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Evaluation of antibacterial activity of mangosteen (*Garcinia mangostana* L.) pericarp extract against rice leaf blight bacteria (*Xanthomonas oryzae* pv. oryzae) at various temperatures and durations of fruit storage

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Abstract. Bioactive contents of mangosteen pericarp can be used as antibacterial agent. This study aimed to examine the antibacterial activity of mangosteen pericarp extract (*Garcinia mangostana* L.) against rice leaf blight bacteria (*Xanthomonas oryzae* pv. oryzae) at different temperature and duration of fruit storage, and to identify the antibacterial bioactive metabolites present in the pericarp extract. The antibacterial experiment was arranged with Randomized Completely Block Design (RCBD) with two factors, namely: storage temperature (0, 13.5, and 27 ºC) and storage duration (1, 7, and 14 days), with 3 replicates. There was significant interaction between storage temperature and storage duration towards the antibacterial activity of mangosteen pericarp extract. The best was showed by the fruits that was kept in temperature of 13,5 ºC for 7 days of storage with inhibition zone of 24.46 mm and the minimum inhibitory concentration (MIC) of 25%. Gas chromatography-mass spectrometry (GC-MS) result showed antibacterial bioactive compounds including (-)-Alpha-Copaene and 2H,6H-Pyran [3,2-B] Xanthen-6-One,5,8-Dihydroxy-9-Methoxy-2,2-Dimethyl-10-(3-Methyl-2-Butenyl)- on the fruits stored in temperature of 0o C and duration of 1 day, and Copaene on the fruits stored during 7 days on temperature of 13,5 ˚C. In conclusion, mangosteen pericarp is a promising source of antibacterial bioactive against Xoo pathogen.

1. Introduction

Mangosteen (*Garcinia mangostana* L.) is a plant species in the Clusiaceae (Guttiferae), known locally as “the queen and the finest of tropical fruits”. Its mature fruit pericarp has been used as a traditional medicine for wound healing, topical infections, and dysentery [1]. Mangosteen pericarp produce secondary metabolites of the xanthone that are useful as antioxidant, antitumor, anti-allergy, anti-inflammatory, antibacterial, and antiviral [1, 2].

Xanthone content acts an antibacterial and gives the potential of the mangosteen pericarp as a natural pesticide to control pathogenic bacteria for plants. These bacteria include *Xanthomonas oryzae* p.v. oryzae (Xoo) which causes leaf blight in rice. Bacterial leaf blight (HDB) is one of the main rice diseases spread in various rice ecosystems in rice-producing countries, including in Indonesia. Pathogen Xoo infects rice plants in all phases of plant growth from the beginning of the nursery until the harvest. Xoo infects rice plants in parts of the leaves through leaf wounds or natural holes in the form of stomata and damages leaf chlorophyll. This causes a decrease in the ability of plants to carry out photosynthesis

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which, if it occurs in young plants, results in death and in generative phase plants, the grain filling becomes less perfect [3]. The HDB attacks in Indonesia cause harvest losses of 21-36% in the rainy season and 18-28% in the dry season [4]. The extent of attacks of HDB in 2006 reached more than 74 thousand ha, 16 ha of which caused harvest abortion [5].

Extraction method is needed to produce ingredients (extracts) containing high bioactive compounds including secondary metabolites which are antibacterial as natural pesticides. In addition to the method of extraction, handling or treatment of fruit before extraction is also a consideration, such as the condition of fruit maturity, age of fruit harvested after blooming, fruit diameter, fruit storage temperature, and duration of fruit storage.

Fruits after harvesting still carry out metabolic processes that cause physical and chemical changes. The physical and chemical changes that can be observed generally consist of changes in color, texture, starch content, protein and organic acids. Changes in chemical composition due to storage not only occur in primary metabolite compounds but also in secondary metabolites such as xanthones and derivatives. Phytochemicals of xanthone derivatives are divided into two main families, namely Clusiaceae, especially prenylated xanthones, and Gentianaceae, especially oxygenated xanthones [6]. Prenylated xanthones, especially α-mangostin, are the compounds most commonly found in the mangosteen pericarp [7, 8].

