3-ACETYL -2,5,7-TRIHYDROXY-1,4-NAPHTALENEDIONE, AN ANTIMICROBIAL METABOLITE FROM THE CULTURE OF ENDOPHYTIC FUNGUS COELOMYCETES TCBP4 FROM Tinospora crispa

3-ACETYL -2, 5, 7-TRIHYDROXY-1, 4-NAPHTALENEDIONE, SEBAGAI ANTIMIKROBIA METABOLIT DARI KULTUR JAMUR ENDOFIT TCBP4

Praptiwi, Yuliasri Jamal, Ahmad Fathoni, Arif Nurkanto, Andria Agusta*
BioScience Laboratory, Botany Division, Research Center for Biology, The Indonesian Institute of Sciences.
Jl. Raya Jakarta-Bogor Km. 46. Cibinong, 16911
*Author for correspondence: bislunatin@yahoo.com

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Abstract

Isolation, identification and testing of antimicrobial activity of secondary metabolites of endophytic fungal culture $TCBP_4$ isolated from bratawali (Tinospora crispa) has been performed. The fungus $TCBP_4$ was cultivated in Potato Dextrose Broth (PDB) for 1 month, media and fungi extracted with ethyl acetate. The extract was separated and purified by several chromatographic techniques, from which 9 fractions were obtained. Fraction 3e was purified again and was obtained 6 fractions ($3e_1$-$3e_6$). Fractions $3e_3$-$3e_6$ were tested against bacteria isolates Staphylococcus aureus Bacillus subtilis,Eschericia coli and yeast isolate Candida albicans by microdillution method. Antimicrobial activity test result showed that fractions $3e_3$-$3e_5$ had better antibacterial activity compared to chloramphenicol as commercial antibiotic. It was indicated by MIC value of the fractions was lower (8 ug/ml) compared with the antibiotic chloramphenicol (16 ug/ml). Fraction $3e_3$ had better antifungal activity compared to commercial antifungal nystatin and cabisidin against C. albicans. GC-MS analysis showed that the chemical constituent of $3e_5$ fraction was identified as 3-acetyl -2,5,7-trihydroxy-1,4-naphtalenedione .

Key words : Tinospora crispa, endophytic fungi, isolation, identification, antimicrobial

Abstrak

Isolasi, identifikasi dan uji aktivitas antimikrobia terhadap metabolit sekunder dari kultur jamur endofit $TCBP_4$ yang diisolasi dari tumbuhan bratawali (Tinospora crispa). Jamur endofit $TCBP_4$ dikultivasi pada media Potato Dextrose Broth (PDB) selama 1 bulan, selanjutnya media dan jamur diekstrak dengan pelarut etil asetat. Ekstrak dipisahkan dan dimurnikan dengan beberapa teknik kromatografi sehingga diperoleh 9 fraksi. Fraksi 3e dimurnikan kembali dan diperoleh 6 fraksi ($3e_1$-$3e_6$). Fraksi $3e_3$-$3e_6$ diuji aktivitas antibakterinya terhadap beberapa isolate bakteri Staphylo-coccus aureus Bacillus subtilis, Eschericia coli dan isolate khamir Candida albicans dengan metode mikrodilusi. Hasil uji antimikrobia menunjukkan bahwa fraksi $3e_3$-$3e_5$ mempunyai aktivitas antibakteri lebih baik bila dibandingkan dengan chloramfenikol, dimana nilai MIC dari fraksi (8 ug/ml) lebih rendah dari antibiotika chloramfenikol (16 ug/ml). Fraksi $3e_3$ mempunyai aktivitas antijamur lebih baik bila dibandingkan dengan antijamur komersial nistatin dan kabisidin terhadap C. albicans. Analisis GC-MS menunjukkan bahwa komponen kimia dari fraksi $3e_5$ diidentifikasi sebagai 3-acetyl -2,5,7-trihydroxy-1,4-naphtalenedione .

