A Phenolic Compound From Active Extract of Endophytic Fungus Isolated From Leaf Stalk of Jambu Bol (Syzygium malaccense)

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A Phenolic Compound From Active Extract of Endophytic Fungus Isolated From Leaf Stalk of Jambu Bol (*Syzygium malaccense*)

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Abstract: *Syzygium malaccense* plant or jambu bol has been known as a medicinal plant. Jambu bol (*S. malaccense*) as a herbal medicinal plant has been widely studied in various countries. However, finding sources of raw materials for new drugs remains a top priority in overcoming various problems, such as resistance and metabolic disorders caused by free radicals.This study aims to identify endophytic fungal species from the plant *S. malaccense* molecularly which has the potential as antibacterial and antioxidant to identify secondary metabolites it contains.The method used for antibacterial testing is the agar diffusion method with the concentration used being 400 ppm with a positive control ratio of tetracycline with a concentration of 30 ppm.Antioxidant testing using the DPPH method. The results were isolated of endophytic fungi from leaves that had potential as antibacterial and Antioxidants is identified as *Daldinia eschsholtzii* producing inhibition zones strong criteria with the highest percentage in YTD3 isolate 17.38±0.89 in *E.coli*, 17.28±0.1 (mm), at *S. typhi*, 17.77±2.73 (mm), *S. aureus* and 17.45±0.54 (mm), in *B. subtilis* and IC_{50} value 33.83.The secondary metabolite compound produced was identified as 3-hydroxy-4-(hydroxy(4-hydroxyphenyl)methyl)dihydrofuran-2-one. Further research is needed, namely testing compounds that have been identified as sources of new drug raw materials.

Keywords: *Syzygium malaccense*, *Daldinia eschsholtzii*, antioxidant, antibacterial, phenolic compound

1. Introduction

Endophytic fungi are found in symbiosis with all types of plants, including medicinal plants (Prasai et al 2021). Jambu Bol has been known as a medicinal plant throughout the world (Arumungan et al, 2014; Batista et al, 2016; Nunes et al, 2016; Fernandes and Rodrigeus, 2018). Endophytic fungi and host plants are thought to be able to synthesize the same metabolites as a host due to coevolution (Ikram et al, 2020). The search for new medicinal ingredients from endophytic fungi and medicinal plants is expected to produce metabolites that have potential as drugs as well (Adeleke and Babalola, 2021; Ibrahim et al, 2021). Species secondary metabolites of endophytic fungi in symbiosis with medicinal plants have been explored for their prospect, such as antibacterial, antioxidant, antimalarial, anticancer, and others (Strobel and Daisy, 2003; Suryanarayanan et al, 2009; Selim et al, 2012; Calcul et al, 2013; Yougen Wu et al, 2015). Isolation endophytic fungi on jambu bol have been known to vary in each organ. Knowledge of species that produce secondary metabolites as antioxidants and antibacterials are important to do as further information in the search for new active ingredients for antibiotics in overcoming bacterial resistance and free radical scavengers to treat degenerative diseases (Sharma et al, 2018). In this study, the isolated species of endophytic fungi that have potential as antibacterial and antioxidant isolates from the leaf stalk of *S. malaccense* will be reported based on the results of molecular tests. Information about the bioprospects of endophytic fungi as a biological manifestation in disclosing their pharmacological effects will ensure their continued use to improve quality of life.

2. Experimental Section

2.1. Sampling

Samples of healthy *S. malaccense* plants, derived from the 3rd leaf stalk, were taken in the Palembang area of South Sumatra in February 2021.

2.2. Isolation and Identification of Endophytic Fungi

Isolation of endophytic fungi on Jambu bol leaf stalk (*S. malaccense*) following the modified method of Aini et al, 2022. Identification of endophytic fungi is carried out based on morphological characteristics of fungi namely colony or macroscopic and microscopic characteristics (Pitt & Hocking, 2009; Walsh, Hayden, & Larone, 2018; Watanabe, 2010).

2.3. Cultivation of endophytic fungi

Each endophytic fungus was isolated and cultivated. Endophytic fungal suspensions were inoculated with 5% (v/v) endophytic fungal spores in 1 L of PDB medium placed in 3 Erlenmeyer tubes. Incubation was done at room temperature for ± 4 weeks. The medium containing secondary metabolites was partitioned in ethyl acetate and evaporated to obtain a concentrated extract (Syarifah et al, 2021).