The temperature and duration of storage of mangosteen fruit is important to be considered in producing mangosteen pericarp extract which has high antibacterial activity. Temperature and storage duration settings in post-harvest handling are needed to maintain product quality and inhibit the rate of metabolic processes so that the chemical content can be maintained, including secondary antibacterial metabolites produced. This study aims to obtain the highest antibacterial activity of mangosteen pericarp extract against bacteria of *X. oryzae* p.v. oryzae (Xoo) causes HDB disease (bacterial leaf blight) in rice with different fruit temperature and duration of storage, and identifies antibacterial compounds from the active extract produced.

2. Material and methods

In vitro study was conducted at the Laboratory of Basic Sciences, Faculty of Agriculture, Siliwangi University, Tasikmalaya. Preparation of mangosteen pericarp extract was performed in Food Technology Laboratory, Faculty of Agriculture, Jenderal Soedirman University, Purwokerto.

2.1. Preparation of mangosteen extract

Fruit of *G. mangostana* were collected from mangosteen plants area belong to farmer in Puspahiang region, Tasikmalaya district, Indonesia in March 2018. After exposing fruits to the treatment of temperature (0, 13.5, and 27 ºC) and duration (1, 7, and 14 days) storage, the pericarp was extracted from mangosteen fruit by drying in shade, and then powdered. The resulting powder was then stored in air tight wide mouth containers. The crude extract of mangosteen pericarp powder was macerated using 95% ethanol placed in an orbital shaker for four consecutive days to remove non-polar substances. The solution obtained was then filtered using a Whatman filter paper. The filtrate was allowed to evaporate in a rotary evaporator with temperature of 55 ºC [9]. The product was stored in *refrigerator* for further use.

2.2. Regeneration and propagation of bacteria

The tested bacteria (the bacteria namely *X. oryzae* p.v. oryzae of high virulent pathotype IV was procured from the culture collection of Indonesian Center for Rice Research, Sukamandi West Java) was propagated in sterilized NA (nutrient agar) medium with oblique position, then incubated at 37º C, overnight. These bacteria from agar media was inoculated aseptically to the flask containing sterilized NB (nutrient broth) medium, and put in the shaker for 24 hours and 37º C. The density of bacteria (number of colonies) in the suspension was counted to 10^6- 10^8 CFU / mL [10].

2.3. In vitro antibacterial test

The evaluation of antibacterial activity was set up in randomized completely block design (RCBD) with two treatment factors of temperature (0, 13.5, and 27 ºC) and duration (1, 7, and 14 days) of mangosteen fruit storage (as source of pericarp extract) with 3 replicates. Amounting 100 µl of bacterial inoculum of *X. oryzae* p.v. oryzae (density was equivalent with number of colonies of 10^6-10^8 CFU/mL) was poured into the vessels/petridish and mixed with 12 ml of NA (Nutrient Agar after melting at 45º C), and allowed to solidified at room temperature. The pericarp extract to be tested was diluted first to reach a concentration of 75% using n-hexane solvent. Paper discs (Whatman No. 1 filter paper, diameter of 0.5 cm) was put on the surface of the agar media, and dropped with 20 µl test extract and allowed until dried before covering the petridish. Antibiotic standard disc (20 µl chloramphenicol 100 mg/L) was also put on the surface of media. The whole of culture vessels was incubated at 37º C for 24 hours. The finding of transparent zone diameter (which was the average from three replicates) around paper discs indicated the inhibition zone of bacterial growth or the presence of antibacterial activities in the pericarp extract and to be compared to antibiotic standard. An analysis of variance was performed to transparent zone diameter data obtained, and significant differences among treatment means were calculated by the Duncan's Multiple Range Test (DMRT) at a probability level of 0.05.

2.4. Identification of MIC

The MIC was determined by a broth dilution method. Mangosteen pericarp extract was dissolved in sodium chloride (NaCl 0.85 %), and subsequent two-fold serial dilutions were performed in the culture medium tube and then poured into petridish. Chloramphenicol was used as a negative control, and was serially diluted in a similar fashion. Medium without extract served as a control for bacterial growth. Each petridish was inoculated with *Xoo* bacteria during the logarithmic phase of growth. The initial density of bacteria was adjusted to 0.5 McFarland turbidity standards (approximately $1x10^8$ colony forming units (CFU)/ml). After 24-hour incubation, MIC was recorded as the lowest concentration that the colony grew ≤ 10 [11].

