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Antibacterial compound from *Euchema spinosum* originated from Tasikmalaya West Java against pathogen bacteria with TLC-bioautography

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ABSTRACT

Streptococcus mutans (Gram-positive) and *Shigella dysenteriae* (Gram-negative) are two types of pathogen bacteria. The use of synthetic antibiotics against both bacteria is known to impact the bacteria's resistance. *E. spinosum* from Tasikmalaya is a potential macroalgae as a source of an antibacterial compound for both bacteria. The research aims to determine the antibacterial metabolite compound from *E. spinosum* originated from Tasikmalaya against *S. mutans* and *S. dysenteriae*. The research was conducted through several stages, starting from phytochemical screening, gradual maceration using hexane, ethyl acetate, and methanol, determination of antibacterial activity, and TLC-bioautography. Phytochemical screening showed that both raw material and extracts contained alkaloids, flavonoids, and steroids. The result showed that hexane, ethyl acetate, and methanol extract could inhibit the growth of *S. dysenteriae* starting from a concentration of 400 µg/mL. However, only ethyl acetate extract can inhibit the growth of *S. mutans*, starting from a concentration of 20 µg/mL. The chromatogram of the hexane extract showed the presence of 6 spots, ethyl acetate extract showed 5, and the methanol extract showed only 4, resulted from the elution system, respectively. The TLC-bioautography against *S. dysenteriae* showed that there was the presence of three clear zones on the ethyl acetate extract, detected as flavonoid, and three clear zones on the methanol extract. The TLC-bioautography against *S. mutans* showed one clear zone on the chromatogram of ethyl acetate extract. According to the $AlCl_3$ spray reagent confirmation test, the active compound was the flavonoid group.

Keywords: *Eucheuma spinosum*, *Streptococcus mutans*, *Shigella dysenteriae*, Tasikmalaya, TLC-bioautography

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INTRODUCTION

E. spinosum is a species of red algae that grows in Indonesian marine areas. One of which is at Cipatujah Coastal, Tasikmalaya, West Java, Indonesia. Cipatujah is the southern coastal area of the city of Tasikmalaya, directly opposite the Indian Ocean. Unlike the other areas, *E. spinosum* originated from Cipatujah is still rarely studied due to the absence of cultivation efforts. However, the Indonesian seaweed production from the *Euchema sp* species was the highest compared to the other seaweed sources in 2015, namely 466,740 dry tons (BPPKP, 2018). Even so, Indonesia was encouraged to become the world's producer of *E. spinosum* seaweed (KKP, 2018). *E. spinosum* is widely used as a source of bioactive protein (Hung et al., 2015; Ramadan et al., 2019; Sugrani et al., 2019), carrageenan (Briones and Sato, 2014; Ulumiah et al., 2019), and also as a source of active secondary metabolites (Anestopoulos et al., 2020; Khatulistiani et al., 2019; Othman et al., 2018). Nevertheless, there is still little information related to specific metabolite compounds that have pharmacological properties.

S. dysenteriae and *S. mutans* are two types of pathogen bacteria that often pose a disease risk for humans, especially in Indonesia. In 2018, Based on Indonesia's health profile data, the incidence of death due to diarrhea was 4.76%. This percentage was the highest level in the last ten years. The report also stated that dental caries was the most common health risk, especially in adolescents (12-17 years). According to the Basic Health Research conducted by the Ministry of Health, data of 2018 reported that 57.6% of the people experienced problems with their teeth and mouth. The primary treatment related to these two diseases was using synthetic antibiotics.

S. dysenteriae (family: *Enterobacteriaceae*) are gram-negative bacteria that cause severe diarrhea (dysentery), also known as shigellosis (El-Gendy et al., 2012; Puzari et al., 2017). *S. dysenteriae* has also been shown to have resistance to several antibiotics, such as the fluoroquinolone class, cephalosporins, azithromycin, chloramphenicol, co-trimoxazole, and ampicillin (El-Gendy et al., 2012; Puzari et al., 2017). *S. dysenteriae* is also able to produce β-lactamase (Ahmed and Shimamoto, 2015; Puzari et al., 2017), forming biofilms layer (Batista et al., 2018), and could perform chromosomal mutations (Ahmed and Shimamoto, 2015). Meanwhile, *S. mutans* (family: Streptococcaceae) are gram-positive bacteria that cause tooth decay. The most common treatment for this condition is using fluoride (Mitsuhata et al., 2013). *S. mutans* can also produce a biofilm layer that makes it resistant to fluoride and synthetic antibiotics (André et al., 2018).

