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Effect of Adding Different Active Substances on Inhibition Zone Diameter and Microstructural Properties of Composite Bioplastics

Nelsy Dian Permatasari[1*], Jatmiko Eko Witoyo[2], Donor Utomo M. Susilo[3], Ayu Rahayu Saraswati[2], Masruri[4], Sudarminto Setyo Yuwono[5], Simon Bambang Widjanarko[5,6]

[1]Department of Food Technology, Politeknik Tonggak Equator Pontianak, Fatimah Street No. 1-2, Pontianak 78111, West Kalimantan, Indonesia
[2]Department of Agroindustrial Technology, Faculty of Industrial Technology Institut Teknologi Sumatera Lampung Selatan,Terusan Ryacudu Street, Way Hui, South Lampung 35365, Indonesia
[3]Department of Agricultural Technology, Pontianak State Polytechnic Pontianak, Jenderal Ahmad Yani Street, Pontianak 78124, West Kalimantan, Indonesia
[4]Department of Chemistry, Faculty of Mathematics and Natural Science Universitas Brawijaya Malang, Veteran Street, Malang 65145, East Java Indonesia
[5]Department of Food Science and Biotechnology, Faculty of Agricultural Technology Universitas Brawijaya Malang, Veteran Street, Malang 65145, East Java Indonesia
[6]Porang Research Center, University of Brawijaya Malang, Veteran Street, Malang 65145, East Java, Indonesia
[*]Corresponding author: nelsypolteq@gmail.com

Abstract
This study identifies the phytochemical compounds in ethanolic extracts of Syzygium myrtifolium leaves, applies it to developing composite bioplastics as a natural antibacterial agent, and compares it with composite bioplastics prepared with sodium benzoate, particularly regarding inhibition zone diameter and microstructure. The results showed that the phytochemicals were identified in the ethanolic extract of Syzygium myrtifolium leaves, like flavonoids, alkaloids, tannins, phenolics, terpenoids, and saponins. LC-MS confirming bioactive in it as auraptenol, calopiptin, quercetin-3-O-β-D-glucuronide, and quercetin-3-O-L-arabinopyranoside. Moreover, in vitro tests showed that composite bioplastics with the ethanolic extracts of Syzygium myrtifolium had inhibition zone diameter against E. coli, similar to those with sodium benzoate added. Additionally, the microstructure of the composite bioplastics with the ethanolic extracts of Syzygium myrtifolium was rougher, irregular, and more porous than those of another. It indicated that the ethanolic extract of Syzygium myrtifolium leaf could be used as a natural antibacterial agent to replace the chemical agent.

Keywords: Composite bioplastics; Inhibition diameter zone; Microstructure; Phytochemical; *Syzygium myrtifolium* Extract.

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INTRODUCTION

The world is currently dealing with severe problems and difficulties because of the large amount of synthetic plastic waste, which amounts to over 400 tonnes annually worldwide and degrades slowly. This trash can indirectly pollute humans because of its poor environmental quality (Fahim *et al.,* 2021). In Indonesia alone, plastic contributes significantly to the total value of the waste, at 18.90% (Artha *et al.,* 2023). To solve this problem, an alternative that can be done is the development of environmentally friendly bioplastics based on local Indonesian natural materials. Durian seed flour (DSF) is one of the Indonesian local natural resources that have a complete composition, including starch, fat, protein, and gum, which can transform into bioplastics (Permatasari *et al.,* 2021; Retnowati *et al.,* 2015). However, the utilization of DSF alone cannot form bioplastics. Commonly, the DSF was combined with other materials to form composite bioplastics, such as starch (Retnowati *et al.,* 2015), or polysaccharides such as glucomannan (GM), from various sources, like konjac (Strnad *et al.,* 2019), sahlep (Fahrullah *et al.,* 2020; Fahrullah and Ervandi, 2022; Kurt and Kahyaoglu, 2014, 2017), or porang (Witoyo *et al.,* 2022; Yanuriati *et al.,* 2017). Furthermore, to improve the functionality of composite bioplastics, it is common to incorporate natural extracts, such as flower extract (Avitabile *et al.,* 2024), leaf extract (Murugan *et al.,* 2024), etc.

