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Quercetin-Containing Extract from Clove *Syzygium aromaticum* L. Endophytic Bacteria, *Fictibacillus phosphorivorans* P1U2, Exhibits Antimutagenic Activity in Yeast *Saccharomyces cerevisiae*

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ABSTRACT

Metabolites from clove (*Syzygium aromaticum* L.) have been extensively studied for various pharmacological uses, including quercetin. Endophytic bacteria isolated from plant tissues can produce similar secondary metabolites as their host plants. However, little is known about the antioxidant activity of the endophytic bacterial extract from clove leaves. Amongst the potential bacterial groups, the genera of *Bacillus* have been widely studied as a source of antioxidants. Thus, this study, we examined the potential antioxidant properties of *Bacillus* sp., isolated from clove leaves. We have successfully isolated ten endophytic bacteria, of which only one isolate (P1U2) was found non-pathogenic based on a hemolytic assay. Based on the 16S rRNA sequence, P1U2 shared the highest similarity to *Fictibacillus phosphorivorans*. The extract of extracellular metabolites of P1U2 contains quercetin compounds, based on LC-MS analysis. The results of the antioxidant activity test using the DPPH assay showed that the P1U2 extract had an IC_{50} value of 161 mg/ml. The supplementation of the extract on culture media reduced the ability of *S. cerevisiae* to decolorize mutagen malachite green and increased cell viability. Furthermore, the addition of 161 ppm extract was able to counteract UV mutagens effects of UV exposure and toxicity of malachite green, significantly increasing the growth of *S. cerevisiae*. Suggesting the bioactivity of the extract in combating mutagens. To the best of our knowledge, this is the first study to report a quercetin-rich extract derived from endophytic bacteria, *F. phosphorivorans*. Thus, further study is required to optimize the synthesis of quercetin from isolate P1U2.

1. Introduction

Free radicals are highly reactive and unstable molecules produced in the body naturally as a product of normal metabolism (Martemucci *et al.* 2022). Cells permanently have reactive oxygen species (ROS) in low concentrations and can accumulate, causing oxidative stress (Rahal *et al.* 2014). Cells will express three antioxidant protein genes, superoxide dismutase (SOD), catalase, and glutathione peroxidase, to overcome ROS accumulation (Pinto *et al.* 2010). Oxidative stress is reported to cause various harmful effects such as cancer and autoimmune and neurodegenerative diseases; therefore, there must be a balance between free radicals and antioxidants.

One of the most sought-after compounds is quercetin which is believed to protect the body from several types of degenerative diseases by capturing free radicals and trapping transition metal ions (Ferrali *et al.* 1997). Quercetin is one of the best flavonols and is mainly extracted from plants, and quercetin is one of the active substances in the flavonoid class, which is biologically very strong.

One of the medicinal plants widely studied with quercetin which has antioxidant activity, is the clove *Syzygium aromaticum* L.. Based on previous research, clove extract was shown to have antiaging, antioxidant activity, and has activity in modulating apoptosis in yeast *Saccharomyces cerevisiae* (Amini *et al.* 2022; Astuti *et al.* 2019; Fauzya *et al.* 2019). The antiaging and antioxidant activities of clove extract were also studied in the *Schizosaccharomyces pombe*

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in upgrading promotes yeast longevity by inducing intracellular oxidative stress response, mitochondria activity, and cell cycle delay (Lesmana *et al.* 2021). Quercetin is a compound contained in plants, such as clove plant. Based on the polyphenol database (http://www.phenolexplorer.eu) states that clove plants have a high quercetin content compared to apples. The content of quercetin compounds in clove plants ranges from 28.40 mg/100 g dry weight. Endophytic fungi is believed to produce bioactive compounds similar to their hosts. Singh *et al.* (2017) stated that endophytic bacteria that live in plants could produce secondary metabolites similar to their host. Thus, application of endophytic bacteria is a source for obtaining quercetin compounds in a short time, does not require large areas of land for its production and the life cycle of bacteria is shorter compared to plants.

Studies of antioxidant activity and other pharmaceutical activities have been widely reported and dominated by bioactive compounds from different plant extracts and endophytic bacteria. However, until now, there has been no reports about endophytic bacteria from clove leaves that have antioxidant and antimutagenic activities. Hence, in this study we managed to evaluate the antimutagenic activity and antioxidant activities of the metabolites produced by clove leaves-endophytic bacterial extract of clove leaf. Cellular activity of the metabolites as antimutagenic agent was determined in cellular levels by using yeast *Saccharomyces cerevisiae*, as model organism.