Kata kunci : Tinospora crispa, jamur endofit, isolasi, identifikasi, antimikrobia

Introduction

Plant has been known as producer of biologically active compounds. The need for active compound if obtained directly from the plant would require biomass from the plant in abundance. However, in recent years has also been realized that the plant also serves as a repository myriad organisms are known as endophytic microbes[1] that live in association with plants. According to Maheshwari[2] endophytic fungi living in plant tissue without causing any signs of to the host plant. Endophytic fungi in general are able to synthesize bioactive compounds that can serve as plant defence, even some of the compounds have been shown to be useful for the discovery of new drug substance. Endophytic fungi have also been reported to have the ability to produce metabolites that are similar to their host plants. Various classes of secondary metabolites such as alkaloids, terpenoids, quinone, derived isocoumarin, phenil-propanoid, phenolic and aliphatic compounds have been isolated from in-vitro cultures of endophytic fungi in the last 20 years.[3]

On the other hand, there is an increase of resistance of pathogenic microbes to commercially antibiotic available. According to Kuswandi[4] in inaugural speech professor at Universitas Gadjah Mada, said that the data in 2010 showed that 79% of *E.coli* strains resistant to ampicillin, whereas 30% of strains were resistant to ciprofloxacin. In 1999-2000 in the United States in case of *S. aureus* infection that 43% were resistant to methicillin. Some harmful bacteria such as *Mycobacterium turberculosis* and *Pseudomonas aeruginosa*, resistant to antibiotics. Increased of resistant micro-bial pathogens populations require new antimicro-bial agents.[5]

One of the medicinal plants that have long been known and used traditionnaly is bratawali (*Tinospora crispa*). Bratawali is a liana plant belonging to Menispermaceae. Genera Menisper-maceae have characteristic compound which is protoberberin alkaloid (berberine, palmatin, jat-rorrhizin) have shown biological activity with a broad spectrum to several diseases. Bratawali extracts have been known to have anticancer activity, antioxidant,[6] antiprotozoa, antimalariak, antiinflammatory, antihiperglicemia,[7] allergenic, antivirus[8] and decrease appetite.[9] Endophytic fungi associated with bratawali plant have also been isolated, one of them is endophytic fungus TCBP4. Endophytic fungus TCBP4 was isolated from bratawali stem collected from Pamengpeuk (West Java). The result from Febryanto[10] showed that extracts of endophytic fungi TCDC2 that isolated from *Tinospora crispa* leaves have inhibitory activity against the growth *S.aureus* and *B. subtilis.* Based on the potential of bratawali as medicinal plant and the potency of endophytic fungi TCBP4 extract then research for isolation and identification of bioactive metabolites and its potential as an antimicrobial has been done. This paper reported the antibacterial activity of pure compound isolated from TCBP4 against several bacteria isolates.

Methods

Material:

Endophytic fungi isolate used in this study was isolated from young stem of bratawali plant, as Bioscience Laboratory collection, Botany Division, Research Center for Biology-Cibinong. Microbial isolates (*S.aureus, B.subtilis, E.coli* dan *C. albicans*) used for antimicrobial activity testing is a collection of Microbiology Division, Research Center for Biology- Cibinong.

Methods:

Scaling up of endophytic fungi TCBP4 cultivation

Pure colonies of endophytic fungi TCBP4 from *Tinospora crispa* were cultured on *Potato Dextrose Agar* (PDA) media taken aseptically in laminar airflow approximately 1x1 cm^2 then cultivated on 2 L *Potato Dextrose Broth* (PDB), then incubated at room temperature under static condition for 1 month.

Extraction, Fractionation dan Purification

After 1 month incubation, the media and endophytic fungi were harvested and extracted with ethyl acetate. Ethyl acetate fraction were separated with separating funnel and concentrated with rotary evaporator. Ethyl acetate fraction analysed by Thin Layer Chromatography (TLC) and eluted with mobile phase dichloromethane: methanol (10:1). The result was observed under UV light at wavelength of 254 and 366 then sprayed with a stain reagent apparition cerium sulphate $Ce(SO_4)_2$.

Furthermore, ethyl acetate extract fractionat-ed by colomn chromatography Sephadex LH-20 and eluted with ethanol. Fractions were monitored by TLC with eluent dichlorometane: methanol (10:1). Fraction 3 still has few spots, fractionated again with silica gel (70-230 mesh) as stationary phase and eluted with increasing polarity, namely

dichlorometane : methanol in the ratio 100:1, 50:1, 25:1, 10:1 and 5:1 and methanol. Fraction 3 were separated into 11 fractions (3a-3k). Fraction 3e still showed some spots purified by preparative TLC with eluent hexane : chloroform : methanol (1:1:0.25). Spots obtained were scraped and dissolved with acetone and filtered with Whatman filter paper. Each component of the spot was analysed again with TLC and eluted with hexane : chloroform : methanol (1:1:0.25). The result was observed under UV light at wavelength of 254 and 366 then sprayed with a stain reagent apparition cerium sulphate $Ce(SO_4)_2$.