2.5. GC-MS analysis of extract components

The resulted active extract that have highest antibacterial activity and the control (the extract obtained from mangosteen fruits stored in temperature of 0º C and duration of 1 day) were analyzed by GC-MS (GC-MSD) in order to identify the antibacterial compounds obtained. This extract was evaporated to dryness and reconstituted in to 2 ml hexane, and then subjected to GC-MS analysis. Chromatographic separation was carried out with instrument GC-MS-Hewlet Packard (HP) type 6890 series (GC) and type 5972 (MSD) instrument with Ultra 2 column (17 m×200 µm x 0,11 µm) and pressure 4.29 psi constant flow. The oven temperature was 50 °C; inlet temperature and pressure respectively were 250º C and 3.82 psi with split model. Mass spectra was taken at 70 eV; the detector was MSD. Helium was used as carrier gas with flow 0.5 ml/min and electronic pressure control on. Samples were dissolved in hexane and injected automatically (HP 6890 Injector, volume of 1 µl).

3. Result and discussion

3.1. Pericarp extract

Mangosteen pericarp extraction produced 9 bottles of extract according to combination treatments (temperatures and durations of fruit storage). Each treatment resulted in different extract weight and percentage of yield. Extraction results showed that the mangosteen peel yielded an average yield (*rendemen*) of 16.22%. Previous study showed that the percentage of *rendemen* of mangosteen pericarp extract ranged from 15.50 to 21.00% [12].

3.2. Antibacterial activity

The inhibition zone of mangosteen pericarp extract of each treatment combination was greater than the chloramphenicol control inhibition zone, except for treatment with a storage time of 14 days at 0° C and

27° C (Figure 1). This clearly proves that mangosteen pericarp extract can inhibit the growth of bacteria of *X. oryzae* p.v. oryzae which belongs to Gram-negative. The inhibitory effect of mangosteen pericarp extract is considered better than chloramphenicol antibiotics.

The presence of this inhibitory effect is determined by several active compounds contained in the mangosteen peel extracted with ethanol 95% of which are xanthone, flavonoid, tannin, terpenoids and saponins. Xanthones are chemical compounds with antibacterial benefits that are strong enough and have the ability to slow cell replication in bacteria and also as high antioxidants in mangosteen pericarp [13]. Saponins function as antibacterials by inhibiting the stability of the cell membrane of bacteria, causing bacterial cells to disintegrate. The cell wall will undergo a very strong stretch and then cause damage to cell membranes which in turn leads to the release of various important components (proteins, nucleic acids, and nucleotides) for the survival of bacteria [14].

Terpenoid is a phenol compound that is lipophilic. The mechanism of action of terpenoids is by damaging the cell membrane. Tannin is able to inhibit protein transport enzymes through cell membranes. Flavonoids are the largest group of phenol compounds that have very active properties to slow the growth of viruses, bacteria and fungi [15]. Flavonoids have a tendency to bind proteins, thus disrupting the metabolic process [16].