2. Materials and Methods

2.1. Isolation of Endophytic Bacteria, Pathogenicity Test, and Morphological Characterization

Clove leaf samples came from the Research Institute for Plants and Medicine, Bogor, with GPS coordinates (6°34'53" S 106°47'22" E). The sample is then subjected to gradual surface sterilization. Clove leaves were washed with running water until clean, clove leaves are taken as much as 2 g, then the leaves are cut into pieces so that the size becomes 5-7 cm. The leaves were soaked in 96% alcohol for 1 minute, 2% NaOCl for 1 minute, and then rinsed with sterile distilled water three times. The sterilized samples were crushed, diluted gradually, and subjected to heat stress at 80°C for 15 minutes to kill non-sporulating bacteria. This procedure is used to distinguish the bacteria *Bacillus* sp. from other bacteria. With the heat treatment, it is hoped that only *Bacillus* sp. bacteria that have spores will live due to exposure to high temperatures. The sample is dripped on a petri dish containing NA media, incubated at 27°C for 72 hours, and until colony growth observed. Endophytic bacteria that grew were tested on blood agar to determine that the bacteria used were not pathogenic and and then the isolates were identified by Gram staining and spore staining, and identified molecularly (Kusumawati *et al.* 2014).

2.2. Identification of Bacteria Based on 16S rRNA Gene

Identification of bacteria was perfomed through DNA extraction method using zymobiomics DNA miniprep kit. The extracted DNA was used as a template to amplify a segment of about 1,500 bp from the 16S rRNA gene sequence using polymerase chain reaction (PCR) with universal primers for bacteria, 63f and 1387r (Marchesi *et al.* 1998), then the PCR results were visualized by electrophoresis on 1% agarose and the amplicons were sequenced through a sequencing service. Nucleotide base sequence results sequences are used to obtain sequence data of relatives through the program BLASTn (http://ncbi.nlm.nih.gov/). Sample sequences, their relatives and selected outgroups were analyzed by creating a phylogenetic tree using MEGA 5.0 software https://www.megasoftware.net.

2.3. The Extraction of Bioactive Compounds

The process of extracting the bioactive compounds and analysis of the percentage yield of the extract was carried out according to Muller *et al.* (2004) with some modifications. One loop of 24-h-old endophytic bacterial isolates was cultivated in 30 ml NB media and incubated using a shaker incubator at 110 rpm for 24 hours at 27°C. One percent of 24-h-old cultures was inoculated into NB media and incubated on a shaker incubator at a speed of 110 rpm for 48 hours at 27°C, after which the cells were separated by centrifugation at 6,000 rpm at 4°C for 15 minutes. The supernatant was mixed with ethyl acetate in a ratio of 1:1 and shaken for 20 minutes, then evaporated using a vacuum evaporator at 40°C. The crude extract obtained was then stored at 4°C for further use.

2.4. The Measurement of Antioxidant Activity using the DPPH Assay

Antioxidant activity test was carried out by means of quantitative use DPPH method (2,2-diphenyl-1-picrylhydrazyl) test. DPPH solution 125 µM (2,5 mg DPPH in 50 ml ethanol) was mixed with a crude bacterial extract diluted in ethanol to make final concentrations of 100, 200, 300, 400, and 500 ppm. The mixture was incubated at room temperature in the dark for 30 minutes, and then absorbance was measured at wavelength 517 nm. Ascorbic acid was set as a standard. The percentage of inhibition was then calculated using a linear regression equation to obtain the 50% Inhibitory Concentration (IC_{50}) (Astuti *et al.* 2021).

