the considerations for using the

molecular marker. The clear of band target appearance above the monomorphic material is 476 bp (Figure 1). This will make it easier to see. Based on the percentage, the value of the band that appears in the positive sample is still low, which shows that 40% or 12 people with T2D sufferers have this band. This value is still very small because the marker may use at least 80% of the sample to have this band.

Previous research shows differences in bands than this study. Six bands show the highest polymorphism where are not found in control samples but found in positive samples. The highest polymorphism is at around 1500 bp in length. However, it was found to be far from the previous. The difference in results is possible because the specifications of each sample origin are different.[17]

The use of the 576 bp must be supported by other research that is mutually reinforcing. To strengthen the genes that are specifically involved, it needs to be confirmed. The use of the 576 bp must be supported by other research. To strengthen the genes that are specifically involved, it needs to be confirmed. The number of presence of 576 bp bands found was 40%. The use of primers that produce different bands can give the best results.

PCR-RAPD analysis was impossible to identify genes specifically. PCR-RAPD method also produces many bands and most of its unnecessary. PCR-RAPD analysis is powerful when several primers that produce statistic significantly different polymorphisms are used together to detect the possibility of a person being exposed to T2D or not. it also can be used as a marker for screening associated gene T2D in certain populations. Validation is recommended for this method. Although a person is genetically at risk, the risk for each person to experience T2D and complications is different.[19] The risk of T2D can be minimized by changing lifestyles.[18] The weakness of this study is the limited information on the personal object detected, such as weight, another disease status, age, etc. so that a deeper discussion cannot be done.

CONCLUSION

The polymorphism analysis comparing T2D sample and controls found significant differences in the 576 bp band (p-value 0.001) and can be used as candidates for molecular markers for these genetic disorders. The use of PCR-RAPD to molecular markers requires support for the other primers that produce specific bands.

DISCLOSURE STATEMENT
The authors declare that they have no conflict of interest.

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SHORT BIOGRAPHY

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Tabel 1. Frequency of polymorphic bands in diabetics and control subjects based on D20 Primer