We have not found any information about the antibacterial activity of *E. spinosum* against *S. dysenteriae* and *S. mutans*. However, *E. Spinosum*, based on several research results, is known to have pharmacological activities as an antibacterial against *B. cereus* bacteria (Rarassari et al., 2016), *S. Aureus* (Safitri et al., 2018; Sugrani et al., 2019), *P. gingivalis* (Mattulada et al., 2018), and *E. Coli* (Sugrani et al., 2019). Therefore, based on its known antibacterial activity, *E. spinosum* has the potential to inhibit the growth of *S. dysenteriae* and *S. mutans* bacteria.

The contact TLC-bioautography is often used in several studies to identify and characterize antibacterial compounds from the natural substance (Al-Saman et al., 2018). This method can selectively determine the antibacterial compounds, even without passing an isolation process (Czernicka et al., 2019). Hence, this research aims to determine the antibacterial activity from *E. spinosum* originated from Cipatujah coastal, Tasikmalaya, West Java, against *S. dysenteriae* and *S. mutans*, and to analyze the active metabolite compounds against both bacteria.

MATERIALS AND METHOD

Materials

The raw material used was *Euchema spinosum*, obtained from Cipatujah Coastal, Tasikmalaya, West Java. The bacteria used included *Shigella dysenteriae* ATCC 13313 and *Streptococcus mutans* ATCC 25175. The media used including nutrient broth (Merck), Salmonella Shigella agar (Himedia), and blood agar media (Merck).

Methods

Raw material determination

The identification of *E. spinosum* took place in the Jatinangor Herbarium with identification sheet number 082/HB/02/2019.

Maceration and phytochemical screening

The *E. spinosum* material was dried first, then macerated gradually using hexane, ethyl acetate, and methanol solvents. Each of the mixtures was then filtrated and concentrated with a rotary vacuum evaporator to produce three viscous extracts, namely hexane, ethyl acetate, and methanol extracts. The raw material and the extracts were then analyzed for their chemical content, including:

Alkaloids: 2 grams of raw material and the extracts of *E. spinosum* were placed in different Erlenmeyer. Each of them was acidified with 2 N HCl and then filtered. The filtrate was then alkalized with 10% NH_4OH and was added with chloroform. The chloroform layer was then separated and tested using Dragendorf reagent and Mayer reagent. The characterization of the alkaloids appeared in two different colors; orange color for Dragendorff reagent and white precipitation for Mayer reagent.

Flavonoids: 2 grams of raw material were added with water and heated. 5 mL of 2 N HCl was added to the mixture, continued by adding a little Magnesium powder, then filtered. After that, amyl alcohol was put into the filtrate and shaken quickly. The presence of flavonoids was seen from the color appearing on the amyl alcohol layer, where it could be red, yellow, or orange in color.

Monoterpenoids and sesquiterpenoids: The raw material and each extract were dissolved in ether and then filtered. The filtrates were evaporated and dried on a drop plate porcelain. Then, 10% vanillin reagent in sulfuric acid was added to the dried extract. The appearance of colors was the indication of positive results.

Steroids: The raw material and each extract were dissolved in ether and then filtered. The filtrates were then added by Lieberman Burchard's reagent. The appearance of green-blue color was the indication of steroids.

Tannins and saponins were analyzed using the method conducted by Safitri et al. (Safitri et al., 2018).

Antibacterial assay from all extracts

The stock solution of *E. spinosum* extract was prepared by dissolving 50 mg extract in 50 mL of 96% ethanol. The stock solution was firstly diluted with water to obtain a variant extract concentration of 20, 40, 60 µg/mL, and they were called tested extracts. Firstly, *S. dysenteriae* ATCC 13313 was cultured on SSA media (Alemu et al., 2019), and *S. mutans* ATCC 25175 was cultured on blood agar (George et al., 2017), both were incubated at 37°C for 24 hours.

