

Potential of Sappan Wood, Purple Cabbage and Beetroot Extract in Sperm Staining

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Abstract

Examination of sperm morphology is an essential criterion for evaluating male fertility. This examination can be done by staining sperm cells with various techniques to facilitate the visualization of sperm cells. Several other methods that can be used to evaluate sperm morphology are Eosin-nigrosin, AgNO₃ staining, Papanicolaou, Diff Quick and Giemsa. However, using these synthetic dyes can harm the environment and water sources because they prefer to decompose. The natural dye derived from environmentally friendly plants expected to minimize hazardous waste. This study aimed to identify and compare the potency of several natural dyes derived from purple cabbage, sappan wood and beetroot, which have not been studied in sperm studies. Sperm was collected from 30 men in the campus area who underwent 3-5 days of abstinence from intercourse in preparation for sampling. The sperm stained with natural dye, and their quality compared with World Health Organization (WHO) standard Papanicolaou. Extracts of sappan wood, purple cabbage and beetroot are made with various mordant alum, ethanol and acetic acid compositions. The study's results showed that the presence of mordant increased the staining quality of sperm with sappan wood and purple cabbage extracts. The use of acid improves the quality of sperm staining with beet extract. There was no different between dyeing with beetroot extract in various solvent compositions and dyeing with Papanicolaou stain to color all sperm components. The results indicated that beetroot extract has a high potential for evaluating sperm morphology.

Keywords

Beetroot, Purple Cabbage, Sappan Wood, Sperm, Staining.

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INTRODUCTION

Globally, infertility is a problem experienced by 8-12% of couples, where male infertility is the main factor contributing to 50% of couples (1). The processes of diagnosing male infertility needs sperm analysis. Sperm morphology examination is one of the essential for evaluating male fertility (2). This examination is done by staining sperm cells with various techniques to facilitate the visualization of sperm cells. In addition, sperm staining could show abnormal sperm morphology. Sperm are considered normal if they are included in the classification of a specific species, including the shape and size of the head, midpiece and tail (3).

The World Health Organization (WHO) examination guidelines are a reference examination to evaluate sperm morphology. Two types of stains methods recommend by WHO, namely Papanicolaou and DiffQuik staining (4). Several other methods that can be used to evaluate sperm morphology are Eosin-nigrosin (5), AgNO_3 (6) and Giemsa (7) staining. However, using these synthetic dyes can be harmful; using them can harm the environment and water sources because they prefer to decompose (8).

Stain with Papanicolaou using hematoxylin as a natural dye and eosin as a synthetic dye. Toxicological information eosin can cause eye irritation, skin redness and pain. Direct contact of eosin with the eye

can cause permanent injury to the cornea by destroying the retinal ganglion cells. Eosin also damages DNA in the digestive organs, causing several diseases in the human body. Inhaling dye reduces pulmonary gas exchange (9). Silver can irritate skin, eyes and has carcinogenic, genotoxic effects. It will bind to purine and pyrimidine bases in DNA, increasing the disruption of normal gene function. Other toxicity of silver ions is hepatic, renal, neurological, and haematological effects (10).

The natural dyes derived from plants, animals and minerals is a sustainable and environmentally friendly resource (11). Dyeing applications have been applied in textile dyeing (12), solar cells, food, and pharmaceuticals (11). Several studies have revealed the potential of natural dyes for histological studies. Tissue coloring can use extracts of henna (13), rosella (14), curcumin (15), saffron (16) and red dragon (17). Mushroom staining using beet extract (18). Identification and differentiation of parasites using extracts of onion skin, walnut husk, and madder roots (19). Identification of grams of bacteria using rosella (20), purple sweet potato (21), and butterfly pea (22) extracts. Sperm morphology studies used extracts of henna, mulberry (23), safflower (23,24), black rice (25), butterfly pea and rosella (24).

Several researchers carried out previous research on sperm coloring using natural dyes. This finding suggests that the black rice

extracted can be used as an alternative dye for human spermatozoa morphology evaluation compared to butterfly pea, fresh roselle, and mulberry extract (24). Extract from black rice is an alternative to stain the nuclei of the sperm head (25). Natural henna is ineffective at coloring sperm. Conversely, extracts from mulberry and safflower have the ability to color sperm (23).