Identification of compound structure by GC-MS

Identification of compound structure from purification process perform by GS-MS varian Saturn 2000 in Analytical laboratory, Research Center for Biology - Cibinong. Type of colomn used was VF-17 MS, 30 mm long with diameter of 0.25 mm. Carrier gas was Helium with a flow rate of 2.0 ml/minute. Injection volume : 5 ul, and injector temperature was 250^{o}C. Column temperature was programmed at 100- 270^{o}C. At early stage, column temperature was constant at 100^{o}C for 3 minutes, then raised to 270^{o}C with the speed of temperature rise was 10^{o}C/minute. This condition was maintained for 18 minutes. Chemical compounds that were detected were identified by comparing mass spectra of target compound with mass spectra in the database[11] (NIST, Wiley).

Determination of Minimum Inhibitory Concentration (MIC)

The media used for antibacterial test was Muller Hinton Broth (MHB) media while for antifungal test was Saboraud Broth (SB) media. Determination of MIC was done by microdilution method in 96 microtiter plate. Stock solution of each fraction that will be used for antibacterial and antifungal test dissolved with dimethyl sulfoxide (DMSO) at the concentration of 512 ug/ml. Microbial isolates used were : Gram positive bacteria (*Staphilococcus aureus* and *Bacilus subtilis*), Gram negative bacteria (*Eschericia coli),* yeast (*Candida albicans*). Well 1st was filled with 100 ul growth media double concentration, while well 2nd – 14th was filled with 100 ul growth media 1x concentration. Furthermore, in well 1st added with 100 ul stock solution, homogenized with micropipette, then 100 ul was taken and added to the second well. The second well was homogenized with micropipette, then 100 ul was taken and added to the third well. The same was done to well 14th. When the process of dilution has been completed, then in each well was added with inoculums of bacteria or yeast. Well 15th was used as positive control for bacterial growth, filled with 100 ul growth media and 100 ul inoculums, while well 16th was negative control containing only 200 ul growth media. This was done in triplicate. Microtiter plate was then incubated in incubator shaker for 24 hours at 35-37^{o}C. Value of MIC was observed visually where there was no growth of bacteria at the lowest concentration observed.

Result

Scaling up and Extraction of Endophytic Fungi Culture $TCBP_4$

Scaling up of endophytic fungi $TCBP_4$ was done because of the previous study showed that its ethyl acetate extract had inhibitory activity against *Staphylococcus aureus* and *Bacillus subtilis*. Endophytic fungi TCBP4 was cultivated in 2 L Potato Dextrose Broth (PDB) medium produce 371.5 mg brownish red extract. Extract was analysed by TLC with dichloromethane : methanol (10:1) as mobile phase. Result was observed under UV light 254, extract further fractionated by chromatography column with Sephadex LH-20 as stationery phase produced 9 fractions, and the weight of the fractions : (1) 55.6 mg, (2) 85.5 mg, (3) 66.9 mg, (4) 13.4 mg, (5) 4.8 mg, (6) 1.6 mg, (7) 1.5 mg, (8) 0.3 mg, dan (9) 4.1 mg. Fraction 3 still had several compounds indicated by several spots based on TLC result. Fraction 3 was fractionated again by chromatography column with Silica gel (70-230 mesh) as stationery phase then eluted with gradually polarity increased. Fractionation of fraction 3 produced 11 fractions with fraction weight consecutively: 0.4, 0.1, 0.4, 0.8, 15.6, 2.1, 9.4, 3.8, 2.5, 1.6, 29.7 mg. Fraction 3e (15.6 mg) was separated and purified further by preparative TLC using n-hexane : chloroform: methanol (1:1:0.25) as eluent produced 6 fractions namely fraction 3e1-3e6. These fractions were used for antimicrobial test to determine MIC value.