The assay of extract activity to *S. dysenteriae* applied the disk diffusion method. First, a small portion of the bacteria was dissolved in nutrient broth to produce turbidity equivalent to the standard of 0.5 Mcfarland (Alemu et al., 2019). Second, the suspension was then smeared on the surface of SSA media. Third, the paper disks were dipped in each test solution, including the tested extracts, 96% ethanol solvent, and 5 µg/mL of cotrimoxazole as the comparator for *S. dysenteriae* assay. Fourth, The disks were then affixed to the SSA surface and incubated at 37°C for 48 hours.

The assay of the extract activity to *S. mutans* was conducted with the agar well diffusion method. First, the *S. mutans* culture was smeared on the surface of the media. Second, wells were made on the blood agar medium using a 6 mm diameter perforator. Third, approximately 50 µL of each concentration of the tested extract, 96% ethanol solvent, and 5 µg/mL of chlorhexidine as the comparator (Jones, 1997) were put into the well using a micropipette. Fourth, the medium containing a tested sample was incubated at 37°C for 48 hours. Each of those assays was done under a laminar airflow environment.

Thin-layer chromatography

Approximately 30 µL of each of *E. spinosum* extracts diluted with each solvent (1 µg/mL) were then spotted onto the activated GF254 TLC plate using a micropipette. The plates were then eluted with the selected eluent combination as shown in Table 1, with an optimized composition than the previous ones.

Table 1. Composition of eluent used in TLC

Extract	The Solvent Mixture	Composition
Hexane	Hexane : ethyl acetate	4:1
Ethyl acetate	Chloroform : ethyl acetate	2:3
Methanol	Chloroform : aseton	1:2

TLC-bioautography

The TLC-bioautography was conducted based on the previous methods (Dewanjee et al., 2015). Each chromatogram was then analyzed using the 254 nm and 366 nm UV lamp then the number of spots that appeared was calculated. The chromatogram containing the well-separated compound was then attached to the surface of the SSA medium for *S. dysenteriae* and blood agar medium for *S. mutans* for 30 minutes. After that, the chromatogram plate was withdrawn from the medium. Subsequently, the medium was incubated at 37°C for 24 hours. Then, the results obtained were observed, and the clear zone that appeared was compared with the chromatogram. Lastly, each spot producing a clear zone was sprayed using $AlCl_3$ and Dragendorff spray reagent.

RESULT AND DISCUSSION

The results of raw material identification stated that the material used was *Euchema spinosum*. The pharmacological activity of *E. spinosum* likely depends on the content of the active chemical compounds, especially the secondary metabolites. *E. spinosum*, based on Phytochemical screening analysis, was known to contain flavonoid, alkaloid, and steroid components (Table 2).

Table 2. Results of phytochemical screening analysis from raw material, extracts of hexane, ethyl acetate, and methanol

Chemical Group	Raw Material	Extract		
		Hexane	Ethyl Acetate	Methanol
Flavonoid	+	+	+	+
Polyphenols	-	-	-	-
Tannin	-	-	-	-
Alkaloid	+	+	+	+
Saponin	-	-	-	-
Monoterpenoid and Sesquiterpenoid	-	+	+	+
Steroid	+	+	+	+

Information: (+) detected (-) undetected

It is proven that the continuous maceration method with the three solvents could engage the essential secondary metabolite compounds in the material, shown by the detection of three metabolite components in the three extracts. Moreover, the maceration process was also able to attract monoterpenoids and sesquiterpenoids. They are known as the constituents of essential oils, previously being undetected at the time of phytochemical screening in the raw material. Several components of essential oils are known to eliminate *S. mutans* and *S. dysenteriae* actively since they can destroy biofilms made by *S. mutans* (Khan et al., 2020; Tofiño-Rivera et al., 2016) and by *S. dysenteriae* (Batista et al., 2018; Kang et al., 2020).