This study aimed to identify and compare the potency of several natural dyes derived from beets, sappan wood and purple cabbage, which have not been studied in sperm studies. Beets contain a purple-red pigment known as betacyanin. Betacyanin is also found in red dragon fruit and has been studied can be a substitute for counter stains for semen smears (23). Sappan wood contains the red pigment brazilein, previously reported to be successful in coloring tissue (26). Purple cabbage contains anthocyanin pigment. The use of anthocyanins in purple cabbage has succeeded in coloring red blood cells, which are usually stained by things like Giemsa (27). Natural dyes extract provides several advantages such as ease of preparation, inexpensive, local availability, and favorable nuclear staining properties.

MATERIALS AND METHODS

This study employs a comparative experimental design aimed at establishing relationships between two or more variables. Specifically, the research compares the

staining quality of sperm using dyes from beetroot, sappan wood, and purple cabbage extracts. The subject selection method involves the use of convenience sampling, allowing the researchers to select readily accessible participants who are available to take part in the study.

The research participants consisted of 30 men (aged 18-35 years) from the Karsa Husada Garut STIKes campus in Indonesia. Prior to sampling, the participants abstained from sexual intercourse for a period of 3-5 days. Ethical clearance for this research was obtained with the reference number 215/ec.01/kepik-bth/IX/2022 from the Health Research Ethics Committee of Bakti Tunas Husada University.

The extraction was made with various compositions from 100 g of beetroot, sappan wood and purple cabbage. The first composition (I): 100 mL of distilled water, 5 ml of ethanol, 10 g of mordant potassium alum and 4 mL of acetic acid. The second composition (II): 100 mL of distilled water, 5 ml of ethanol and 4 mL of acetic acid. The third composition (III): 100 ml of distilled water, 5 mL of ethanol and 10 g of mordant Potassium alum. After that, the container was wrapped in aluminium foil overnight in the extraction process. The extract supernatant was filtered using Whatman filter paper number 1 and stored in a dark place until use.

In sperm staining, 10-20 μ L of semen smeared on a clean glass slide. Air-dried

sperm smeared and fixed in 95% ethanol for Papanicolaou staining and natural dye using sappan wood, purple cabbage and beetroot extracts. Papanicolaou quick stain was used as a control recommended by WHO 2010 criteria. For natural dyes, sperm was soaked with the natural extract for 15 minutes at room temperature and washed with tap water. Evaluation of the staining results in the form of background color and three sperm components, including the head, midpiece and tail.

The scoring system assigns scores based on the color appearance of each sperm component: 0 for pale or poor color, 1 for unclear color, 2 for clear color, and 3 for very clear color (24). Repeated sperm smear slides of each stain were evaluated by three technician experts in semen analysis who have worked for more than five years. The staining results were viewed under a microscope with a total 1000x magnification.

The evaluation of sperm staining quality involved assessing the morphology of spermatozoa, assigning a scoring value. A statistical analysis was conducted to compare the staining outcomes achieved with natural dyes and Papanicolaou (PAP) standards

RESULTS

The results of sperm morphology staining with Papanicolaou staining can be seen in Figure 1. This dye can color the sperm parts, such as the head, midpiece and tail of sperm appeared blue with various staining qualities.

Sperm staining with sappan wood extract showed a red color on the sperm. The use of solvents with three different compositions to evaluate the quality of the staining results with sappan extract can be seen in Figure 2 a-c. Sperm staining with purple cabbage extract showed a blue color in the sperm.

The assessment of staining quality using three different solvent compositions in conjunction with purple cabbage extract is illustrated in Figure 3a-c. Sperm staining with beetroot extract resulted in a purple coloration of the sperm.

The utilization of solvents with three distinct compositions to assess the quality of staining outcomes using beet extract is depicted in Figure 4a-c. The establishment of the scoring system for staining with sappan wood, purple cabbage, and beetroot on sperm can be observed in Figure 5.

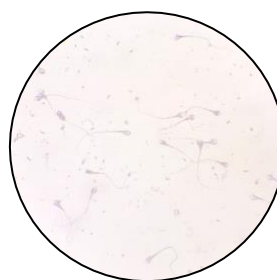


Figure 1. Sperm staining result with Papanicolaou stain observed by 1000x magnification.