Table 1. Weight of fractionation of fraction 3e of ethyl acetate extract TCBP4

No	Fraction	Colour	Weight (mg)
1	$3e_1$	Reddish yellow	1.1
2	$3e_2$	Red	1.4
3	$3e_3$	Orange	3.2
4	$3e_4$	Reddish yellow	1.4
5	$3e_5$	Red	6.2
6	$3e_6$	Pale Orange	2.3

Table 2. MIC value of fraction 3e of endophytic fungi TCBP4 extract

Sample	MIC (ug/ml)			
	S.aureus	***B.subtilis***	***E.coli***	***C.albicans***
Fraction $3e_3$	**8**	8	64	16
Fraction $3e_4$	**8**	8	64	32
Fraction $3e_5$	**4**	8	64	32
Fraction $3e_6$	16	16	64	32
Chloramphenicol	16	8	8	-
Erythromycin	0.06	0.03	32	-
Nystatin	-	-	-	32

Note : - not tested

Determination of Minimum Inhibitory Concentration (MIC)

Fraction used for antimicrobial testing were fraction $3e_3$-$3e_6$, whereas microbial used were *S.aureus*, *B.subtilis*, *E.coli* and *C.albicans* with the number of colonies were 1.27 x 10^9, 5.09 x 10^9 , 5.6 x 10^8 dan 8 x 10^6 respectively. MIC value of fraction $3e_3$-$3e_6$ were in Tabel 2.

Result in Table 2 showed that MIC values of fractions $3e_3$,$3e_4$ and $3e_5$ were lower than MIC value of chloramphenicol.

Identication of the compound structure by GC-MS

Identification of compounds contained in the fraction $3e_3$ and $3e_5$ perform by GC-MS. Identification of compounds only done to $3e_3$ and $3e_5$ fraction because these fractions had better antimicrobial activity compared with other fractions (Table 2). Result of fraction $3e_3$ perform by GC-MS (Fig. 1) showed a single peak with retention time at 33.23 minute. MS spectrum of the compound had a base peak 274 m/z (Fig. 2).