Flavonoids are metabolite compounds that have antibacterial activity, especially against *S. mutans* and *S. dysenteriae*. Extracts from several herbs containing flavonoids are known to actively inhibit the growth of *S. mutans* (Jiang et al., 2020) since they can penetrate the film membrane of *S. mutans* (Veloz et al., 2019). Flavonoids could form complex compounds with the constituent compounds of the bacterial cell wall (extracellular protein). It would inhibit the activity of essential enzymes in bacteria (tyrosyl-RNA synthetase enzymes, β-ketoacyl acyl carrier protein synthase I and III, topoisomerase IV) and inhibit the adhesion process. They also could damage the biofilm layer of bacteria, depolarize membranes, inhibit the synthesis of DNA, RNA, and membrane protein (Farhadi et al., 2019). Zou et al. in their research succeeded in isolating several flavonoids from *Selaginella moellendorffii* Hieron, being able to damage the biofilm layer, namely 5-carboxymethyl-4'-hydroxyflavone-7-O-β-D-glucopyranosid, 2-(2-(3.4-dihydroxyphenyl)-7-hydroxy-4-oxo-4H-chromen-5-yl) acetic acid, (S)-2-(7-hydroxy-2-(4- hydroxyphenyl)-4-oxochroman-5-yl) acetic acid (Zou et al., 2016).

Apart from flavonoids, alkaloids and steroids are signified to have antibacterial activity, especially against *S. mutans* and *S. dysenteriae*. Xu et al. successfully isolated several alkaloid groups such as Fructigenine A, Fructigenine B, and Brevicompanine G from *Pleosporales sp* (marine fungi) that actively inhibit the growth of *S. dysenteriae*. Xu also successfully isolated the steroid class, namely Ergosta-4,6,8 (14), 22-tetraen-3-one, and (22E) -Ergosta-5,22-dien-3-one that are also known to actively inhibit the growth of *S. dysenteriae* (Xu et al., 2018). Meanwhile, alkaloids can damage the peptidoglycan constituent components in bacterial cells, causing the cell wall layer to not forming appropriately. In addition, alkaloids also inhibit the activity of the topoisomerase enzyme in bacteria (Singkoh et al., 2019). Some of the alkaloids from Rhodophyta algae are the indole alkaloid group (Pérez et al., 2016).

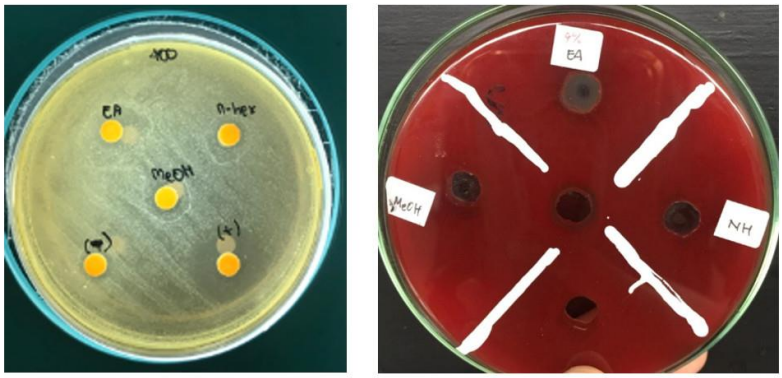
Antimicrobial activity

Initially, all hexane, ethyl acetate, and methanol extract concentrations did not show the inhibition growth activity against *S. dysenteriae*. However, after the assay was continued with an increase in the concentration measure, all extracts finally showed the inhibition zone starting from a 400 µg/mL concentration (Figure 1). However, only ethyl acetate extract was particularly active against *S. mutans* bacteria and gave an inhibition starting from 20 µg/mL (Table 3 and Figure 1).

Table 3. Clear zone diameter of each extract concentration against *S. Mutans* and *S. dysenteriae* Bacteria

ppm	*S. mutans*			*S. dysenteriae*		
	hexane	**Ethyl acetate**	**methanol**	**hexane**	**Ethyl acetate**	**methanol**
20	-*	7.1 + 0.14	-*	-*	-*	-*
40	-*	7.9 + 0.28	-*	-*	-*	-*
60	-*	9.15 + 0.21	-*	-*	-*	-*
400	-**	-**	-**	7.70 + 0.07	7.78 + 0.11	8.8 + 0.07
800	-**	-**	-**	7.30 + 0.64	7.30 + 0.64	7.8 + 0.07
1600	-**	-**	-**	8.88 + 0.11	7.85 + 0.14	7.25 + 0.07

*not detected
**not assayed



(a) (b)