| No	Polymorphic band size (bp)	Number of Sample		% Sample		P-Value
		Control	DMT2	Control	DMT2	
1	174	6	2	20.0	6.7	0.129
2	255	7	10	23.3	33.3	0.390
3	312	9	7	30.0	23.3	0.559
4	353	28	29	93.3	96.7	0.554
5	405	26	27	86.7	90.0	0.688
6	476	28	28	93.3	93.3	1
7	576	1	12	3.3	40.0	0.001
8	623	11	12	36.7	40.0	0.791
9	716	27	28	90.0	93.3	0.640
10	784	26	29	86.7	96.7	0.161
11	944	29	29	96.7	96.7	1
12	1163	27	29	90.0	96.7	0.301
13	1519	6	9	20.0	30.0	0.371
14	2109	30	30	100.0	100.0	1
15	2381	10	15	33.3	50.0	0.190
16	3677	2	4	6.7	13.3	0.389
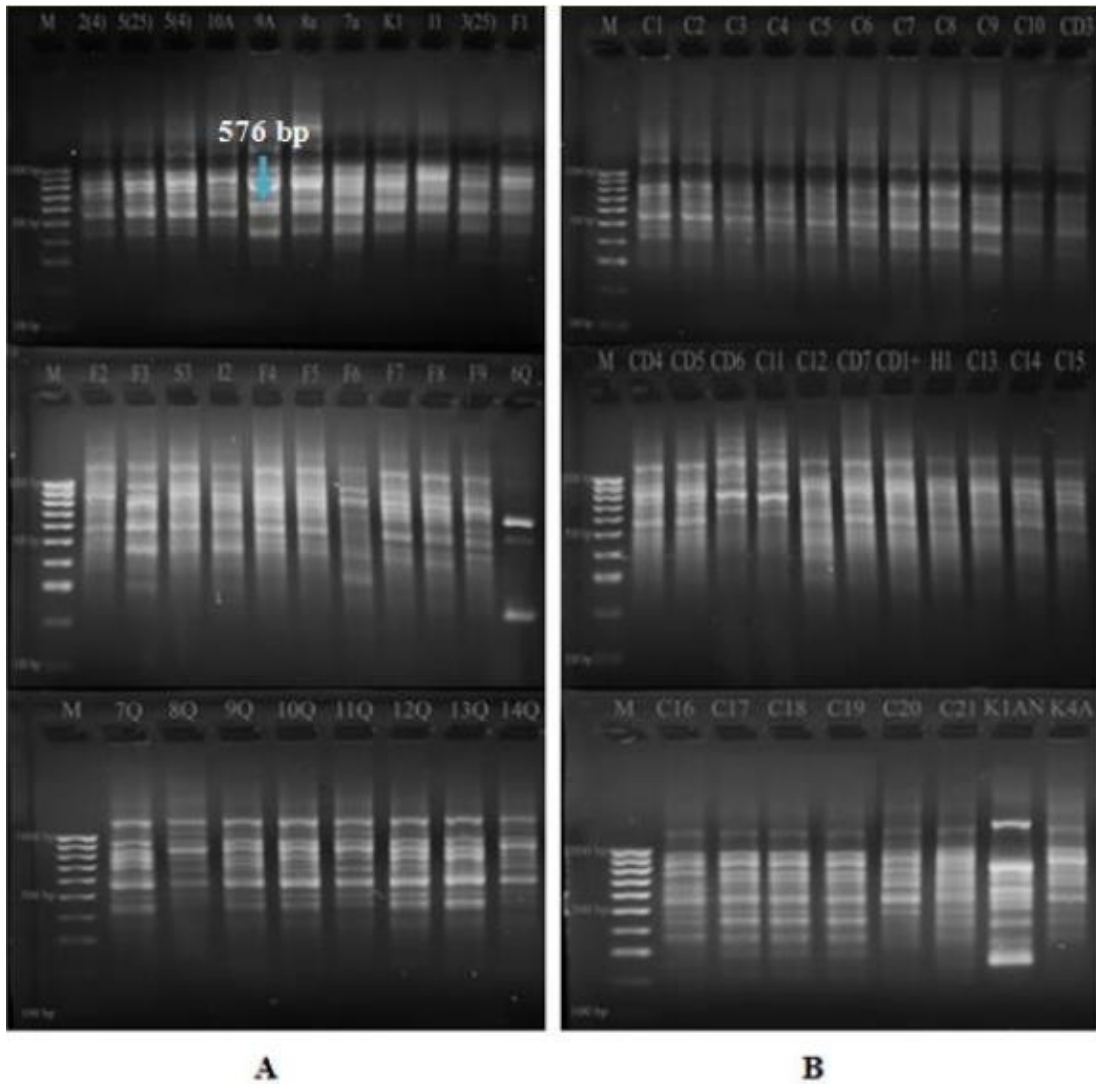
Figure 1. RAPD amplification products generated by primer D20. electrophoresis in 1.2% agarose gel, M is 100 bp DNA ladder

A: positive T2D, B: Control

Original Research

OPEN ACCESS

The effect of temperature and storage time of cuccal swabs on FGA and D13S317 loci with the STR PCR method

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HIGHLIGHTS
- The evidence in the crime scene that DNA checks can perform has various characteristics
- FGA and D13S317 are short tandem repeat loci recommended by the FBI in human identification
- Temperature and storage time affect the quality of DNA in the buccal swab

ARTICLE INFO

A B S T R A C T

The samples used for forensic DNA analysis in living individuals are usually blood and buccal swabs, however blood collection requires an invasive method that can cause discomfort, thus a buccal swab can be a good choice for individuals examined, especially children. This study aimed to determine the effect of temperature and storage time of buccal swabs on the quantity of DNA as material for DNA examination in the forensic field. This study was a laboratory experimental to determine the effect after treatment. Buccal swab samples were 48 and divided into 2 temperature groups, namely room temperature (RT) and 4°C. The division of the temperature groups was also observed with time differences, namely 1, 3, 5, 7 days. EDNA extraction used the DNAzol method and DNA quantification used a Spectrophotometer. The PCR process was carried out with STR primers FGA and D13S317 loci. The visualization stage used acrylamide gel and silver staining.