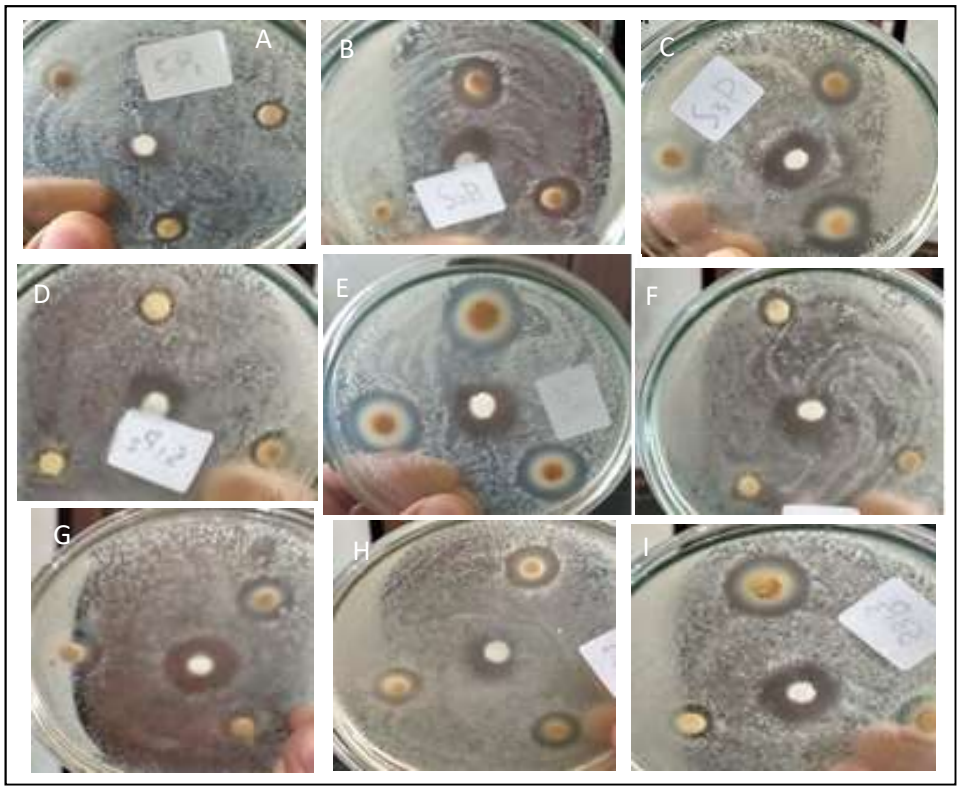


Figure 1. Growth inhibition zone of *Xanthomonas oryzae* p.v. oryzae produced by mangosteen pericarp extract of various temperature and time storage of fruit. The extracts were obtained from fruit storage of temperature and duration respectively: 0 ºC and 1 day (A), 13.5 ºC and 1 day (B), 27 ºC and 1 days (C), 0 ºC and 7 days (D), 13,5 ºC, and 7 days (E), 27 ºC and 7 days (F), 0 ºC and 14 days (G), 13.5 ºC and 14 days (H), and 27 ºC and 14 days (I).

Poeloengan and Praptiwi [10] reported that mangosteen peel extract with the highest concentration of 50% formed a growth inhibition zone in the tested Gram-positive bacteria, but in Gram-negative only a partial inhibition zone occurred. This happens probably due to differences in cell wall composition in Gram-positive and negative bacteria. Single-cell Gram-positive bacterial cell walls with 1-4% lipid

content, whereas in the three-cell cell-negative Gram-negative bacteria consisted of lipoproteins, outer membranes of phospholipids and lipopolysaccharides, and lipid content in cell walls ranged between 11-22%. The outer phospholipid membrane causes antibacterial chemical components that are difficult to penetrate the cell wall of gram-negative bacteria. However, an increase in extract concentration is known to increase the antibacterial activity of the extract. Therefore, in this experiment, a larger extract concentration was used, which was 75%. This proved successful with the large diameter of the inhibition zone formed.

Antibacterial activity of mangosteen peel extract indicated by the inhibition zone is influenced by the interaction between temperature and time storage of mangosteen fruit. The highest antibacterial activity was shown by 7 days storage treatment at 13.5° C with an inhibition zone of 24.46 mm. The combination treatment that had the lowest antibacterial activity was shown by the storage treatment at 27° C for 14 days with an inhibitory zone of 9.44 mm, not significantly different from the storage treatment for 7 days at the same temperature (Table 1).

Table 1. Antibacterial zones of inhibition produced by mangosteen pericarp extract of various temperature and time storage of fruit against *Xanthomonas oryzae* p.v. oryzae

Temperature and Duration of Fruit Storage	Inhibition Zone Diameter (mm)
0 ºC; 1 day	15.06^{c}
13.5 ºC; 1 day	14.67^{c}
27 ºC; 1 day	18.58^{d}
0 ºC; 7 days	12.78^{bc}
13.5 ºC; 7 days	24.46^{e}
27 ºC; 7 days	9.59^{a}
0 ºC; 14 days	12.10^{b}
13.5 ºC; 14 days	13.60^{bc}
27 ºC; 14 days	9.44^{a}

The values followed by same letters are not significantly different according to DMRT Test ($\alpha = 0.05$).