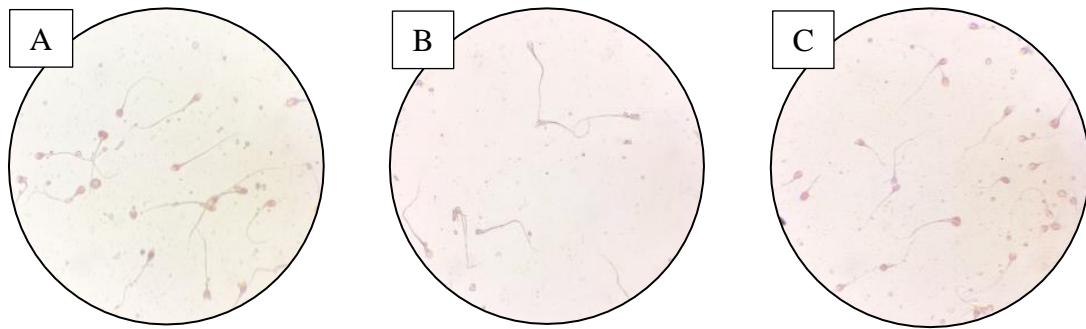


Figure 2. Sperm staining results of sappan extract at 1000x magnification. (A) Mixed solvents of mordant, ethanol and acetic acid. (B) Mixed ethanol and acetic acid. (C) Mixed mordant and ethanol.

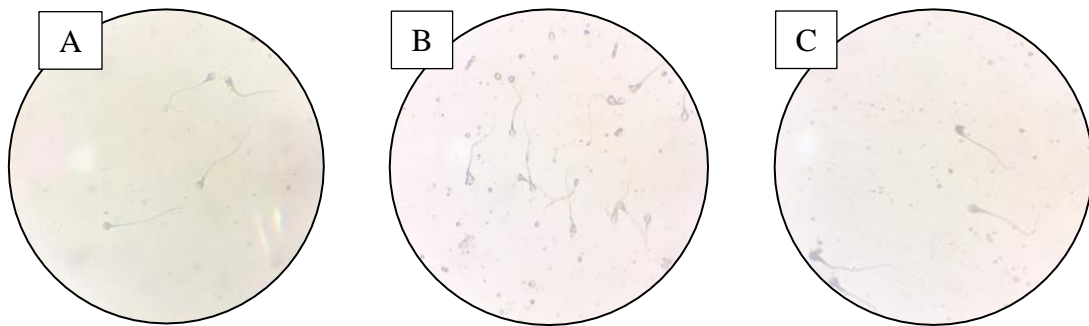


Figure 3. Sperm staining results of purple cabbage extract at 1000x magnification. (A) Mixed solvents of mordant, ethanol and acetic acid. (B) Mixed ethanol and acetic acid. (C) Mixed mordant and ethanol.

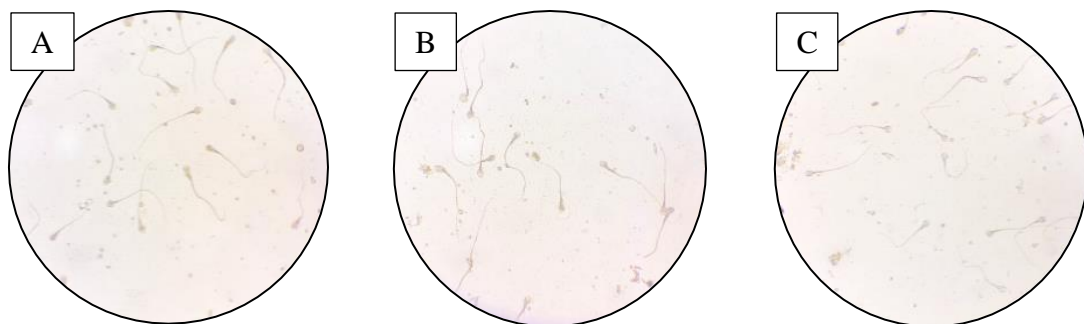


Figure 4. Sperm staining results of beetroot extract at 1000x magnification. (A) Mixed solvents of mordant, ethanol and acetic acid. (B) Mixed ethanol and acetic acid. (C) Mixed mordant and ethanol.