2.5. Decolourization Test and UV Toxicity Analysis on Cells

Decolourization malachite green test was carried out using the method (Biradar *et al.* 2017) and UV toxicity analysis on yeast using the technique (Masuma *et al.* 2013) with some modifications. Yeast culture of S. cerevisiae grown in yeast extract peptone dextrose (YPD) media for 24 hours was reacted with malachite green 10 ppm as a mutagenic agent, and added endophytic bacteria extract at various concentrations (80.5, 161, 322, and 644 ppm). Malachite green 10 ppm was added to each medium. All yeast cultures (control and clove extract treatment) were incubated at 110 rpm in an incubator. Samples were centrifuged at 4,000 rpm for 10 minutes. This analysis was carried out by observing turbidity using a spectrophotometer at a wavelength of 620 nm with an interval of 0 hours and 12 hours in duplicate. Yeast treatment without bacterial extract was used as a control, and ascorbic acid 3.82 ppm was used as a standard, then further treatment was given exposure to UV light at a wavelength of 200-280 nm for 15 minutes, the method used was the same as giving malachite green, the difference was only in given mutagenic substance. The yeast culture of *S. cerevisiae* grown in media (YPD) for 24 hours was reacted with extracts of endophytic bacteria at some of the same concentrations as the malachite green treatment, and then exposed to UV-C light for 15 minutes. The samples were then incubated for 12 hours.

2.6. Cell Viability Analysis

S. cerevisiae was analyzed for its viability after exposure to malachite green and UV light. Aliquot of 100 µl liquid culture medium in the decolorization mutagenic effect of malachite green experiment and UV exposure. In terms of In this case, 100 µl of liquid culture medium in the decolorization experiment at 0 and 12 hours was taken and diluted into physiological solution until 10^{-4}. Dilution 10^{-4} taken 100 µl and spread into a petri dish containing yeast extract peptone agar (YPA). The plate was then incubated at room temperature (33±2°C). Colonies growing on the media were counted in duplicate.

2.7. Liquid Chromatography-Mass Spectrometry Analysis

A crude bacterial extract (10 mg) was prepared and diluted in 5 ml of ethanol. the LC-MS analysis was carried out using the Xevo G2-XS QTof Quadrupole Time-of-Flight Mass Spectrometry instrument, using an electrospray ionization with high sensitivity and information on complex compounds. Water added with 0.1% formic acid (A) and acetonitrile added 0.1% formic acid were used as eluents. The sample temperature was 20°C, and the column temperature was 40°C. Unifi software was used to analyze the results.

3. Results

3.1. Bacterial Characteristics and Identification

A total of 10 isolates of endophytic bacteria were isolated from clove leaves (*S. aromaticum* L.). However, only one isolate was non-pathogenic based to the hemolytic assay (Table 1). Based on observations of the morphological characteristics endophytic bacteria, isolate P1U2 has yellow colonies, circular in shape, with bacilli-shaped cells, and the characteristics of the cell wall isolate P1U2 belongs to Gram-positive bacteria, and has spores (Figure 1).

Table 1. Hemolytic activity of endophytic bacteria of clove leaves

Isolate code	Hemolysin production
P1U1	+
P1U2	-
P2U1	+
P2U2	+
P3U1	+
P3U2	+
P4U1	+
P4U2	+
P5U1	+
P5U2	+

(+) = clear zone formed, (-) = no clear zone formed

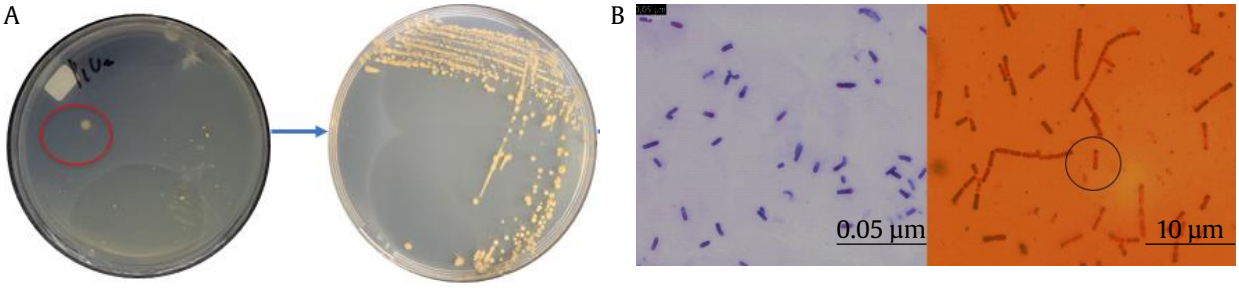

Figure 1. Clove leaf endophytic bacteria *S. aromaticum* (A) colony form of P1U2 isolate on a nutrient agar plate (B) gram staining of P1U2 isolate bacteria (gram-positive bacteria) (c) spores staining of P1U2 isolate (1,000× magnification)