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2.4. Antibacterial and Antioxidant Activity Test

Antibacterial activity test using agar diffusion method and NA (Nutrient agar) medium with a concentration of 400 ppm; and Tetracycline 30 ppm. The test bacteria used were *Salmonella thypi* (ATCC1408), *Escherichia coli* (Ina CCB4), *Staphylococcus aureus* (Ina CCB5), and *Bacillus subtilis* (Ina CCB4). Observations and measurements of the inhibition zone were carried out for 24 hours based on modifications from Giuliano, Patel, and Kale-pradhan 2019. Antioxidant activity test in this study used the modified DPPH (1,1-Diphenyl-2-picrylhydrazyl) based Metasari, Elfita, Muharni, & Yohandini (2020) method.

2.5. Isolation of Bioactive Coumpound

Isolation of bioactive compounds followed the procedure described by Muharni (2014) with slight modifications. Concentrated EtOAc extract (1.0 g) from broth cultures was chromatographed on a silica gel column (70-230 mesh, 30 g) and eluted with gradient solvent systems, n-Hexane-EtOAc, and EtOAc-MeOH. The chemical structure of the compound was determined by spectroscopic methods which included: ^{1}H-NMR, ^{13}C-NMR, HMQC, and HMBC.

3. Results and Discussion

3.1. Endophytic fungi isolated from the petiole of *S. malaccense*

Use either SI (MKS) or CGS as primary units. (SI units are strongly Isolation of endophytic fungi from leaf stalks of *S. malaccense* produced 3 isolates and coded YTD1, YTD2, and YTD3. The isolates from the molecular test, are the isolates with the potential to be identified as *Daldinia eschsholtzii* species (fig 2). The results of macroscopic and microscopic observations are shown in table 1.

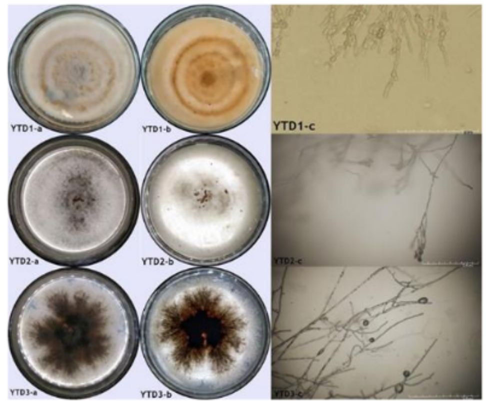


Fig. 1. Isolates of endophytic fungi isolated from leaf stalks *S. malaccense*, there were 3 isolates YTD1, YTD2, and YTD3, macroscopic and microscopic observations, namely a. surface colonies, b. reverse colony, c. Hyphae and spores.

3.2. Screening of antibacterial and antioxidant test results of endophytic fungi isolates from leaf stalks of *S. malaccense*

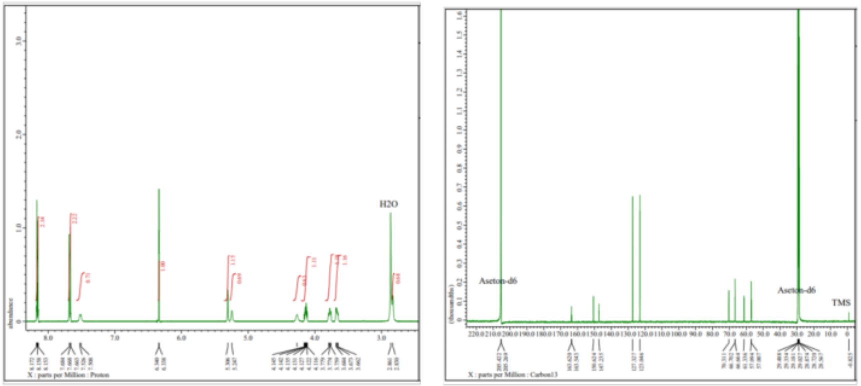
The following are the results of antibacterial test screening on *Eschcericia coli*, *Salmonella thypi*, *Staphylococcus aureus*, *Basilussubtilis* bacteria at a concentration of 400 ppm and the antibiotic used as a comparison is tetracycline with a concentration used of 30 ppm as shown in table 3. The antibiotic used was tetracycline, the criteria for the antibacterial test were based on the percentage of antibacterial activity of the extract compared to standard antibiotics (%). Inhibition zone (mm) extract/ inhibition zone (mm) tetracycline: *** strong (≥ 70%), **moderate (50-70%), and *weak (< 50%). The results of the antioxidant test using the DPPH (1,1-Diphenyl-2-picrylhydrazyl) method are presented in table 2. in this antioxidant test.