One of the part plants in Indonesia that can be used as raw material to produce natural extracts for application in composite bioplastics is *Syzygium myrtifolium* leaf, which is widely used as an ornamental plant. Several studies reported the *Syzygium myrtifolium* leaf and its extract contained bioactive compounds like phenolics, tannin, flavonoids, steroids, terpenoids, alkaloids, saponin, and anthraquinone (Jena *et al.,* 2021; Nursyafni *et al.,* 2023; Wahyu *et al.,* 2021). Moreover, in vitro studies reported by Ahmad et al. (2022), who exhibited that the extract of *Syzygium myrtifolium* leaf possessed antioxidant, antibacterial and antiviral properties. Furthermore, another option to enhance the functional activity of bioplastics, such as their anti-bacterial properties, is to use chemical agents widely used in food products as preservatives, such as sodium benzoate. Chen and Zhong (2018) reported that incorporating sodium benzoate in food can inhibit the growth of the bacteria. In our previous studies, combining the ethanolic extract of *Syzygium myrtifolium* leaf into composite bioplastics made from DSF-porang flour exhibited antibacterial properties (Permatasari *et al.,* 2022a). However, the specific bioactive compounds responsible for the antibacterial effects of the ethanolic extract of *Syzygium myrtifolium* leaf have not yet been definitively identified. Additionally, there is limited research comparing the effectiveness of natural antibacterial agents, such as the ethanolic extract of *Syzygium myrtifolium* leaf, with chemical antibacterial agents, like sodium benzoate, in inhibiting bacterial growth. Additionally, there is limited research comparing the effectiveness of natural antibacterial agents, such as the ethanolic extract of *Syzygium myrtifolium* leaf, with chemical antibacterial agents, like sodium benzoate, in inhibiting bacterial growth. So, this research offers a significant innovation in developing sustainable bioplastics. Identifying the bioactive component in *Syzygium myrtifolium* leaf extract using LC-MS/MS techniques and observing an inhibition zone diameter compared to sodium benzoate, have proven the potency of natural extracts in inhibiting bacterial growth. This approach not only reduces the risk of chemical contamination in the final product but also opens up opportunities for developing bioplastics with broader antimicrobial properties. Furthermore, microstructural analysis is also essential for understanding the fundamental relationships between the internal structure of bioplastics and their macroscopic properties. The microstructure affects the permeability of gases and liquids through the bioplastic (Attallah *et al.,* 2021).

MATERIALS AND METHODS

Material

Durian seed flour obtained from our previous research, with durian seeds collected from West Kalimantan (Permatasari *et al.,* 2022b), porang flour was obtained from the Pilot Plant Laboratory, Faculty of Agricultural Technology, Universitas Brawijaya, and continued with purification using 50% ethanol in 3 cycles (Witoyo *et al.,* 2022). *Syzygium myrtifolium* was obtained from the Materia Media Laboratory, Batu City, East Java. Bacteria *E. coli* and agar medium were purchased from the Laboratory of Food Microbiology and Agricultural Products, Department of Food Science and Biotechnology, Faculty of Agricultural Technology, Universitas Brawijaya. *E. coli* was selected as the test microorganism because it is one of the many pathogens contaminating food that can lead to food diseases. The chemicals within the pro-analysis specifications were obtained from Merck, Germany, through the Food Chemistry and Biochemistry Laboratory, Department of Food Science and Biotechnology, Faculty of Agricultural Technology, Universitas Brawijaya, and distilled water was obtained from a local chemical store in Malang City.

Tool

Hotplate stirrer (MS-H-Pro), stirring rod, thermometer, beaker glass (Pyrex brand), spatula (Pyrex brand), blender (Philips Brand, Indonesia), rotary evaporator (IKA HB-RB10 digital), petri dish, incubator, local tray dryer, and desiccator.