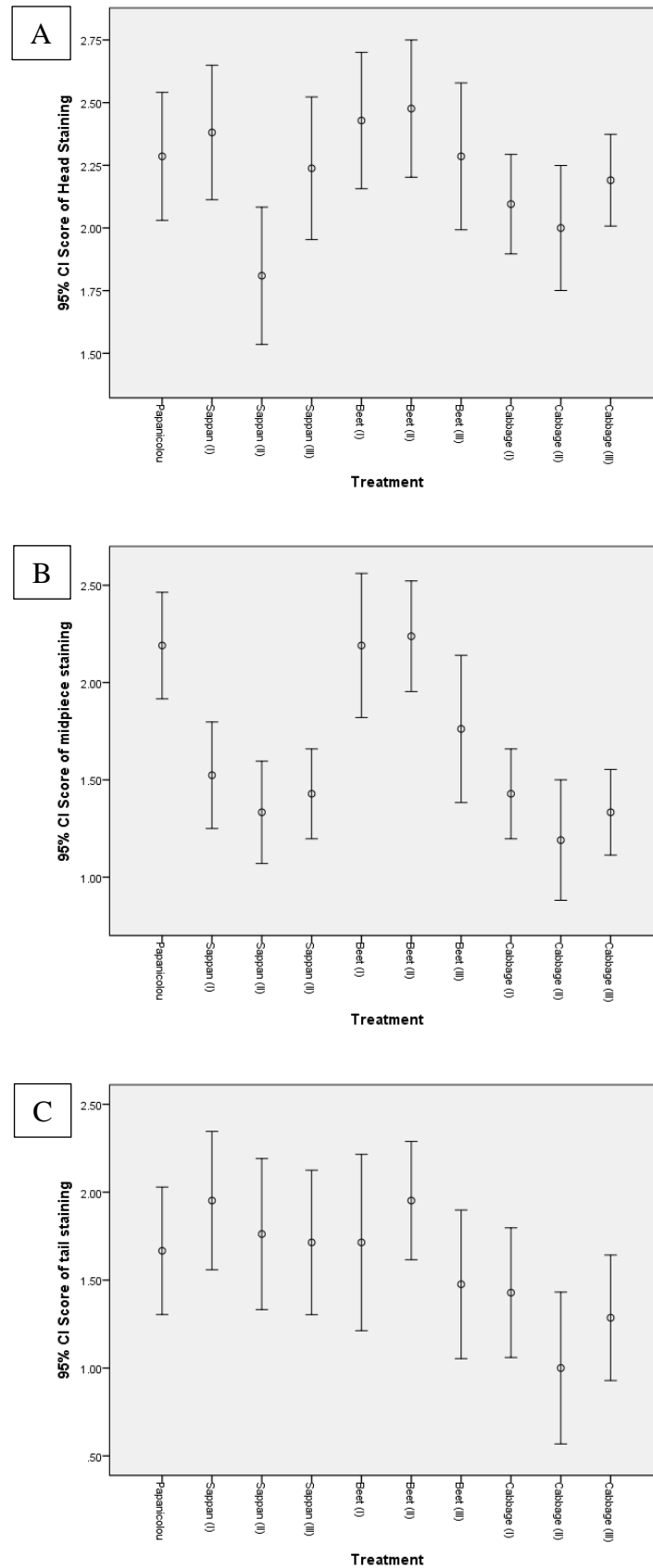


Figure 5. Mean Score results of sperm staining the 95% Confidence Interval (CI). (A) Head. (B) Midpiece. (C) Tail.

Based on Figure 5, the results of the mean score of Papanicolaou staining, the composition of sappan extract I-III, beet extract with composition I-III and purple cabbage extract with composition I-III in each part of the sperm, head (a), midpiece (b) and tail (c). For head staining results, each staining has a mean score of >2 , except for sappan staining with composition II which has a mean score of <2 . For the staining results in the midpiece, the beet extracts in compositions I and II had a mean score >2 , similar to the results of the Papanicolaou staining. Whereas midpiece staining had a

mean score <2 for staining using beet extract with composition III, sappan extract in composition I-III and purple cabbage extract in composition I-III. For tail staining results, the mean score for staining of sappan extract with compositions I-III, and beet extracts within compositions I and II is the same as Papanicolaou or higher. While the mean score for staining beet composition III and purple cabbage composition I-III has a mean score below Papanicolaou. Based on the means score, it can be seen that the staining results with beet extract with compositions I and II have better staining than the others.