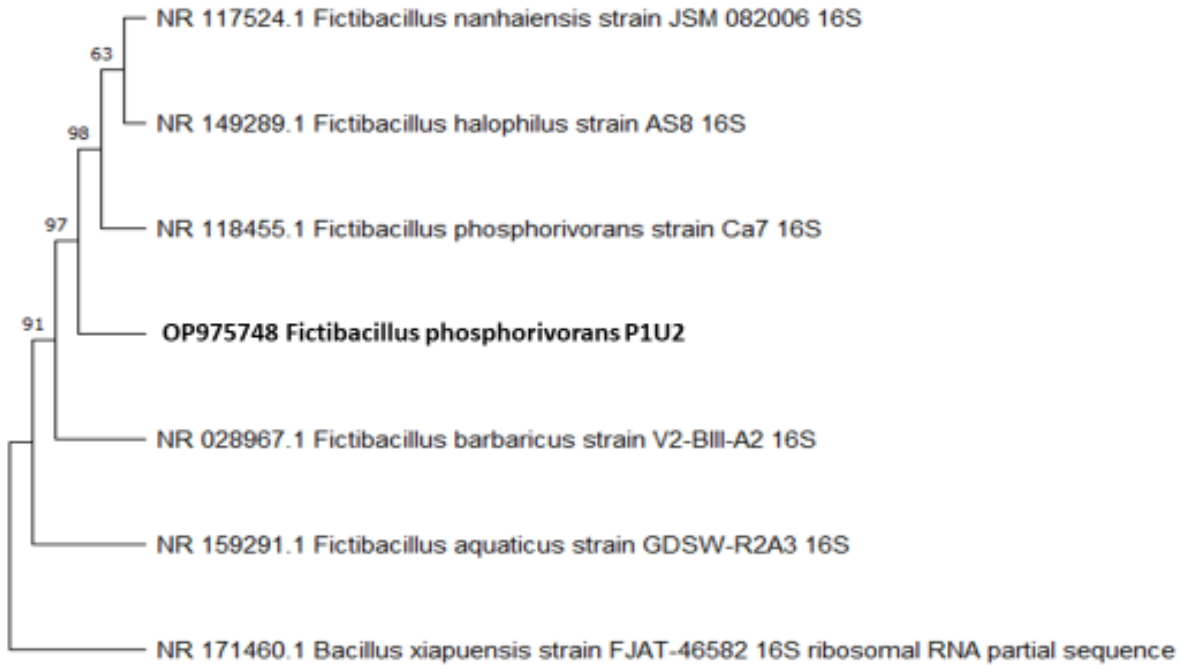

Figure 2. Phylogenetic tree of the P1U2 isolate was constructed using the Neighbor-Joining method with a bootstrap value of 1,000 replications

Bioinformatics analysis grouped isolate P1U2 in the genus Fictibacillus. The BLAST results with the Gene Bank data showed hih similarities of isolate P1U2 with Fictibacillus phosphorivorans with a similarity value of 99.65%. (Figure 2). The 16S rRNA sequence of the P1U2 isolate has been deposited to GenBank with accession number OP975748.

3.2. Extraction of Bacterial Metabolites

A 2.389 g of crude bacterial extract was obtained from 3,000 ml of culture isolate P1U2, with a yield of 0.079% (w/v). The extract is in powdered form and greenish-yellow colour.

3.3. Metabolite Profile of Bacterial Extract

LCMS analysis revealed that five dominant compounds were found in the extract, including quercetin, aeruginosine, linolenic acid, euparin, and benzofuran (Figure 3). The properties of the aeruginosine compound obtained from the LCMS analysis showed that the compound's molecular weight was 607.2 m/z, and the formula was $C_{32}H_{44}N_0O_6$