Research Stages

Preparation of Ethanoic Extract of *Syzygium myrtifolium* Leaves

The process of making ethanolic extract of *Syzygium myrtifolium* leaf refers to the method described by Ahmad *et al.* (2022). The leaves of the *Syzygium myrtifolium* are sorted and dried at a temperature of 40°C for 2 days, reduced in size using a blender, and sifted until they pass a 120 mesh sieve. *Syzygium myrtifolium* leaf powder is macerated using 96% ethanol with a ratio of 1:10 b/v at room temperature for 24 hours. The maceration results are filtered and evaporated at a temperature of 40°C using a rotary evaporator until a concentrated extract is obtained. The concentrated extract is ready for qualitative analysis, LC-MS testing, and applications in composite bioplastics.

Manufacturing of Composite Bioplastics with the Addition of Different Active Substances

The manufacture of composite bioplastics is based on the method described by Kurt (2019) with some modifications. First, 0.5 grams of durian seed flour is dissolved in 100 ml of distilled water. This mixture is stirred until homogeneous using a magnetic stirrer at 100 rpm and then heated to 75 °C. Once the temperature reaches 75 °C, the following ingredients are added to the durian seed flour solution, such as 5% v/v glycerol, 1% b/v porang flour, and either an ethanolic extract of *Syzygium myrtifolium* leaves or 25% b/v sodium benzoate. The composite mixture solution is then reheated to 81 °C and maintained at that temperature for 15 minutes while stirring at 500 rpm. Next, the composite bioplastic solution is poured into a petri dish, achieving a thickness of 2 mm. The dish is then placed in an oven at 50 °C and dried until the weight is constant, typically around 48 hours, and the composite bioplastic is ready for further testing.

Analysis Procedure

Qualitative Analysis of Ethanolic Extract of *Syzygium myrtifolium* Leaves

The qualitative analysis of ethanolic extract of *Syzygium myrtifolium* chemically refers to the method described by Nursyafni *et al.* (2023).

Qualitative Analysis of Ethanolic Extract of *Syzygium myrtifolium* Leaves Using LC-MS/MS

The viscous ethanolic extract of *Syzygium myrtifolium* leaves was analyzed for bioactive compounds using the LC-MS/MS (Liquid Chromatography Tandem Mass Spectrometry) method on the Xevo G2-XS QTOF (Waters) in the Chemistry laboratory at the National Research and Innovation Agency in Serpong. The operating mode used was TOF MSE, equipped with an electrospray ionization (ESI) source in positive ion mode. The mobile phase for this analysis consisted of 0.1% formic acid (FA) in water and a mixture of methanol and water at a ratio of 1:9, with a total flow rate of 0.6 mL/min. Before injection, 0.5 grams of the viscous ethanolic extract of *Syzygium myrtifolium* leaves was dissolved in 10 mL of methanol, homogenized, and then filtered using a 0.22 µm GHP/PTFE membrane filter, as suggested by the Sudradjat and Rahayu (2022) method. After this, a sample volume of 10 µL was injected into LC-MS/MS for analysis. The UNIFI software was utilized to identify the bioactive compounds by comparing the sample readings with the mass spectrum in the UNIFI library.

Testing of Inhibition Zone Diameter of Composite Bioplastic

The composite bioplastic inhibition zone diameter testing procedure refers to the Wang *et al.* (2019) method. The bacterial suspension was cultured at a particular concentration on the agar medium. Bioplastics cut to a specific size and sterilized using UV light were placed on the plate agar and incubated at 37°C for 12 hours. After 12 hours of incubation, the diameter of the composite bioplastic inhibition zone was measured. The test was performed three times to determine the composite bioplastic's inhibition zone diameter.

Testing of Microstructure of Composite Bioplastics

The microstructure testing procedure for composite bioplastics involves cutting the material into 0.5 x 0.5 cm pieces and placing them on a carbon-plated and gold-plate coated before observation. Then, the composite bioplastics were examined using a Scanning Electron Microscope (SEM) (Instruction Manual: FEI type Inspect S50) at a magnification of 500x.

Data Analysis

The inhibition zone diameter data for composite bioplastics with varying active compounds were analyzed using a paired t-test in Minitab 17, applying a 95% confidence level. Qualitative data, including phytochemicals, LC-MS of ethanolic extract from *Syzygium myrtifolium* leaf, and the microstructures of the bioplastics, were analyzed descriptively by comparing them with information from previous literature.