Table 2. Antioxidant test of endophytic fungi isolates from leaf stalks of *S. malaccense*

No	Samples	An antioxidant test (IC_{50})	Categories
1	Isolat YTD1	206,96 ± 2,676	In active
2	Isolat YTD2	95,32±0,832	active
3	Isolat YTD3	33,83 ± 0,068	strong

Table 1. Morphology of endophytic fungi isolates from leaf stalks of *S. malaccense*

Isolates	Macroscopic observations	Microscopic observation	Genus/ species
YTD1	Colony Surface: white edge, center brown to gray. reverse: brownish-white edge, dark center, texture; cottony, spreading, zonate pattern	Conidiophores lacking. Conidia blastospores, lateral directly on hyphae, forming spore masses on hyphae, hyaline, cylindrical, 1-celled. Septate Hyphae	*Aureobasidium sp.*
YTD2	Colony Surface: white edges, yellow spots, tawny middle, and yellow spots. Flip: white edge, middle brownish-yellow spots, texture; cottony, spread, pattern forming Zonate	conidiophores are hyaline in color, vesicles are round to semicircular, phialids are formed directly on vesicles or on metulae, Conidia are globose to semicircular, pale green, and spiny.Septate hyphae	*Aspergillus sp.*
YTD3	Colony surface: brownish-white edges, black center. reverse: brownish-white edge, black center	Hyphae are septate, have a single peritechia structure, round/oval. Septate hyphae	*Daldinia eschsholtzii*



A

Fig 3. The ^{1}H-NMR (A) and ^{13}C-NMR (B) spectra of compound 1 (^{1}H-500 MHz; ^{13}C-125 MHz in Aceton)

Based on the results of the molecular examination, it is known that the endophytic fungal species isolate YTD3 which has potential as antibacterial and antioxidant has a number of nitrogen bases in the sequence Assembly Sequence 559b: AGGTGAACCT GCGGAGGGAT CATTACTGAG TTATCTAAAC TCCAACCCTA TGTGAACTTA CCGCCGTTGC CTCGGCGGGC CGCGTTCGCC CTGTAGTTTA CTACCTGGCG GCGCGCTACA GGCCCGCCGG TGGACTGCTA AACTCTGTTA TATATACGTA [illegible]TCTGAATG CTTCAACTTA ATAAGTTAAA ACTTTCAACA ACGGATCTCT TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT TGCGCCCATT AGTATTCTAG TGGGCATGCC TGTTCGAGCG TCATTTCAAC CCTTAAGCCC CTGTTGCTTA GCGTTGGGAA TCTAGGTCTC CAGGGCCTAG TTCCCCAAAG TCATCGGCGG AGTCGGAGCG TACTCTCAGC GTAGTAATAC CATTCTCGCT TTTGCAGTAG CCCCGGCGGC TTGCCGTAAA ACCCCTATGT CTTTAGTGGT TGACCTCGAA TCAGGTAGGA ATACCCGCTG AACTTAAGCA TATCAATAA.

The phylogenetic results from the nitrogen base sequences above were obtained as shown in Figure 2. The species referred to in the YTD3 fungi isolate is homology to the strain species MW898677 *Daldinia eschscholzii*. Fungi species *Daldinia eschscholzii*, found as safrophytes in wood. *Daldinia eschscholzii* as an endophytic fungal species isolated from *Musa paradisiaca* leaves showed antibacterial activity against *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Escherichia coli* but not as an antifungal (for biorelevance) (Victor, Moses, Eze, Festus, & Charles, 2020). Microscopic observation showed that septate hyphae had a Perithecia structure with a diameter of 0[illegible].4 mm, belonging to the Ascomycet group (there were Asci 160–195×7–9μm; Ascospores 10–14 (–15.5) × 5–6.5 m, straight germ slit spore-length, grows well on PDA medium and mycelia growth temperature is between 20-40 C (optimum 30 C)(Yuyama, Pereira, Maki, & Ishikawa, 2013). *Perithecium* [illegible] found (singular, called perithecia), single or more in number, but at maturity is provided with a pore (ostiole) through which the ascospores escape those that produce their asci in an open ascocarp, called an apothecium (singular. Apothecia) those that form their asci directly in a cavity (locule) within the stroma. The stroma itself thus forms the wall of the ascocarp [illegible] such species. Conidiophores, hyaline, mononymous or synnematous, nodulisporium-like branching patterns with dichotomous or trichotomous branches are present. Conidiogeneous cells were 2.8–3.1×2.5–2.9μm, cylindrical, and smooth. Conidia were 4.8–6.4×2–3.8 m, holoblastic, hyaline, ellipsoid to obovoid, aseptate, and smooth to finely roughened with flattened base (Wutthiwong et al., 2021).