The results of this study prove that there is an effect of temperature and storage time of buccal swab samples. The longer the treatment time, the lower the DNA level. With statistical analysis, it is obtained p-value of <0.005, it can be concluded that there are significant differences in DNA levels at the temperature and storage time treatments of the buccal swab sample. The results of DNA visualization at the FGA and D13S317 loci using the STR PCR method in this study can still be detected and can be used as a reference for examination in forensic cases.

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1.   INTRODUCTION

DNA stores all the information about genetics. *Polymerase Chain Reaction* (PCR) technique invention caused quite revolutionary changes in various fields. The results of the application of this PCR technique are called DNA *fingerprint* which is a description of the pattern of DNA pieces from each individual; because every individual has different DNA *fingerprint*.[1,2,3] DNA fingerprint that is widely used as an individual identity is Short Tandem Repeat (STR). STR is an area that does not code in nuclear DNA and consists of 2-7 nucleotide sequences that are arranged repeatedly. Each STR locus has a polymorphism in the form of a difference in the number of repetitions in its partner allele and by using 13-20 STR loci, a person's identity can be determined.[4,5,6]

Any part of human body can be taken as a specimen because every nucleated cell in a person's body has identical DNA sequences, wherein a child receives essentially the same amount of genetic material from his/her biological mother and father (Mendel's law of inheritance).[7,8] So far, specimens (samples) that are widely used in DNA testing to identify are blood/blood spots, sperm spots, vaginal swabs, buccal swabs.[9,10] In general, the main choice used for DNA testing is blood. Blood specimens can be obtained from venous, arterial or capillary blood but this procedure requires an invasive procedure that can be uncomfortable for the individuals examined, and is impractical if used for large sampling.[11]

A buccal swab examination can be a good and convenient option for individuals examined, especially toddlers or children. Besides, samples from buccal swabs are more economical, practical, and easier to ship because they can be spared from the risk of tube rupture that can occur in delivery with blood specimens, but there are no specific standards regarding how long the temperature and storage time of buccal swab samples for DNA delivery so as to produce genomic DNA of good quality and an adequate amount for DNA examination. Hence this research on the effect of temperature and storage time of the buccal swab samples on the quality of DNA is the first step that will be used as a reference for future studies. This study aimed to obtain the optimal temperature and storage time and to produce good quality genomic DNA in sufficient quantities so that it can be used for STR PCR analysis.[12,13]

2.   MATERIAL AND METHOD

Research design

This study was a laboratory experimental, which is a method used to determine the effect after treatment in a study.

Research population and sample

The sample of this study is the DNA of male or female volunteers. The volunteers have previously filled out the *Inform Consent* form. Sample analysis was carried out at the Human Genetic Laboratory of the Institute for Tropical Diseases, Universitas Airlangga. This study used 48 samples of buccal swabs and divided into 4 groups of storage time with 12 samples each, namely 1, 3, 5, 7 days. Each storage time group has 2 temperature categories, namely 6 samples for room temperature and 6 samples for temperature of $4°C$. The research sample is the DNA of male or female volunteers. The volunteers have previously asked for approval to take buccal swabs as materials for research purposes at the Human Genetic Laboratory of the Institute for Tropical Diseases, Universitas Airlangga.

Research materials and tools

DNA isolation from buccal swabs using an organic method, namely DNAzol.[14,15] The extracted DNA would be quantified using a UV spectrophotometer[16,17,18] In the PCR process using STR primers with FGA (5'-GGCTGCAGGGCATAACATTA-3 'and 5'-ATTCTACGATTTGCGCTTCAGGA-3'), D13S317 (5'-ATTACAGAAGTCTGGGGGATGTGGAGGA-3 'and 5'-GGCAGCCCAAAAGACAGA-3') loci.[2,19] DNA amplification via PCR was carried out with the following protocol: FGA (Gene Ampr. PCR System 9700 Thermal Cycler, Promega Corp. 2001): initial denaturation; $96°C$ for 2 minutes,