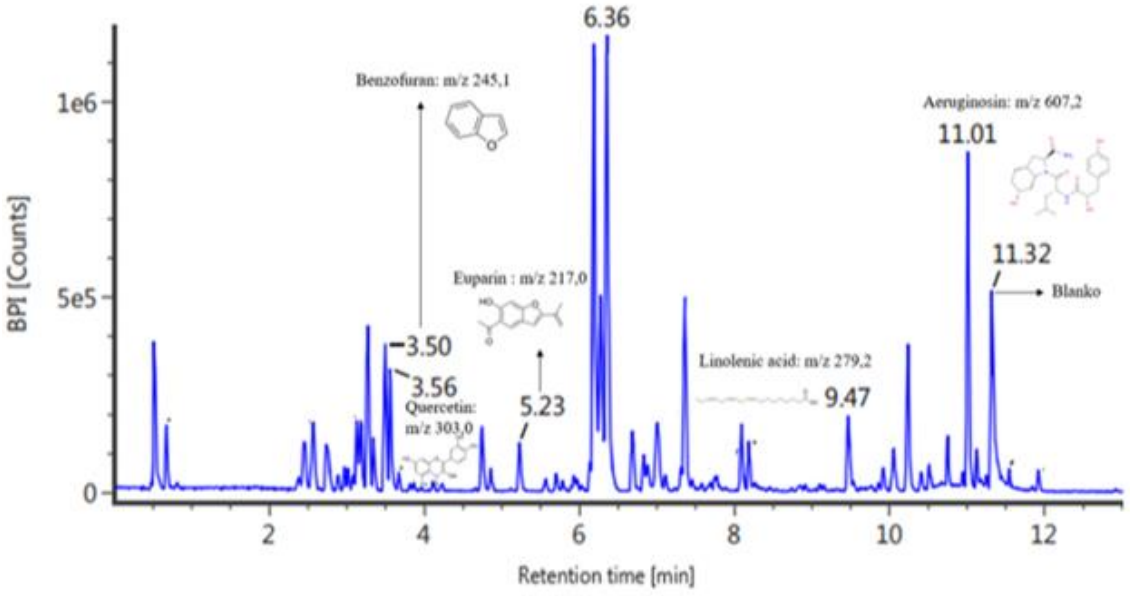

Figure 3. The metabolite profile of P1U2 extract based on LC-MS analysis

with a retention time of 11.01 min. The following compound, quercetin, has a molecular weight of 303.0 m/z, and the formula is $C_{15}H_{10}O_7$, with a retention time of 3.56 min. The linolenic acid compound has a molecular weight of 279.2 m/z, and the formula is $C_{18}H_{32}O_2$, with a retention time of 9.47 min. The compound euparin has a molecular weight of 217.0 m/z, and its formula is $C_{25}H_{22}O_5$, with a retention time of 5.23 min. The compound benzofuran has a molecular weight of 245.1, and the formula is C_8H_6O with a retention time of 3.50 min.

3.4. Antioxidant and Antimutagenic Activity of Bacterial Extract

Based on the IC_{50} value, the crude extract of P1U2 isolate had low antioxidant activity (Table 2). The results obtained from the antioxidant test using the DPPH radical are: IC_{50} value of the extract samples. The IC_{50} value is the activity value of the compound for stabilize or degrade as much as 50% of the radical compounds and compounds the test target, and the smaller the IC_{50} value of a test compound, the compound are increasingly effective as free radical scavengers. The results of the decolorization study showed a decrease in the percentage of decolorization of malachite green by *S. cerevisiae* with the addition of P1U2 extract (Figure 4). The highest and lowest decolorization percentages respectively namely the

Table 2. Antioxidant activity (IC_{50}) of P1U2 extract based on the DPPH test

Sample	IC_{50} value (ppm)
P1U2 extract	161.00
Ascorbic acid	3.82

treatment without the addition of extract control with a value of 100% and addition of 644 ppm extract with a value of 42% It is worth noting that the addition of ascorbic acid had a decolorization value of 90%.

The addition of P1U2 extract in the decolorization process and cell viability effect of the toxic agent malachite green can increase the viability of yeast cells compared to without the addition of the extract (Figure 5). Based on these results, P1U2 extract treatment significantly increased the viability of *S. cerevisiae* cells. The concentration of P1U2 extract of as much as 644 ppm suppressed yeast growth at 12 hours. Since malachite green has been reported to be one of the genotoxic agents, thus this result indicates the potential antimutagenic activity of P1U2 extract.

In addition to malachite green treatment, UV has also been reported to cause DNA mutation. Interestingly, adding P1U2 extract could increase yeast cell viability exposed to UV radiation (Figure 6). Adding P1U2 extract, 80.5 ppm to 161 ppm, could maintain cell viability for up to 12 hours.