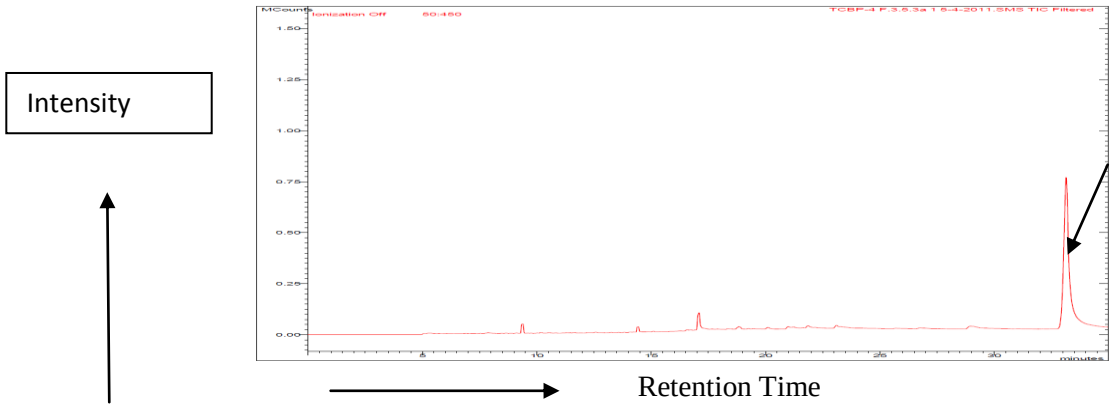

Figure 1. GC chromatogram of fraction $3e_3$

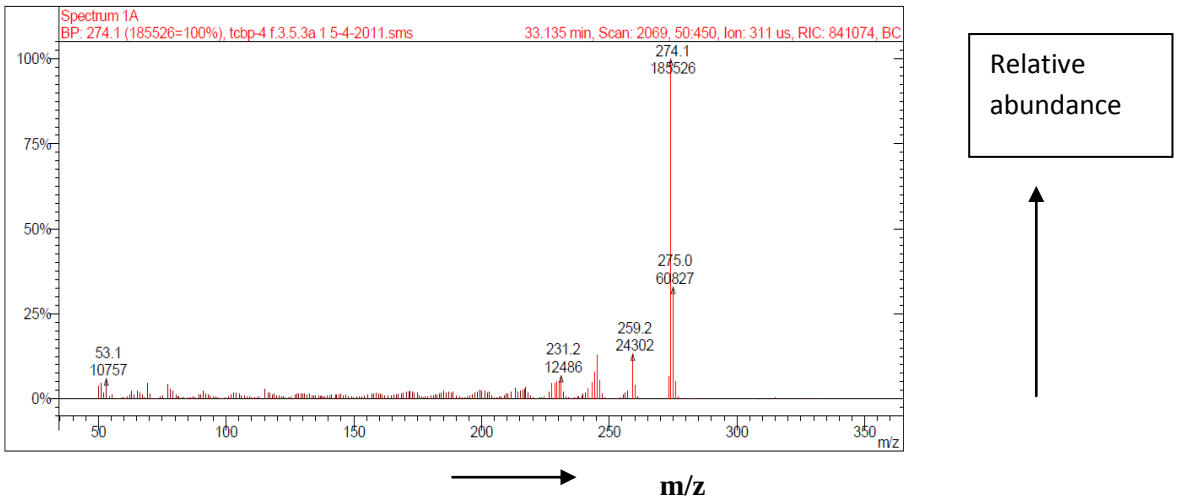

Figure 2. MS spectrum of fraction $3e_3$

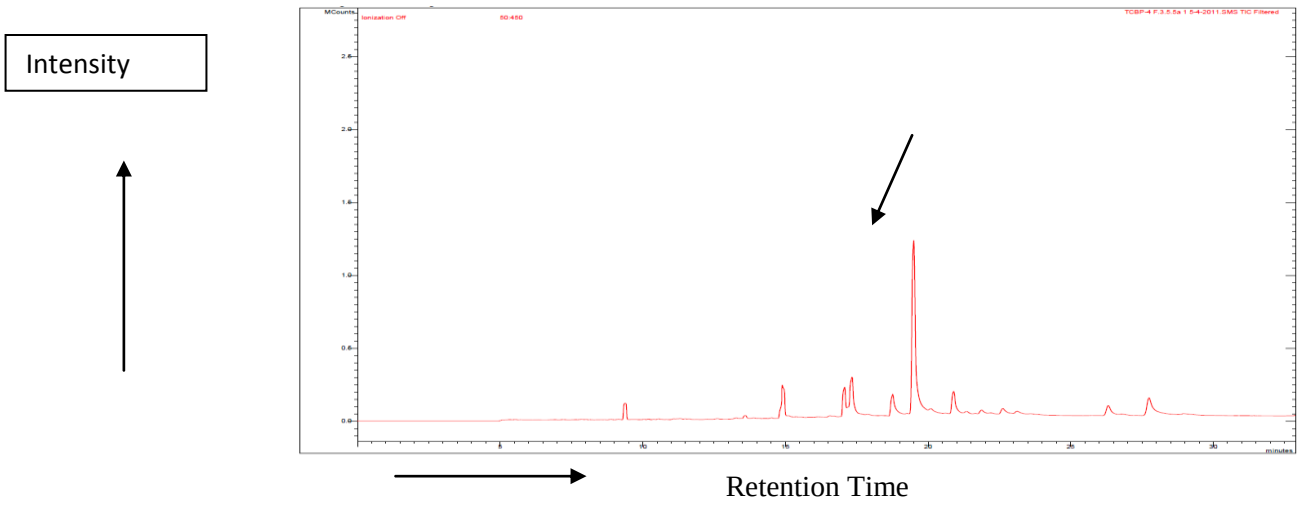

Figure 3. GC chromatogram of fraction $3e_5$

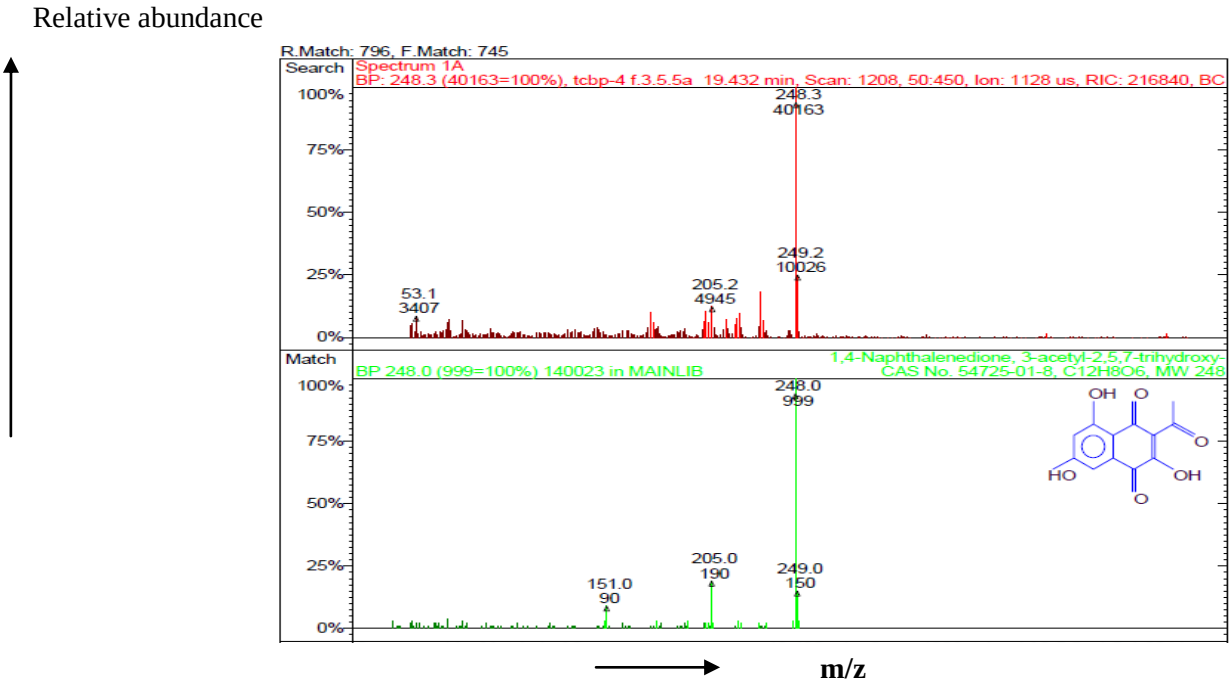

Fig 4. Mass spectrum comparison between fraction $3e_5$ (over) with 3-aceryl -2,3,5-trihydroxy-1,4-naphtalene

Analysis of fraction $3e_5$ by GC-MS (Fig. 3) also indicated high peak compound with retention time at 19.53 minute and some small peaks as impurities, and suspected that compound with high peak was potential as an antibacterial.

MS spectrum of this compound had a base peak at 248 m/z (Fig. 4).

Discussion

Comparing the mass spectrum of fraction $3e_3$ with NIST Library showed there was no resemblance to the mass spectrum of the existing data. Therefore it was suspected that compound in the fraction $3e_3$ was new compound that has to be identified its chemical structure. While the mass spectrum of fraction $3e_5$ with NIST Library showed that it was resemble to the mass spectrum of 3-acetyl -2,5,7-trihydroxy-1,4-naphtalenedione, with the molecular formula $C_{12}H_8O_6$. Its structure had 1,4 naphtoquinone structure. Naphtoquinone is secondary compound used as a dye[12]. Several compounds from this class as alkanin, shikonin and its derivative have biological activity as anti-inflammatory, antibacterial, antifungal, antioxidant, and antitumor[13]. Based on the literature search, the structure of this compound is identical to the structure of the compound spinochrome M (2,7 dihydroxy-3-acetyl-naptazarin)[14] which is naphta-quinone isolated from the pigment of sea urchin *Echinometra oblonga, Blainville* and *Colobo-centratus atratus* Linn[15].