denaturation; 90℃ for 1 minute, Annealing; 60℃ for 1 minute, Extension; 70℃ for 30 seconds; cycle 25 times. D13S317(Gene Ampr. PCR System 9700 Thermal Cycler, Promega Corp. 2001): Initial denaturation; 96℃ for 1 minute, Denaturation; 94℃ for 30 seconds, Annealing; 60℃for 30 seconds, Extension; 70℃ for 45 seconds; 30 times cycle, final extension 60℃ for 30 seconds. Electrophoresis in this stage was using Polyacrylamid Gel Electrophoresis with silver staining.[20],[21]
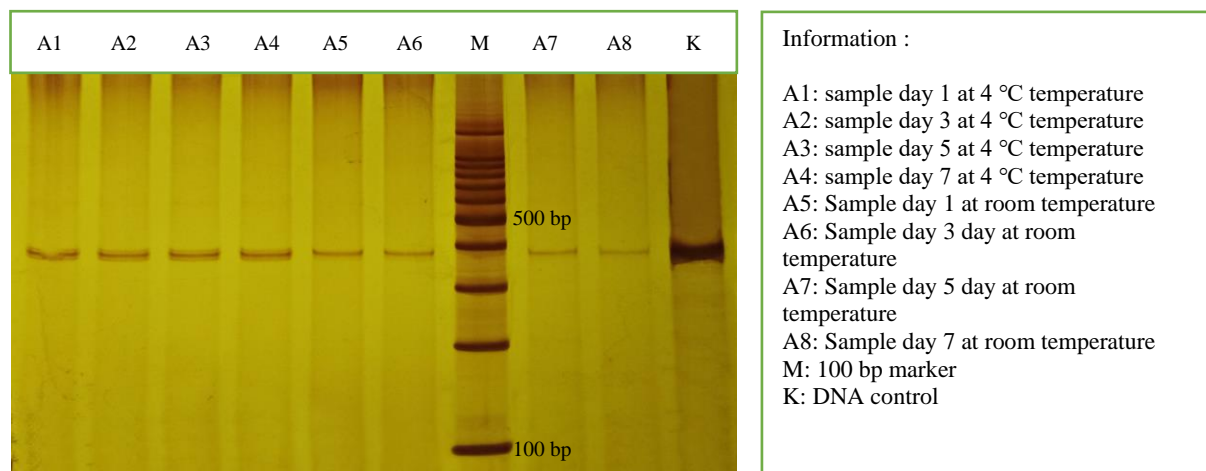
3.   RESULTS AND DISCUSSION

The results of the mean measurement of DNA levels using a UV-Visible Spectrophotometer for the research samples from the buccal swabs are as follows

Table 1. The results of mean DNA levels isolated from buccal swab samples

Treatment		Mean DNA levels (ng/ul)
Room temperature (RT)	1 day	1966.416667
	3 days	1773.333333
	5 days	1519
	7 days	1455.416667
Temperature 4℃	1 day	2996.583333
	3 days	2307.666667
	5 days	1969.333333
	7 days	1593.083333

Table 1, it is shown a tendency of decreasing DNA levels from buccal swab samples that have been through treatment time of 1, 3, 5, 7 days at room temperature and 4℃. The decrease in DNA levels in this study indicate an effect on the length of treatment time, resulting in damage to the DNA structure. DNA damage caused by abnormal exposures, is caused by the irreversible breakdown of DNA hydrogen bonds. This condition results in damage to the purine - pyrimidine pair in DNA, where this purine - pyrimidine pair is the main component in the DNA structure. Another factor is the condition of the old sample and the presence of protein associations, such as (mentioned in) the study conducted by Hansen.[22] However, with this decrease in levels, the remaining DNA levels still allow for DNA profiling, which is at least 50 ng. In principle, the minimum level of DNA that can be used in DNA analysis depends on the need and the type of examination being performed. In this study, the DNA levels obtained from the buccal swab samples ranged from 1519-2996 ng/ul, so that it was still possible to carry out DNA analysis tests as was done by Fattorini.[18]

Information :

A1: sample day 1 at 4 °C temperature
A2: sample day 3 at 4 °C temperature
A3: sample day 5 at 4 °C temperature
A4: sample day 7 at 4 °C temperature
A5: Sample day 1 at room temperature
A6: Sample day 3 day at room temperature
A7: Sample day 5 day at room temperature
A8: Sample day 7 at room temperature
M: 100 bp marker
K: DNA control