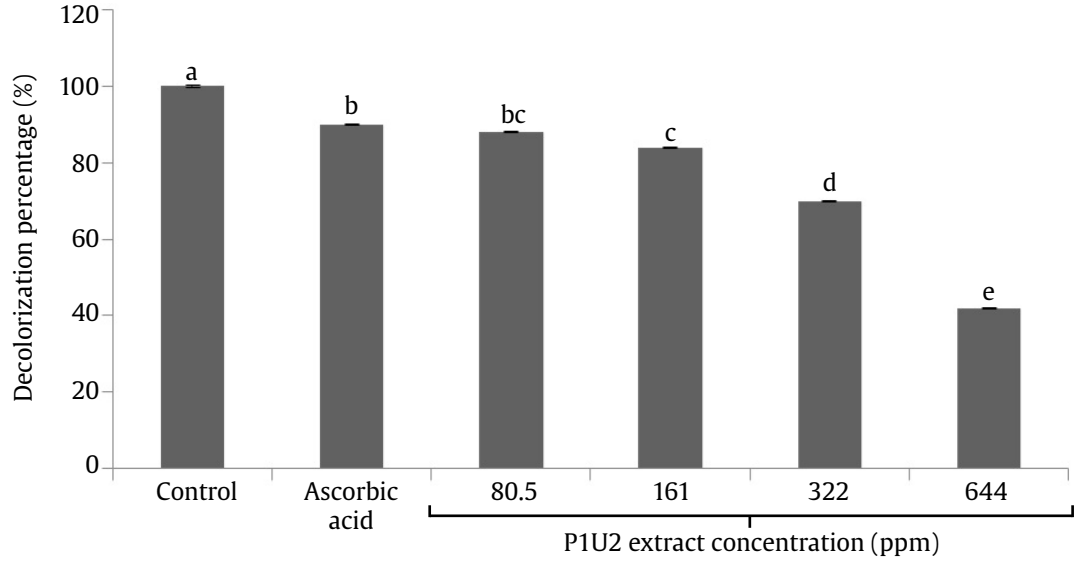

Figure 4. The effect of P1U2 extract on the decolorization percentage of toxic compound malachite green (15 ppm) by *S. cerevisiae* after incubation for 12 hours. Ascorbic acid was designated as positive control (3.82 ppm), and without the addition of extract were used as controls. Statistical analysis were determined by ANOVA

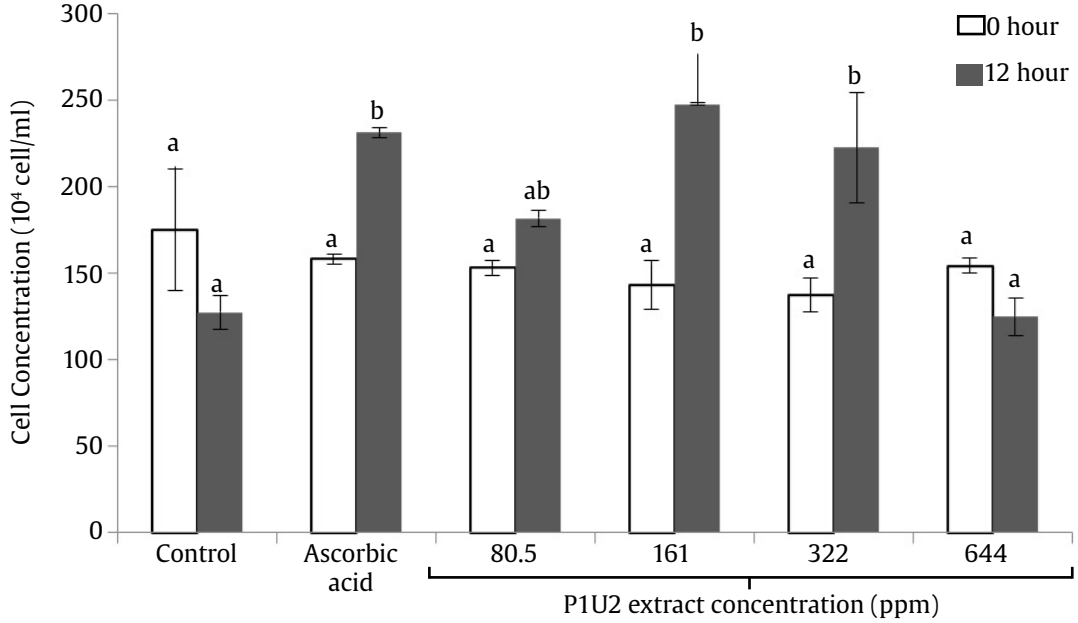

Figure 5. The effect P1U2 extract on the cells viability of yeast *Saccharomyces cerevisiae* which were exposed to mutagen, malachite green Ascorbic acid was designated as positive control (3.82 ppm), and without the addition of extract were used as controls. Differences were determined by ANOVA, followed by the Tukey range test ($p<0.05$)