The optimal temperature and duration of fruit storage increased/maintained the quality of the active compounds contained in the pericarp of the fruit. The temperature of 13.5 ºC was categorized as the optimal temperature for fruit storage, in accordance with the results of a previous study proposed by Suyanti and Setyadjit [17] that the optimal temperature for storing mangosteen fruit ranged from 12-14 ºC. The study of Isro'illa [18] proved that the highest saponin levels possessed material stored for 7 days at a temperature treatment of 11 ± 1 ˚C, was higher than the treatment temperature of 29 ± 1 ˚C and 39.5 ± 0.5 ˚C. Roza et al. [19] revealed that the longer storage time, the total phenol content of the mangosteen peel decreases.

3.3. Minimum inhibitory concentration (MIC)

Table 2 shows that the combination of storage treatment at 0 and 13.5 °C for 1 day and at a temperature of 13.5 °C for 14 days has a MIC value of 50%, while the combination of storage treatment at 27 °C for 1 days and at a temperature of 13.5 °C for 7 days had a MIC value of 25%. The other treatment combinations did not show any bacterial inhibitions to the diluted extract, but it was possible that the MIC value of the other treatment combinations was 75%, which was the extract concentration when disc diffusion testing where mangosteen peel extract could still show antibacterial activity.

The MIC value of mangosteen pericarp extract against *Xoo* bacteria is high because the *Xoo* bacteria are Gram-negative bacteria which are composed of 3 layers of cell walls. To be able to penetrate the defenses is needed a high concentration of extract. Unlike the case with Gram-positive bacteria which

have a single-layered cell wall, making it easier to penetrate by antibacterial extract substances even in lower concentrations. This theory is supported by previous study which showed the MIC value of mangosteen pericarp extract on gram-positive bacteria namely *Staphylococcus aureus* and *Staphylococcus epidermidis* only 2% [10]. The magnitude of the MIC value is also influenced by the interaction of each treatment factor, as referred to the results of antibacterial activity tests through disc diffusion method.

Table 2. Minimum inhibitory concentration (MIC)

Conc.of Dilution	Extract According to Fruit Storage Treatment								
	s_1p_1	s_2p_1	s_3p_1	s_1p_2	s_2p_2	s_3p_2	s_1p_3	s_2p_3	s_3p_3
-	K-	K-	K-	K-	K-	K-	K-	K-	K-
50%	-	-	-	+	-	+	+	-	+
25%	+	+	-	+	-	+	+	+	+
12.5%	+	+	+	+	+	+	+	+	+
6.25%	+	+	+	+	+	+	+	+	+
3.13%	+	+	+	+	+	+	+	+	+
1.56%	+	+	+	+	+	+	+	+	+
0.78%	+	+	+	+	+	+	+	+	+
0.39%	+	+	+	+	+	+	+	+	+
+	K+	K+	K+	K+	K+	K+	K+	K+	K+

K- : negative control (Chloramphenicol)
\K+ : positive control (*aquadest*)
\ (-) : test media with growing bacterial colonies of ≤10
(+) : test media with growing bacterial colonies of >10
Temperature and duration of fruit storage: 0 ˚C and 1 day (s_1p_1), 13.5 ˚C and 1 day (s_2p_1), 27 ˚C and 1 days (s_3p_1), 0 ˚C and 7 days (s_1p_2), 13,5˚C, and 7 days (s_2p_2), 27 ˚C and 7 days (s_3p_2), 0 ˚C and 14 days (s_1p_3), 13.5 ˚C and 14 days (s_2p_3), and 27 ˚C and 14 days (s_3p_3).

3.4. Identification of antibacterial compounds using GC-MS

Identification of metabolites with GC-MS on the pericarp active extract of the highest antibacterial activity (from fruit stored at 13.5 ºC for 7 days) and active extract from control treatment (from fruit stored at 0 ºC and for 1 day) found in addition to the same compounds, different compounds were also found. The name and number of types of compounds were thought to be due to the influence of different fruit storage temperatures and duration (Table 3, Figure 2 and Figure 3).