Figure 1. The results of the FGA locus (322 - 444 bp) STR amplification in sample A
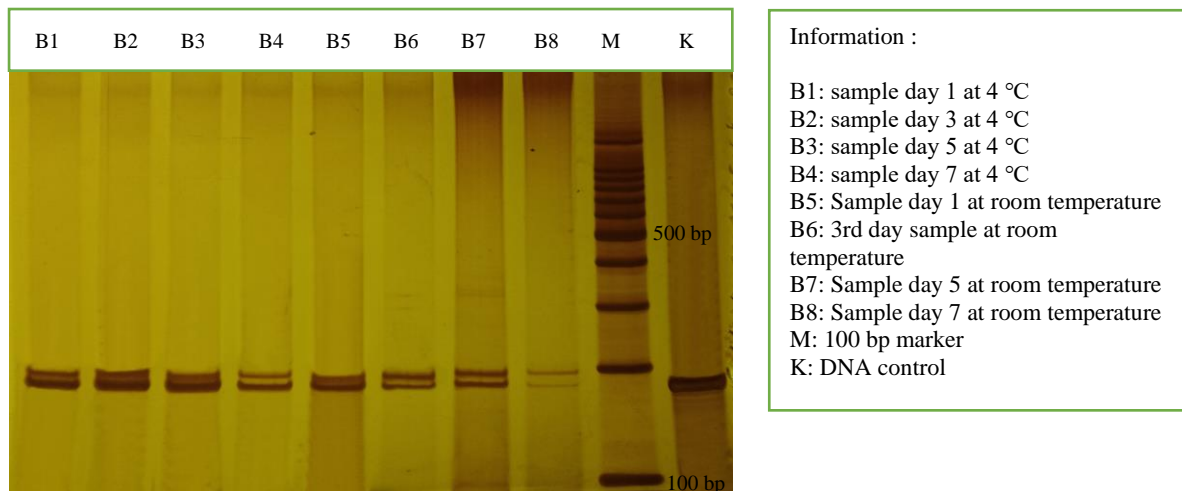
Figure 2. The result of the D13S317 locus (169-201 bp) STR amplification in sample B

The complete results of the detection of FGA and D13S317 loci on the buccal swabs DNA as an effect of temperature and storage time can be seen in the table 2.

Table 2. The results of detection of buccal swab DNA by STR PCR examination of the FGA and D13S317 loci

Treatment		FGA		D13S317	
		Detected	Undetected	Detected	Undetected
Room temperature	1 day	6	0	6	0
	3 days	6	0	6	0
	5 days	6	0	6	0
	7 days	6	0	6	0
Temperature 4 ℃	1 day	6	0	6	0
	3 days	6	0	6	0
	5 days	6	0	6	0
	7 days	6	0	6	0

The results of examining the effect of temperature and storage time on buccal swabs DNA in the FGA and D13S317 loci using the STR PCR method can be seen in Table 2. In all samples in this study, all are detectable. The visualization of PCR results using polyacrylamide gel in Figure 1 shows that all samples are detected in all treatments on the FGA locus (range between 322 - 444 bp) and in Figure 2 on the D13S317 locus (range between 169 - 201 bp). These results are in line with the research conducted by Manamperi et al that the D13S317 locus has a high polymorphic level based on the Patternity Index, Power of Discrimination, and Matching Probability.[23]

CONCLUSION

Analysis of DNA identification in this study concluded that there is an effect of temperature and storage time of the buccal swabs on DNA levels. The longer the treatment time, the lower the DNA level is. This can be seen in the results of Kruskal Wallis analysis which obtain a p-value of 0.001 ($p < 0.05$). The effect of temperature and storage time of buccal swabs is not too disturbing to the DNA at the FGA and D13S317 loci with the STR PCR method. For further research, it is necessary to carry out PCR amplification of the buccal swab DNA at another STR locus and continue with a longer time so that it can be further utilized more widely in the field of forensic identification.

DISCLOSURE STATEMENT

The authors declare that they have no conflict of interest.

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