RESULTS AND DISCUSSION

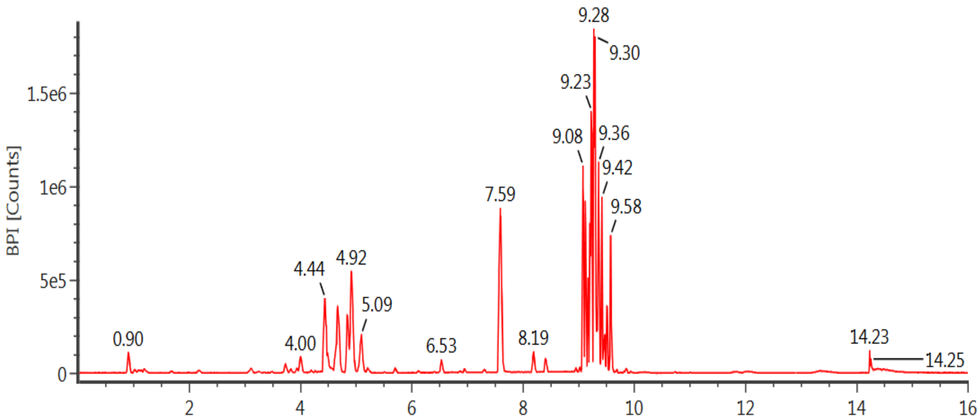
1. Qualitative Analysis of Ethanol Extract of *Syzygium myrtifolium* Leaves

The ethanolic extract of *Syzygium myrtifolium* leaf contains several important phytochemical compounds, including flavonoids, alkaloids, tannins, phenolics, triterpenoids, and saponins, as indicated by the qualitative tests shown in **Table 1**. These findings align with previous research by Wahyu *et al.* (2021) and Nursyafni *et al.* (2023). Wahyu *et al.* (2021) reported that the ethanolic extract of *Syzygium myrtifolium* contains flavonoids, tannins, and steroids, although it did not detect alkaloids, triterpenoids, or saponins. Additionally, Nursyafni *et al.* (2023) also successfully qualitatively identified bioactive compounds in the ethanolic extract of *Syzygium myrtifolium*, including flavonoids, alkaloids, tannins, steroids, terpenoids, and saponins.

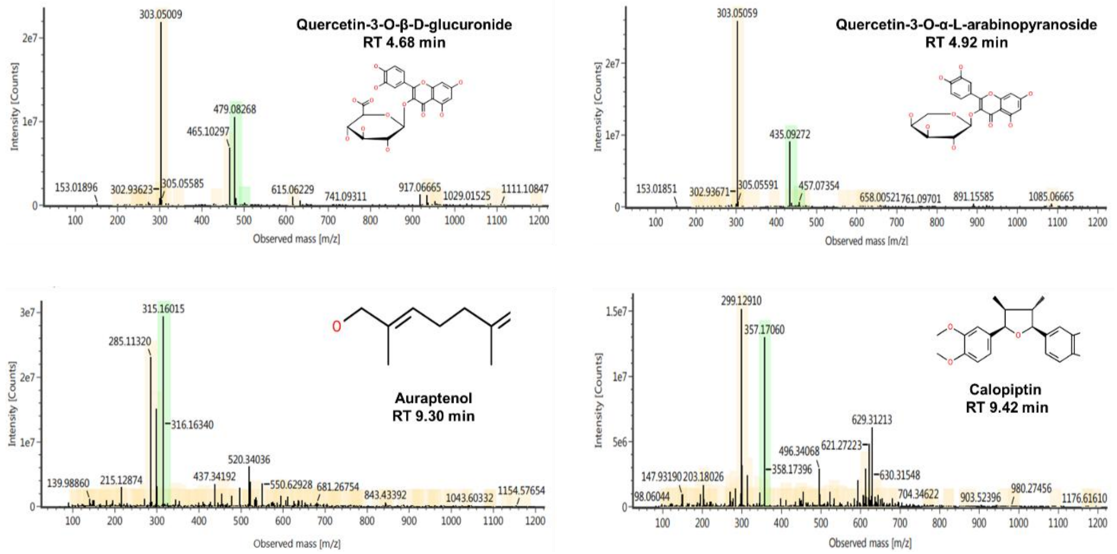
Table 1. Qualitative Analysis of Ethanolic Extract of *Syzygium myrtifolium* Leaf