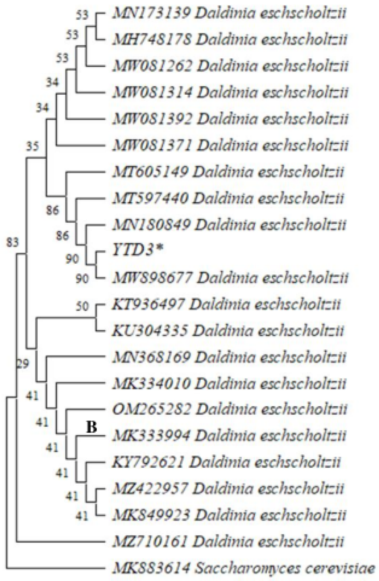


Fig 2. Phylogenetic tree of YTD3

3.3. Identification of compounds that have the potential as antibacterial and antioxidant in AD3 isolate isolated from the leaf stalk of *S. malaccense*

The [1]H-NMR spectrum of compound 1 (Figure 3A) showed the presence of six proton signals including two dublet signals in the aromatic chemical shift with the integration of 2 protons and the ortho-bond constant (J = 8.0 Hz).This indicates that compound 1 is a para-substituted aromatic compound, so it has two pairs of equivalent protons. In addition, there are five signals on the chemical shift δ_H 6.5 ppm including indicating the presence of sp3 proton groups on oxygenated carbon. The five signals appear at δ_H 6.34 (1H, d, J= 1Hz); 5.31 (1H,s); 4.14 (1H, m); 3.78 (1H, m); and 3.67 ppm (1H, m). Based on the analysis of the [1]H-NMR spectrum, compound 1 was identified as a para-substituted aromatic compound with nine protons attached to a carbon atom.The [13]C-NMR spectrum of compound 1 (Figure 3B) showed the presence of 11 signals. Four carbon signals are in the aromatic region, a characteristic of para-substituted aromatic compounds. Two aromatic carbon equivalent signals were characterized by the presence of high-intensity signals at δ_C 123.8 and 127.3 ppm. It was also seen that the presence of aromatic oxyaryl carbon in the low field was δ_C 147.2 ppm. The lowest field carbon appears at δ_C 163.6 ppm for carbonyl esters. In addition, there are three oxygenated carbon signals that appear in the δ_C 60.0 – 71.0 ppm area and one tertiary carbon signal at δ_C 57.1 ppm. The analysis of the proton and carbon NMR spectra were confirmed by the data on the HMQC spectrum shown in Figure 4 and Table 4. The HMQC spectrum showed seven 1H-13C correlations through one bond. Proton signals at δ_H 3.78 (1H, m) and 3.67 ppm (1H, m) showed a correlation to the same carbon atom at δ_C 61.3 ppm indicating a cyclic methylene group. Thus, compound 1 in addition to having a substituted benzene ring, also has a lactone ring with a methylene group on the ring.

The HMBC spectrum (Fig. 5) showed a [1]H-[13]C correlation through two or three bonds. The aromatic proton signal at δ_H 8.16 ppm is correlated through three bonds with its equivalent aromatic carbon (δ_C 123.0 ppm) and quaternary aromatic carbon at δ_C 150.6 and 147.2 ppm. The aromatic proton at δ_H 7.67 ppm correlates via two or three bonds with three aromatic carbons at δ_C 123.0; 127.3; and 147.2 ppm and oxygenated carbon at 70.3 ppm C). The oxygenated methine proton at δ_H 5.31 ppm correlates with two aromatic carbons, namely through three bonds with equivalent aromatic carbon (δ_C 127.3 ppm) and two bonds with quaternary aromatic carbon (δ_C 150.6 ppm). The correlation indicates that the oxygenated methine group is directly attached to the aromatic ring and is para-substituted with a hydroxyl group. Furthermore, the correlation of two methylene protons (δ_H 3.78 and 3.67 ppm) to the same carbon atom is at δ_C 57.1 and 70.3 ppm. The spectrum also shows the correlation of the oxygenated proton at δ_H 5.34 ppm to the carbonyl ester carbon atom via two bonds. The 1D and 2D NMR spectral data for compound 1 are shown in Table 4.