Table 1. Statistical Analysis of Sperm Staining with Papanicolaou, Sappan Extract, Purple Cabbage and Beetroot

Control dye	Natural dye	Sig. Head	Sig. Midpiece	Sig. Tail
Papanicolaou	Sappan Extract (composition I)	1,000	0.035	1,000
	Sappan Extract (composition II)	0.313	0.001	1,000
	Sappan Extract (composition III)	1,000	0.006	1,000
	Purple Cabbage Extract (composition I)	1,000	0.006	1,000
	Purple Cabbage Extract (composition II)	1,000	0.000	0.713
	Purple Cabbage Extract (composition III)	1,000	0.001	1,000
	Beet Extract (composition I)	1,000	1,000	1,000
	Beet Extract (composition II)	1,000	1,000	1,000
	Beet Extract (composition III)	1,000	1,000	1,000

Statistical analysis of the sperm component staining results is presented in Table 1. A significant difference ($p < 0.05$) was observed between the effects of sappan and purple cabbage extracts when compared to Papanicolaou staining specifically for the midpiece. However, there were no significant differences ($p > 0.05$) in staining between sappan extract, purple cabbage extract, and

Papanicolaou stain for the head and tail components, except for the midpiece. No significant variations in staining were observed among different solvent compositions of beetroot extract and The utilization of the Papanicolaou stain, in relation to the coloration of all sperm components, did not yield statistically significant differences ($p > 0.05$). The

findings indicate that beetroot extract can serve as a means to assess sperm morphology.

DISCUSSION

Two types of dyes can be used, namely synthetic and natural dyes. The use of natural dyes is considered to be able to increase the economic value of local materials, reduce the use of synthetic dyes and reduce negative impacts on humans and the environment (28). Hematoxylin is a natural dye used in biological staining and is the gold standard dye in histological examination and sperm staining (PAP method). Due to some drawbacks (26), it proposed natural plant substitutes for hematoxylin. This research focuses on using natural dyes of sappan wood, purple cabbage and beets. The composition of the solvent used consisted of distilled water, ethanol, alum and acetic acid (composition I), and variations were made without alum (composition II) and without acetic acid (composition III). The results of sperm staining with PAP control are shown in Figure 1, whereas staining with beetroot extract, sappan wood extract and purple cabbage can be seen in Figures 3-4.

Based on Figure 5a, the results reveal that potassium alum is a crucial component of natural staining from sappan extract staining. The staining result with the solvent composition added with alum produced a higher staining score than without alum.

Alum functions as a mordant having a positive charge enabling it to bind to anionic tissue areas used to repair or increase the intensity of cell or tissue staining (29). Similarly, the hematoxylin-containing Papanicolaou stain requires a mordant to oxidize the hematoxylin before it can be applied as a stain for spermatozoa (24).

The inclusion of acetic acid improves the staining process, as the combined action of the two enhancing agents in the coloring procedure proves more effective than using mordant alone for beet staining, as illustrated in Figure 5b. The data indicates that the pH conditions of the extracts yielded improved nuclear staining due to appropriate acidification. This is in line with several studies on acid beet staining on mycological preparations (18), histological preparations (27), onion bulb tissue preparations (30), sperm (24), also staining with black rice, butterfly pea flowers are acidic in staining sperm (24,25).

The quality results of staining with reagent III compositions on sappan and purple cabbage extracts free of acetic acid showed good color compared to compositions without mordant. The solvent extraction with composition III reduces the use of acetic acid. The extraction process using alcohol produces a slightly acidic PH, so it still supports the coloring process.

These natural dyes contain hydroxyl or phenolic groups, which are responsible for

the coloring ability of sperm (23). This phenolic group is found in dyes extracted from beets, sappan wood and purple cabbage. Beets generally contain betalain pigments (23). Brazilin is a pigment component found in sappan wood extract (31). Phenolic compounds in purple cabbage, one of which is anthocyanin pigment (32). These pigments can color sperm.