Compound Identification	Result	Literature
Flavonoid	+	+*,**
Alkaloid		-*
Meyer	+	+**
Dragendrof	+	+**
Bouchardat	+	+**
Tanin/Phenol	+	+*,**
Terpenoid		
Steroid	-	+*,**
Triterpenoid	+	-* +**
Saponin	+	-* +*

Description: + (present) and – (absent)
Source:* (Wahyu *et al.,* 2021), ** (Nursyafni *et al.,* 2023)



(A)



(B)

Figure 1. LC-MS Profile of Ethanolic Extract *Syzygium myrtifolium* Leaf (A) and Tentative Compounds Successfully Identified in Ethanolic Extract *Syzygium myrtifolium* Leaf (B)

The ethanolic extract of *Syzygium myrtifolium* leaves revealed four tentative compounds, including quercetin-3-O-β-D-glucuronide, quercetin-3-O-L-arabinopyranoside, auraptenol, and colopiptin. Each compound is identified by its retention time and m/z, as illustrated in **Figure 1**. Specifically, quercetin-3-O-β-D-glucuronide was detected at a retention time of 4.68 minutes with m/z of 469.08, while quercetin-3-O-L-arabinopyranoside appeared at a retention time of 4.92 minutes with m/z of 435.09. Quercetin-3-O-β-D-glucuronide (QG) is one of the quercetin flavonols that is found in numerous herbal plants (Yin et al., 2019), which have various bioactivities, such as anti-viral, anti-inflammation, anti-melanogenesis, moisturizing, antioxidant, and anti-bacterial (Ha *et al.,* 2022; Mehrbod *et al.,* 2021; Yu *et al.,* 2023). Furthermore, quercetin and conjugated quercetin have antibacterial and antioxidant properties (Elansary *et al.,* 2020; Jaisinghani, 2017; Sun *et al.,* 2021). Moreover, auraptenol was identified at a retention time of 9.30 minutes with m/z of 315.16, while calopiptin was noted at 9.42 minutes with an m/z of 357.17. Auraptenol is a compound that belongs to the coumarin group of organic substances (Patel, 2024), with numerous pharmacological activities have been recorded, such as antidepressants, antitumors, and anti-bacterial effects (Gu *et al.,* 2014; Liu *et al.,* 2020; Tan *et al.,* 2017).

2. Inhibition Zone Diameter of Composite Bioplastic

The inhibition zone diameter of the composite bioplastic with the addition of different active compounds is shown in **Table 2**. The results of the test using the t-pair test showed that the addition of ethanolic extract *Syzygium myrtifolium* (BPE) and sodium benzoate (BPB) had no significant effect ($p > 0.05$) on the inhibition zone diameter of the composite bioplastic. These results indicate that ethanolic extract of *Syzygium myrtifolium* leaves can be used as an alternative to replace sodium benzoate as a natural antibacterial agent that can inhibit bacteria *E. coli* on bioplastics composites. The inhibition mechanism of *E. coli* is caused by bioactive compounds in the ethanolic extract of *Syzygium myrtifolium* leaf (**Table 1** and **Figure 1**), which inhibit the formation of proteins in synthesizing bacterial cell walls by acting as ligands. Inhibition by bioactive compounds results in the performance of alanine racemase (Alr) and enolpyruvate transferase (EPT), which has the function of synthesizing bacterial cell walls is inhibited, resulting in the non-formation of D-ala so that the cell wall is not formed completely. The absence of D-ala has implications for inhibiting the glycoprotein formation process, so no polypeptides are formed that can protect the bacterial cell wall of *E. coli*, thus causing the lysis of the walls of bacteria *E. coli,* which has implications for decreased performance or inactivation of bacteria *E. coli* (X. Chen *et al.,* 2019; Mezoughi *et al.,* 2021). Studies in *silico* conducted by Permatasari et al. (2022c) exhibited that bioactive compounds, particularly flavonoids, exhibited inhibition in the catalytic regions of alanine racemase (Alr), specifically at the amino acid residue denoted as TYR255. In the case of transglycosylase, inhibition was observed in the active site, particularly at the residues identified as GLU478. Nguyen and Bhattacharya (2022) also reported that quercetin possesses the property to significantly disrupt the bacterial cell membrane integrity, thereby inhibiting bacterial growth, including *E. coli*. In another study Wang *et al.* (2018) also claimed that the inhibition mechanism of quercetin and their conjugates damage the structure of bacterial cell walls and cell membranes of *E. coli*, leading to an increase in the permeability of their structure. This condition also leads to the endochylema content releasing and affects ATP activity, thus protein synthesis of *E. coli* bacteria decreases, thereby affecting protein expression in the cell, and eventually, the cell lysed and experiences death.