Table 3. An antibacterial test screening of endophytic fungi isolates from leaf stalks of *S. malaccense*

No	Samples	An antibacterial test screening (mm)				Categories
		E.coli	***S. thypi***	***S. aureus***	***B. subtilis***	
1	Antibiotic	20.7±1.00 100	22.2±0.93 100	21.6±1.02 100	20.5±1.08 100	Strong
2	YTD1	16.51±0.64 79.77***	15.58±0.16 70.15***	17.26±0.31 79.9***	14.89±0.75 72.63***	Strong
3	YTD2	14.57±0.29 70.37***	16.24±0.32 73.14***	15.91±1.03 73.63***	16.09±0.63 78.47***	Strong
4	YTD3	17.38±0.89 83.94***	17.28±0.1 77.84***	17.77±2.73 82.28***	17.45±0.54 85.1***	Strong

Table 4. The NMR data of compound 1, recorded at [1]H-500 MHz; [13]C-125 MHz in aceton

No. C	δ_C ppm	Type of C	δ_H ppm (ΣH, multiplicity, *J* (Hz))	HMBC
2	163.6	C		
3	66.7	CH	6.34 (1H, d, 1.0 Hz)	163.6
4	57.1	CH	4.14 (1H, m)	

5	61.3	CH_2	A. 3.67 (1H, m) B. 3.78 (1H, m)	57.1; 70.3
6	70.3	CH	5.31 (1H, s)	127.3; 150.6
1'	150.6	C		
2'	127.3	CH	7.67 (1H, d, J= 8.0 Hz)	70.3; 123.0; 127.3; 147.2
3'	123.0	CH	8.16 (1H, d, J= 8.0 Hz)	123.0; 147.2; 150.6
4'	147.2	C		
5'	123.0	CH	8.16 (1H, d, J= 8.0 Hz)	123.0; 147.2; 150.6
6'	127.3	CH	7.67 (1H, d, J= 8.0 Hz)	70.3; 123.0; 127.3; 147.2

Based on the spectrum analysis of ^{1}H-NMR, ^{13}C-NMR, HMQC, and HMBC, it can be explained that compound 1 has a para-substituted benzene ring between the hydroxyl group and the oxygenated methine group. This oxygenated methine group binds to the 3-hydroxy--butyrolactone ring. Thus, the proposed chemical structure of compound 1 is 3-hydroxy-4(hydroxy(4-hydroxyphenyl)methyl)-γ-butyrolactone as shown in Figure 6.