Table 3. Antibacterial compounds identified by GC-MS from active extracts that have the highest antibacterial activity compared to control (fruit stored at 0 ºC for 1 day)

Mangosteen Pericarp Extract	RT	Quality	Antibacterial Compounds	Content (%)
Fruits stored in temperature of 0 ºC and duration of 1 day	13.740	98	(-) - Alpha-copaene	3.69
	38.315	99	Stigmasterol	5.01
	39.336	99	Gamma-Sitosterol	3.51
	42.218	49	2H,6H-Pyran [3,2-B] xanthen-6-one,5,8-dihydroxy-9-methoxy-2,2-dimethyl-10-(3-methyl-2-butenyl)-	7.77
Fruits stored in temperature of 13.5 ºC and duration of 7 days	13.713	99	Copaene	4.20
	38.225	99	Stigmasterol	4.18
	39.218	99	Gamma-Sitosterol	2.20

Alpha copaene is a hydrocarbon sesquiterpene found in the bark of trees producing essential oils of *Kielmeyera coriacea* Mart. & Zucc which shows a high antimicrobial activity against Gram-positive and Gram-negative bacteria. One of them from the oil content released can provide significant antimicrobial activity against the anaerobic bacteria Prevotellanigercens [20]. The γ (gamma)-Sitosterol and Stigmasterol is also metabolite which have significant antibacterial activity and are found in large amounts in parts of *E.odoratum* leaves [21].

The 2H, 6H-pyran [3,2-B] xanthen-6-one-5,8-dihydroxy-9-methoxy-2,2-dimethyl-10- (3-methyl-2-butenyl)- is metabolite secondary xanthones that can be found on mangosteen plants, especially the peel (pericarp) of the fruit. In study of Putra, it was identified that xanthones are a key antimicrobial component whose presence is most abundant in the mangosteen peel studied with percentage of 33.87% [22]. Xanthones consisting tricyclic aromatic rings substituted with various phenolic, methoxy, and isoprene groups. Xanthones have other derivative compounds such as 9-hydroxycalabaxanthone, 3-isomangostin, gartanin, 8-desoxygartanin, γ-mangostin and methoxy-β-mangostin [23].

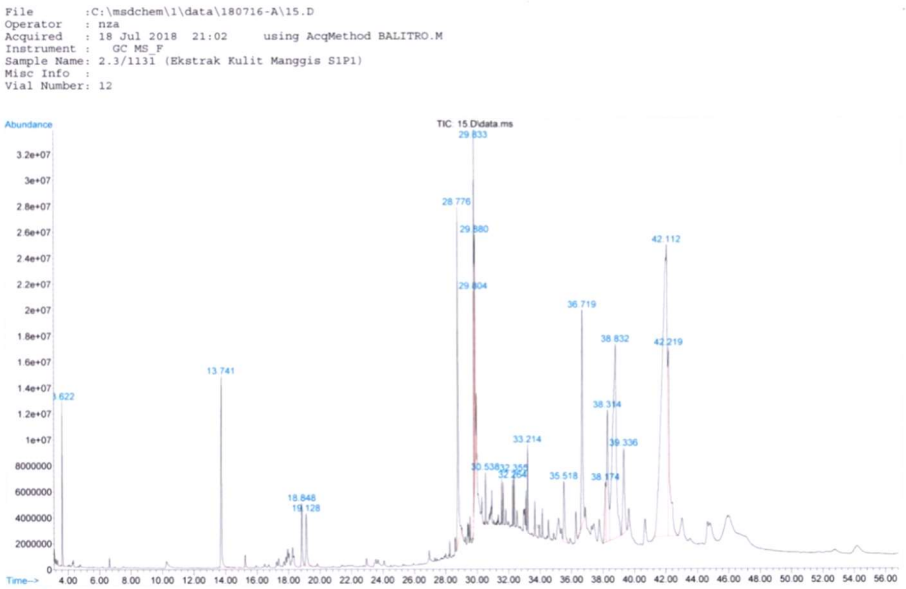


Figure 2. Chromatogram from GC-MS active extract of s2p2 (pericarp from fruit stored at 13.5 ˚C for 7 days) which showed the highest antibacterial activity