Figure 1. The inhibition zone from methanolic (MeOH), ethyl acetate (EA), and hexane (NH) extract; (a) concentration of 400 µg/mL of each extract against *S. dysenteriae* on SSA medium with cotrimoxazole as a positive control, (b) 20 µg/mL of each extract against *S. mutans* on blood agar medium with chlorhexidine as a positive control

Although the phytochemical screening showed the same class of compounds present in each extract, the polarity of these compounds was different. Hydrophilicity and lipophilicity are known to affect the antibacterial activity of a compound. Lipophilic extracts from the Rhodophyta division have been proven to inhibit the growth of several bacteria (Cortés et al., 2014). Lipophilic compounds such as flavonoids and terpenoids are known to be able to interact with bacterial cell walls so that they can damage membranes (Dharmautama et al., 2019; Medina-Flores et al., 2016). Lipophilic flavonoids also actively inhibit the growth of both gram-positive and gram-negative bacteria (Farhadi et al., 2019). As an example, Apigenin (4′,5,7-Trihydroxyflavone) could reduce the virulence level of *S. mutans* (André et al., 2018). Hydrophilic compounds are also known to actively resist several microbes including the glycoside group, such as aminoglycosides (Nweze et al., 2020), flavonoid glycosides (Zou et al., 2016), and surfactants (Anestopoulos et al., 2020). Hence, the three extracts were continued to the examination using the TLC-bioautography analysis against *S. dysenteriae* bacteria. Meanwhile, the ethyl acetate extract was the only one subjected to the TLC-bioautography assay against *S. mutans*.

TLC-bioautography

TLC analysis of the three extracts resulted in that hexane extract eluted with hexane: ethyl acetate (4:1) showed at least 6 spots. Methanol extract eluted with chloroform: acetone eluent (1:2) showed the presence of 4 spots, and ethyl acetate extract eluted with chloroform: ethyl acetate eluent (2:3) showed 6 spots (Figure 2). Unfortunately, the elution systems could not reproduce the Rf spot with precision, where the elution at different times produces the same number of spots, but with diverse Rf. Each extract was known to contain many compounds with different polarities. It shows that *E. spinosum* is rich in compounds that might likely produce pharmacological activity, especially antibacterial.

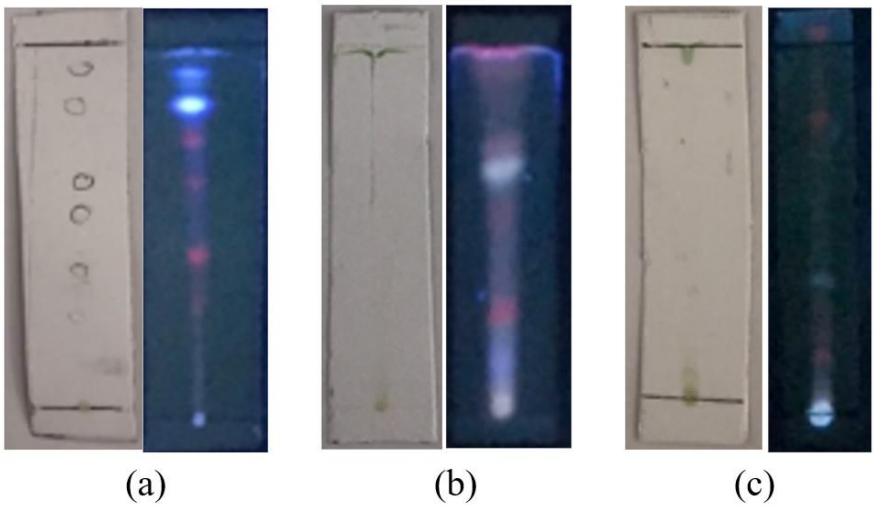


Figure 2. Chromatogram under visible conditions and under UV lamp 366 nm: (a) hexane extract, (b) ethyl acetate extract, and (c) methanol extract

Bioautographic TLC assay against *S. dysenteriae* showed a clear zone given by methanol and ethyl acetate extracts (Figure 3), while hexane extract showed no clear zones. However, in the methanol extract, a clear zone appeared on the spot with an Rf between 0.2 to 0.5. It means that the compound was relatively more polar than the other compounds.