Betalain consists of two parts, namely betacyanin and betaxanthin. (32). Betacyanins, red to purple pigments contain cyclo-3,4-dihydroxyphenylalanine (cyclo-DOPA) residues, while betaxanthins, yellow-orange pigments contain amino acid or amine residues. The most common betacyanin is betanin. Betanin contained in beets is betanidin 5-O- β -glucoside (33).

In this study the extraction of beetroot was carried out with distilled water and alcohol with variations in the addition of acetic acid and alum, the extract was purple in color, and the extract was obtained. Water and alcohol extracts from beets contain a mixture of betaxanthin, betanin and betanidin.

The process of adding acid lowers the pH to 7 causing a hypochromic shift and a hypochromic effect (decreased absorption intensity). This hypochromic effect is accompanied by a slight hyperchromic effect (increased absorption intensity), in the 575–650 nm range, and the color changes from red to purple. Betalains allow forming metal

complexes which can lead to hypochromic (27).

Sappan wood extract predominantly contains brazilin, protosappanin B, and a small portion of brazilein. Brazilin with hematoxylin has similar molecular structures. In hematoxylin staining, a hematoxylin formulation containing a haematein and aluminium complex is called hemalum. Brazilin has more than one hydroxyl group which acts as a ligand to form metal complexes. The presence of brazilin complexing on the metal causes a bathochromic shift in the spectrum of brazilin because the metal has a pair of electrons for the O atom donor in brazilin (27).

When the pH of the sappan wood extract changed to acid with the addition of acetic acid, a hypochromic effect was observed with a shift towards shorter wavelengths, namely, the color of the dye changed to pale yellow. The extract is pink in color, and when mixed with various mordants it forms a different color, one of which is the addition of alum mordant, which causes the extract to change color to a red color (31).

Purple cabbage is one of the primary sources of anthocyanin pigments, around more than 10 g per kg (32). Over 30 anthocyanin pigments contain cyanidin-3-diglucoside-5-glucoside derivatives in non-acylated or acylated forms, mainly acylated by P-coumaric, sinapic, and ferulic acids (34). Cyanidin is an anthocyanin as a

flavonoid which becomes a dye through the chromophore and auxochrome groups it contains. Acylated anthocyanins have the advantage of being more stable to temperature and light, having greater antioxidant capacity, and having a wider color spectrum than un-acylated ones. With these properties, it is an excellent candidate as a natural pigment (32).

In addition, this result may be obtained for several reasons, including the different characteristics of sperm as a single cell compared to neural tissue as a solid mass of cells and differences between sperm sample preparations compared to fixed tissue, which is a multi-step process that can affect. The proportion of dye concentration and exposure time of spermatozoa to dye, which can affect the coloring (35)

This study represents the pioneering effort in exploring the dye capabilities of beetroot, sappan wood, and purple cabbage extracts for sperm staining. All three dyes exhibit potential for effective staining and can serve as alternative options for staining sperm of different qualities.

Notably, the results of beetroot staining demonstrated comparable quality to that achieved with Papanicolaou staining, with no significant difference ($p > 0.05$). These findings strongly suggest the utility of beetroot extract for evaluating spermatozoa morphology. The emergence of such alternative staining options holds promise in

terms of reducing experimental costs and mitigating environmental pollution, as they are devoid of carcinogenic azo compounds.

CONCLUSIONS

The research findings lead to the conclusion that sappan wood, purple cabbage, and bua beet extracts are viable options for staining sperm components, encompassing the head, midpiece, and tail. The introduction of mordant and acid amplifies the coloration effectiveness of sappan extract and purple cabbage. Notably, among the tested extracts, beetroot extract demonstrates the highest proficiency in achieving optimal staining results for sperm analysis.

AUTHOR CONTRIBUTIONS

Mamay: concept and research design, data interpretation, manuscript preparation and revision. Ernawati: administration of research, preparation of research subjects. Astari Nurisani: laboratory analysis and data collection.

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CONFLICT OF INTEREST

There are no conflicts of interest.

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