Table 2. Inhibition Zone Diameter of Composite Bioplastic with Addition of Different Active Compounds

Types of Bioplastics	Inhibition Zone Diameter (mm)
Bioplastics with the addition of ethanolic extract *Syzygium myrtifolium* (BPE)	15.33±0.45[a,*]
Bioplastics with sodium benzoate (BPB)	16.15±0.28[a]
P-value	0.188

Description: The data is the average of 3 replications ± standard deviation. Different letters in the same column indicate a noticeable difference ($p < 0.05$) based on paired t-tests.
Source:* (Permatasari *et al.,* 2022a)

3. Microstructure of Composite Bioplastics

The appearance of composite bioplastic microstructures with the addition of various active substances is illustrated in **Figure 2**. In **Figure 2 A1**, the surface of the bioplastic material (BPE) is rough and irregular, with numerous granules present. These granules consist of starch from durian seed flour and porang flour that have yet to be fully homogenized or gelatinized during bioplastic manufacturing. This phenomenon may be attributed to uneven distribution during homogenization, which prevented complete gelatinization during heating. As a result, the interaction between the starch components in durian seed flour and the elements in porang flour, particularly glucomannan, did not occur optimally. The result of this study was in line with the research reported by Fahrullah and Ervandi (2022), who stated that the imperfect homogenization process in the solution whey with KGM during film production causes aggregation in the microstructure of the film whey-KGM. In **Figure 2 A2**, a cross-section of the BPE has an irregular surface structure, and several cavities are observed. The many interactions of the constituent polymers in the BPE matrix, such as cellulose, fat, protein, and starch, cause irregular BPE (Retnowati *et al.,* 2015). Moreover, the composite bioplastics with more cavities and porous microstructures tend to have low tensile strength and elongation, thereby it easy to break (Fahrullah and Ervandi, 2022; Wang *et al.,* 2010). Dias *et al.* (2010) added that the homogeneity of the film matrix is positively correlated with increased polymer integrity and improved mechanical properties of the film.

Figure 2 B1 illustrates that BPB has a smoother surface than BPE (**Figure 2 A1**). BPB is not porous or cracked. However, some durian seed flour starch or porang flour granules have yet to gelatinize fully. Moreover, the cross-section of BPB (**Figure 2 B2**) reveals a more compact surface than BPE (**Figure 2 A2**). This observation indicates that the sodium benzoate added to the bioplastic has been wholly dissolved, resulting in a more homogeneous and smoother surface than that of BPE. According to Cagri *et al.* (2004), sodium benzoate added to the film will still be active when it is finished. This statement is supported by the data in **Table 2**, which demonstrates that adding sodium benzoate to bioplastics can inhibit the growth of *E. coli*, as indicated by an inhibition zone diameter of 16 mm. This result suggests sodium benzoate retains its antibacterial properties even after bioplastic production. Furthermore, this evidence implies that the bioplastic made from a mixture of durian seed flour and porang flour effectively preserves the bioactivity of sodium benzoate throughout bioplastic production.