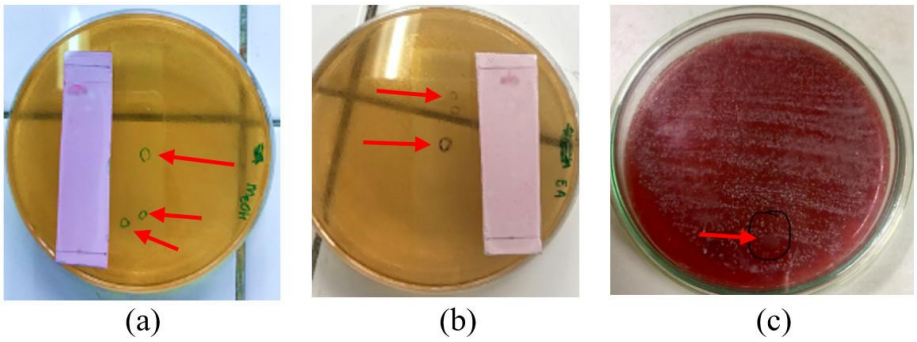


Figure 3. TLC-bioautography results; (a) methanol extract on SSA medium, (b) ethyl acetate extract on SSA medium (response to *S. dysenteriae* growth), (c) ethyl acetate extract in blood agar medium (response to *S. mutans* growth)

These compounds were probably flavonoids glycosides that can act as a surfactant capable of penetrating bacterial cell wall membranes. The spray reagent assay on the spot showed negative results (Figure 4) where none of the compounds reacted with the spray reagents, but the phytochemical screening confirmed the appearance of flavonoids, proving the possibility of flavone glycosides within. As we know, flavone glycosides with sugar at C5 would not react with $AlCl_3$ reagents (Harborne, 1973).

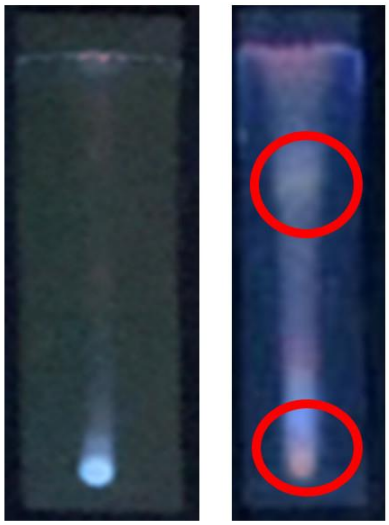


Figure 4. The results of spraying with $AlCl_3$ spots on (a) methanol extract chromatogram below 366 nm UV lamp, and (b) ethyl acetate extract chromatogram below 366 nm UV lamp

As for the ethyl acetate extract, a clear zone appeared on the spot with an Rf between 0.5-0.8. It indicates that the polarity of the compound was semipolar, almost non-polar. The spray reagent assay showed that the spot producing the clear zone reacted with $AlCl_3$ (observed under 366 nm UV lamp lighting). This compound was likely flavonoids of the flavone type, evidenced by its ethyl acetate-soluble nature resulting in a positive outcome in the phytochemical screening and with the $AlCl_3$ reagent (Harborne, 1973). On the other hand, the hexane extract showed no clear zone at all. In this extract, the components of the compound were very non-polar, so it was likely that this compound was unable to penetrate the bacterial cell wall.

The ethyl acetate extract assay against *S. mutans* showed a clear zone at the starting point. It indicates that the compound has a semipolar relative to polar nature. Analysis with $AlCl_3$ showed a positive result in the spot area (Figure 4). The information about the specific active compound needs to be further clarified through the isolation stage to determine which flavonoid groups having an activity to inhibit the growth of both *S. dysenteriae* and *S. mutans*.

CONCLUSION

It is proven that *E. spinosum* extract can inhibit the growths of *S. dysenteriae* and *S. mutans* bacteria, but only *E. spinosum* in ethyl acetate extract could inhibit both bacteria. All of the tested extracts might have the ability to inhibit the growth of *S. dysenteriae* starting from 400 µg/mL, where only ethyl acetate extract could inhibit *S. mutans* growth starting from 20 µg/mL. The class of compounds predicted to have antibacterial activity against the two tested bacteria were flavonoids.

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CONFLICT OF INTEREST

The author declared that there is no conflict of interest in this research. The data could be published by the author completely.

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