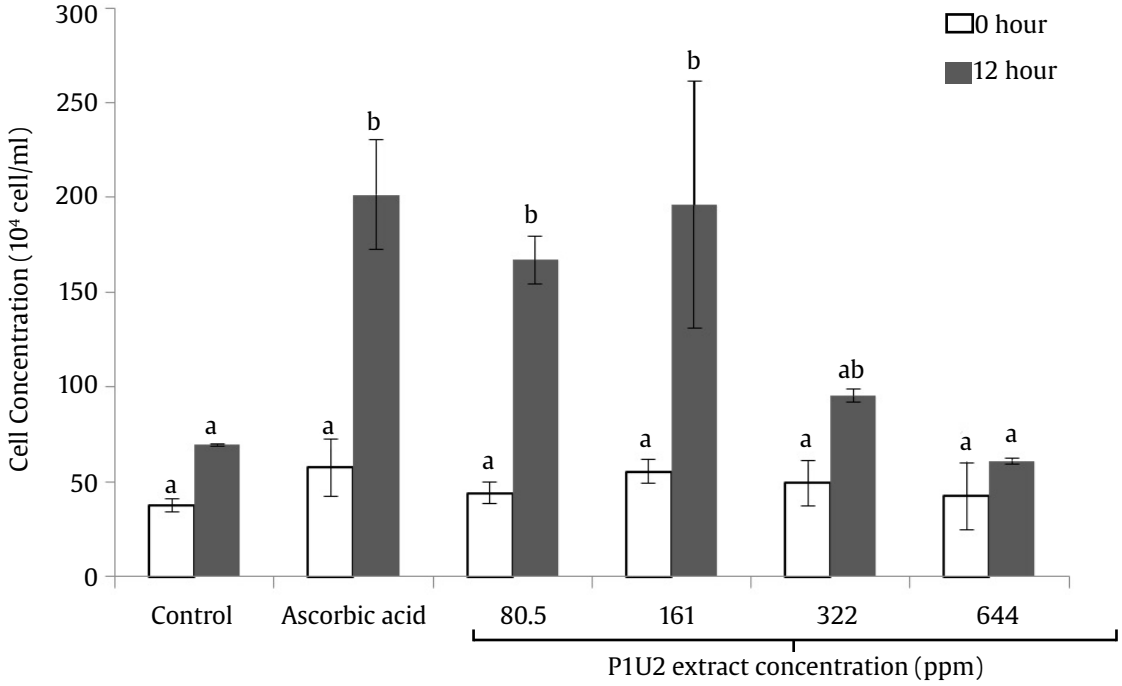

Figure 6. The effect P1U2 extract on the cells viability of yeast *Saccharomyces cerevisiae* which were exposed to mutagen, UV exposure at wavelength 200-280 nm on the growth of *S. cerevisiae* cells with the addition of P1U2 extract, ascorbic acid positive control (3.82 ppm), and without the addition of extract was used as controls. Differences were determined by ANOVA, followed by the Tukey range test ($p<0.05$)

4. Discussion

Endophytic bacteria are symbiotic microorganisms that live in plant tissues and do not cause adverse effects that harm the host plant (Nxumalo *et al.* 2020). P1U2 isolate from clove leaves colonised by a diversity of endophytic bacteria that grew in color, colony shape, and different growth rates. Bhore dan Sathisha (2010) stated that endophytic bacteria in one plant generally consist of several genera and species. The diversity of endophytic bacteria in a plant is also influenced by plant growth conditions, especially soil environmental conditions. In some cases, plants with the same type or species have endophytic bacteria that are not always the same. In some plants, endophytic bacteria are specific and typically inhabit these plants.

Based on the results of LC-MS analysis, aeruginosin, quercetin, linolenic acid, euparin, and benzofuran compounds were found to have potential antioxidant activity. Quercetin is one of the most effective flavonoid antioxidants by showing the ability to prevent oxidation of low-density lipoprotein (LDL) by counteracting free radicals in the form of superoxide anions and hydroxyl (Ozgen *et al.* 2016). Quercetin is a compound in plants, such as the clove plant known in the polyphenol database (http://www.phenol-explorer.eu), containing quercetin of 28.40 mg/100 g of dry weight. This result is compared with apples, known for their relatively high quercetin content with a ±2.35 mg/100 g dry weight (http://www.phenol-explorer.eu). When compared, the quercetin content available in clove plants is more elevated than in apples.