Based on result in Table 2 showed that fraction $3e_3$,$3e_4$ and $3e_5$ have better antibacterial activity than chloramphenicol against *S.aureus*. This is shown by lower MIC value of the fractions compared with MIC value of chloramphenicol, it means that these fraction need lower concentration (8 ug/ml) to inhibit the growth of *S. aureus* compared with chloramphenicol (16 ug/ml) as commercial antibiotics. Antibacterial activity of these fractions ($3e_3$ – $3e_5$) was similar to chloramphenicol to inhibit the growth of *B.subtilis*, only fraction $3e_3$ has better antifungal activity when compared with nystatin and kabisidin as commercial antifungal against *C. albicans.* The MIC value against Gram negative bacteria *E.coli* was greater than positive control erythromicin and chloramphenicol. This may be due to the composition of the cell wall of Gram negative bacteria more difficult to be penetrated by the active compound of the tested fractions, because its cell wall is more complex than Gram positive bacteria.

Conclusion

Chemical compound in the fraction $3e_3$ was suspected to be new compound, while chemical compound in the fraction and $3e_5$ was identified as 3-acetyl -2,5,7-trihydroxy-1,4-naphtalenedione, with the molecular formula $C_{12}H_8O_6$. The antibacterial activity of fraction $3e_3$,$3e_4$ and $3e_5$ were better than chloramphenicol against *S.aureus*, but their activity were similar to chloramphenicol against *B.subtilis*. The fraction that had better antifungal activity against *C. albicans* was fraction $3e_3$.

Suggestion

Further study need to be done to identify the chemical compound of fraction $3e_3$, while further antimicrobial test should be done by using more isolates of pathogenic microbes.

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