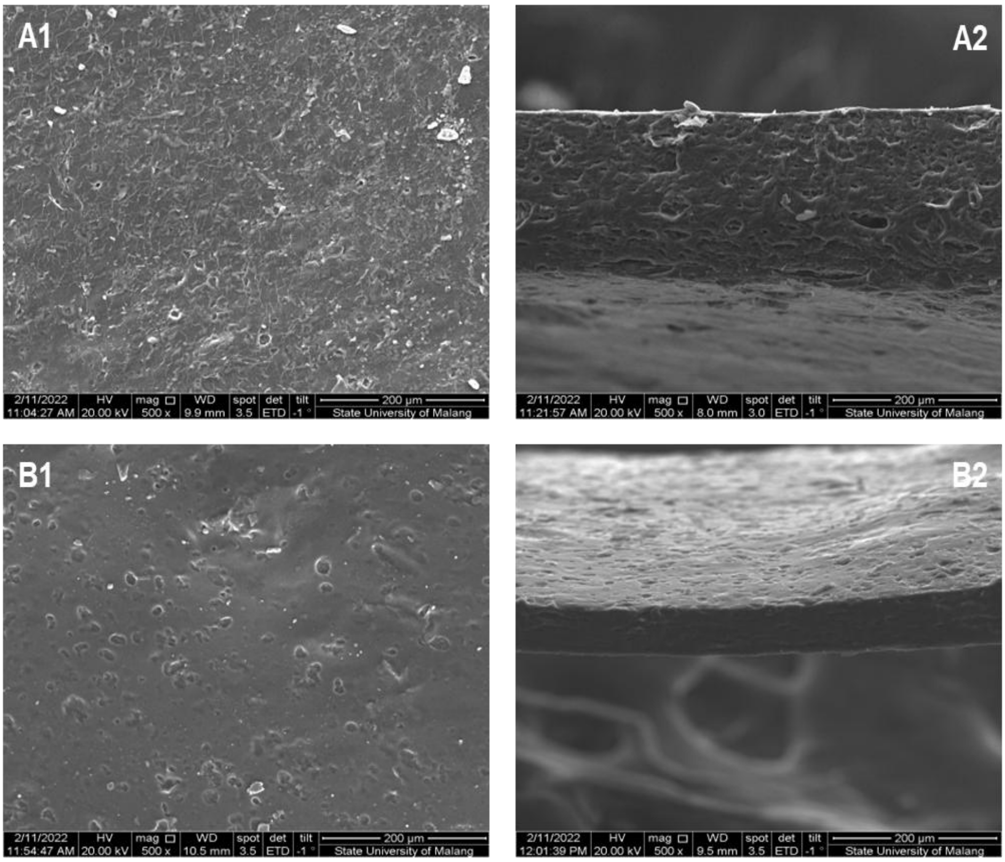


Figure 2. Microstructure of Bioplastics with the Addition of Different Active Compounds. **(A1)** BPE at 500x Magnification, **(A2)** Cross Section of BPE at 500x Magnification (Permatasari *et al.,* 2022a) and **(B1)** BPB at 500x Magnification, and Cross Section of BPB at 500x Magnification

CONCLUSION

The study successfully identified bioactive compounds in the ethanolic extract of *Syzygium myrtifolium* leaf, including flavonoids, alkaloids, tannins, phenolics, triterpenoids, and saponins, through chemical qualitative tests. LC-MS analysis confirmed the presence of four tentative bioactive compounds: auraptenol, calopiptin, quercetin-3-O-β-D-glucuronide, and quercetin-3-O-L-arabinopyranoside, each with different retention times. *In vitro* studies showed that adding an ethanolic extract of *Syzygium myrtifolium* leaf to composite bioplastics exhibited a similar inhibition zone diameter to sodium benzoate when inhibiting *E. coli*. The microstructure of the composite bioplastics containing the ethanolic extract of *Syzygium myrtifolium* leaf appeared rough, irregular, and more porous than those containing chemical agents like sodium benzoate. These results suggest that ethanolic extract of *Syzygium myrtifolium* can be a natural antibacterial agent and potentially substitute sodium benzoate.

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