The study of quercetin-producing microbes is very limited. Quercetin is mainly found in fruits and vegetables. However, previous studies reported the presence of quercetin produced by endophytic bacteria, including *Streptomyces artropurpurea* (Priyanto *et al.* 2014) and *S. antibiotics* (Nageh *et al.* 2020). Quercetin has also been synthesized via biotransformation of the enzymes from *Bifidobacterium* mixed with Salicornia herbacea (Ozgen *et al.* 2016). To our knowledge, this is the first report regarding the presence of quercetin in clove endophytic bacteria.

In addition to quercetin, the P1U2 extract also contains linolenic acid. Linolenic acid compounds were found in the *Syzygium paniculatum* in the myrtaceae family with levels of (4.85%) and had antioxidant activity (Okoh *et al.* 2019). Euparin compounds also exhibit antioxidant and antifungal activities (Jaime *et al.* 2013). Aeruginosine compounds derived from cyanobacteria also have anti-inflammatory activity (Kapuścik *et al.* 2013), benzofuran compounds have

antioxidant activity (Miao *et al.* 2019), and benzofuran was also found in *Syzygium cumini* L. (Ary *et al.* 2021).

The IC_{50} value of the P1U2 extract was higher than that of ascorbic acid control. The ascorbic acid used in this analysis works as a comparison in the measurement of antioxidant activity, due to ascorbic acid or vitamin C is known as a strong antioxidant and can react with hyperperoxide (Cao *et al.* 2019). These results show that the antioxidant ability of the P1U2 extract was weaker than the positive control. Based on testing on the P1U2 extract, it was found that the IC_{50} value is 161 ppm, while ascorbic acid has an IC_{50} value of 3.82 ppm. However, the IC_{50} values of the isolate P1U2 were still relatively high in comparison with ascorbic acid. The antioxidant activity of the extract is lower than that of the ascorbic acid can be caused by the presence of various active compounds in the extract, which are inhibits the radical scavenging process.

Crude extract P1U2 isolate added to the decolorization process decreased the percentage of decolorization by *S. cerevisiae*; this is due to reduced levels of oxidative stress by reducing the induction of color-degrading enzyme activity by yeast. Phugare *et al.* (2010) explained that S. cerevisiae could decolorize malachite green textile dye. According to Biradar *et al.* (2017), the addition of extracts containing antioxidant compounds in the decolorization process can reduce the percentage of decolorize by *S. cerevisiae* by reducing the induction of dye-degrading enzymes such as lignin peroxidase, laccase, NADH-DCIP reductase, and MG reductase and extracts containing antioxidants can protect cells from oxidative stress, compared to cells lacking antioxidant activity. At a dose of 10 ppm, malachite green can also cause a decrease in cell viability <20%. Biradar *et al.* (2017) research showed that adding malachite green toxicity causes the yeast lag phase, usually 4 hours to 40 hours, and does not continue to the cleavage phase. UV radiation can be used in mutagenesis studies. In yeasts, exposure to UV radiation with short wavelengths causes damage to DNA (Hauser *et al.* 2019). As seen in the control without P1U2 extract, according to Suharyono *et al.* (2009), UV rays will be absorbed by proteins and nucleic acids, which fail cell metabolic processes, and cells will die. The addition of P1U2 extract has antioxidant effectiveness in counteracting UV mutagens.

In conclusion, the active ingredient from Fictibacillus phosphorivorans P1U2 extract exhibits low antioxidant activity yet highly potential antimutagenic activity. P1U2 extract too has the ability as an antimutagenic agent against malachite green compound and UV exposure. P1U2 bacterial extract contains notable antioxidant compounds, including quercetin, euparin, linolenic acid, and benzofuran. Suggestions for further research further research regarding the mechanism of quercetin production from P1U2 isolates needs to be carried out. So far, quercetin is known to be produced and extracted from plants or from recombinant Escherichia coli bacteria. Because of that The study of the genomic level and the potential for quercetin production from P1U2 isolate is very important conducted.

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