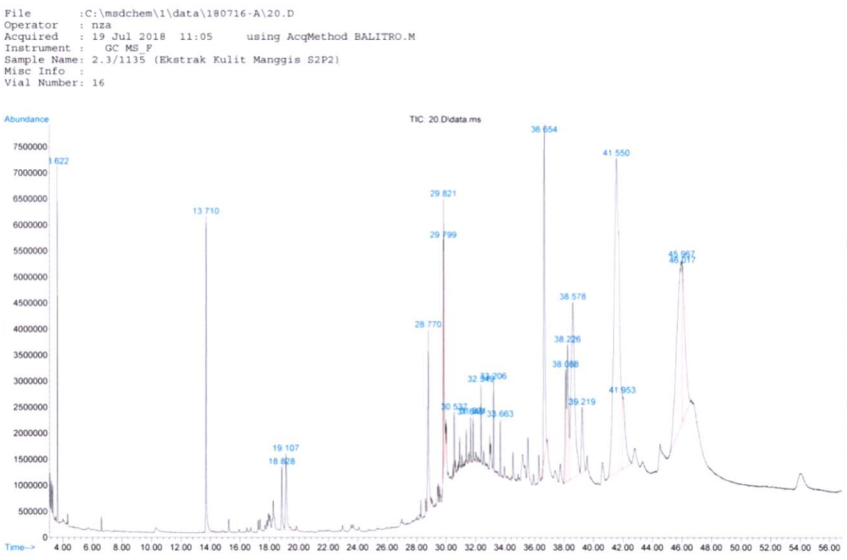


Figure 3. Chromatogram from GC-MS active extract of s2p2 (pericarp from fruit stored at 13.5 ˚C for 7 days) which showed the highest antibacterial activity.

The stability of bioactive compounds in mangosteen fruit pericarp is also influenced by temperature and storage time. The majority of bioactive compounds in mangosteen peel are classified as phenolic and flavonoid compounds, including xanthones and their derivatives, anthocyanin pigments and others. Total phenol content of mangosteen peel without being stored had a concentration of 195.51 mg/g, much higher than the mangosteen peel stored for 10 days (81.17 mg / g) and 20 days (71.02 mg/g) [19]. The longer storage decreased the phenol content.

Compound of 2H, 6H-pyran [3,2-B] xanthen-6-one-5,8-dihydroxy-9-methoxy-2,2-dimethyl-10- (3-methyl-2-butenyl) - as well as are α-mangostin and the other xanthones, their stability is also influenced by temperature and storage time. Narulita has tested the stability of α-mangostin at a temperature of 45 ± 5 ºC and humidity of 75 ± 5% for 21 days [24]. The test results showed that the levels of α-mangostin decreased by 31.11% and significantly affected ($p \leq 0.05$) [24]. According to Anindya, xanthones contained in mangosteen fruit pericarp are bioflavonoids [23]. The mechanism of bioflavonoids as antibacterial is by poisoning the protoplasm, damaging and penetrating the cell wall and precipitating bacterial cell proteins even at very low concentrations.

4. Conclusion

A significant interaction was found between temperature and storage duration of fruit on the antibacterial activity of mangosteen pericarp extract (*Garcinia mangostana* L.). Mangosteen fruit stored for 7 days at a temperature of 13.5 ºC showed the best antibacterial activity with an inhibition zone diameter of 24.46 mm and a minimum inhibitory concentration (MIC) value of 25%. The results of Gas chromatography-mass spectrometry (GC-MS) showed the presence of antibacterial compounds including (-) - alpha - copaene and 2H, 6H - pyran [3,2 - B] xanthen - 6 - one, 5,8 - dihydroxy - 9 - methoxy - 2,2 - dimethyl -10 - (3 - methyl - 2 - butenyl) -, stigmasterol dan gamma-sitosterol in the mangosteen pericarp extract the fruit is stored at 0 ºC for 1 day, and copaene, stigmasterol dan gamma-sitosterol in the mangosteen pericarp extract whose fruit is stored for 7 days at temperature 13.5 ºC. To find out which component of the compound is the most dominant in giving antibacterial effect, further investigation is needed.

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