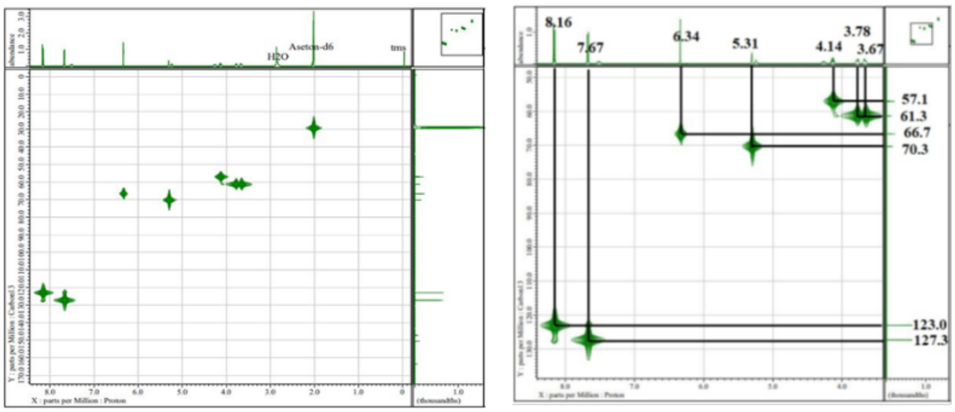


Fig 4. The HMQC spectra of compound 1

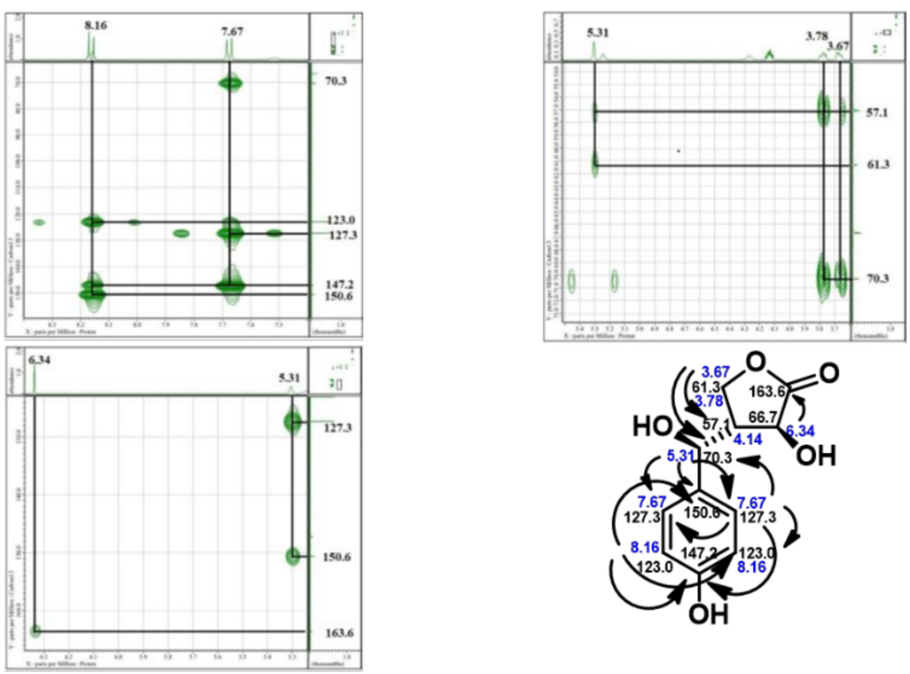


Fig 5. The HMBC spectra of compound 1

Several compounds that have been isolated from the genus and species Daldiniasp and *Daldinia eschscholtzii*, including in the genus Daldinia, 2 cyclopentenones compounds have been isolated from Daldinia sp, which have the potential as antiviral with strong criteria and antibacterial with moderate criteria (Gu et al, 2020). The isolate of the fungus Daldiniaeschscholtzii from the Dendrobiumchrysotoxum plant in China obtained 5 benzopyran derivative compounds, namely (1) (R)-2,3-dihydro-2,5-dihydroxy-2-methylchromen-4-one,(2). (2R, 4S)-2,3-dihydro-2-methyl-benzopyran- 4,5-diol, (3). (R)-3-methoxyl-1-(2,6-dihydroxy phenyl)-butane-1-one, (4). 5-O-α-d-ribosyl-5-hydroxy-2-methyl-4H-chromen-4-one, and (5). 7-O-α-d-ribosyl-2,3-dihydro-5-hydroxy-2-methyl-chromen-4-one called Daldinium A, the compound has activity as antimicrobial, anti-acetylcholinesterase, nitric oxide inhibition, anticoagulant, photodynamic antimicrobial and glucoseuptake of adipocytes (Hu et al, 2017). Species D. eschscholtzii, has the ability to adapt to the environment and various hosts in producing various active secondary metabolites, namely daldinone F, galewone, lactone helicascolide C, cytochalasin, polyketides 8-O-methylnodulisporin F, nodulisporin H, dalesindoloids A, indochromins A and B, benzopyran-naphthalene hybrid daldinsin, and new lactone, 8-hydroxylhelicascolide A (Li et al, 2021).

4. Conclucions

The species *Daldinia eschscholtzii* that has been isolated from the leaf stalk of the plant S. malaccense has secondary metabolites that have the potential as antibacterial and antioxidant with strong categories, but further testing needs to be done on the compound 3-hydroxy-4(hydroxyl (4-hydroxyphenyl) methyl) - γ - butyrolactone, which has been isolated, so that it can be developed in the next process.

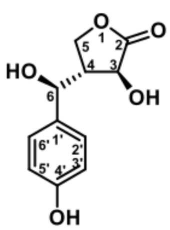


Fig 6. The structure of compound 1 as 3-hydroxy-4(hydroxy(4-hydroxyphenyl)methyl)-γ-butyrolactone

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