Ergosterol Isolated from Agaricus blazei Murill N-Hexane Extracts as Potential Anticancer MCF-7 Activity

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ABSTRACT
Extracts and some of the Agaricus blazei Murill isolates have potential anticancer. Ergosterol isolate from Amauroderma rude can also inhibit the growth of MDA-MB-231 cancer cells through apoptotic pathways by increasing FOXO3 expression, while its potency against MCF-7 cells has not been reported. The purpose of this study was to isolate, determine the structure, determine the anticancer activity of MCF-7 cells, and the isolate mechanism by apoptosis from one of isolates the n-hexane A. blazei Murill extracts. This research method includes the isolation of compounds from A. blazrei Murill extract by chromatography method guided using Bioactivity Guided Isolation. The structure elucidation of structure isolates used UV, NMR and MS spectroscopy. Anticancer activity test using the MTT cytotoxic test. Eludation of UV, NMR and MS structures showed a ergosterol. The anticancer activity test showed IC50 values of 43.10 µg/mL with the strong cytotoxic category. The mechanism of action is to increase apoptosis induction through inhibition of the cell cycle in the G2/ M phase. The conclusion of the isolated compound was ergosterol with an IC50 value of 43.10 µg/mL with an increased apoptosis induction mechanism through inhibition of the cell cycle in the G2/ M phase.

Key words: Agaricus blazel, Murill extract, Ergosterol, MCF-7 cells, Apoptosis.

INTRODUCTION
Agaricus blazei Murill is a low-level plant which is a functional food and has the potential to have activities both in vitro and in vivo, including reducing blood sugar, lowering blood pressure, reducing blood cholesterol, reducing arteriosclerosis,7-11 antioxidant, antiviral, antimutagenic, antiproliferative, antiparasitic, antitumor, anti-inflammatory, and immunostimulatory activities both in vivo and in vitro.9,14,22-25 Other names for Agaricus blazei Murill are A.subrufescens, A.sylvaticus, and A.brasiliensis.13 Several studies have been carried out related to the activity of the extract as an anticancer, namely research on 50% ethanol extract of Agaricus blazei Murill that can inhibit the growth of Hela cells with an IC50 value of 19.44 µg/mL.17 Agaricus blazei Murill extract can also inhibit myeloma cells and leukemia cells20-24. Agarit isolates from Agaricus blazei Murill can also inhibit the proliferation of leukemia cancer cells U937, MOLT4, HL-60, and K-562 with IC50 values of 2.7; 9.4; 13.0; and 16.0 µg/mL.25 Blazein isolate from Agaricus blazei Murill extract can also induce the death of human lung LU99 and stomach KATO III cancer line at a concentration of 200 µg/mL.26 Polysaccharides obtained from the isolation process of Agaricus blazei Murill extract have the potential to inhibit osteosarcoma cell proliferation (HOS cell line) at a concentration of 100 µg/mL. Agaricus blazei Murill extract can inhibit proliferation in DU145 and PC3 prostate cancer cells 400 µg/mL and 800 µg/mL.27 Hot water extract of Agaricus blazei Murill also inhibits the growth of pancreatic cancer cells.28 Several previous studies have also shown that the isolated compounds associated with the divine mushroom isolate have anti-cancer mechanisms, such as β-D-glucan and ergosterol isolate. β-D-glucan can cause apoptosis in human ovarian cells which will involve the p38 MAPK (Mitogen Activated Protein Kinase) pathway by translocation of apoptotic activator from the cytosol to mitochondria, cytochrome c is formed which then activates caspase 9.10,12-20 stated that β-D-glucan isolate from the fungus Botryosphaeria rhodina can also increase apoptosis, oxidative stress, mRNA expression for p53, p27, and Bax (Bcl-2 Associated X-protein), activated AMP-protein kinase (Adenosine monophosphate protein kinase), transcription factor FOXO3a (Forkhead Box O3), Caspase3, and decreased p70S6K (Ribosomal protein S6 kinase beta-1) in MCF-7 cells. Ergosterol isolated from Amauroderma rude can suppress the growth of breast cancer cells through the apoptotic pathway by increasing the expression of FOXO3.13 Previous studies showed that the activity test of n-hexane, dichloromethane, chloroform, ethyl acetate, and butanol extracts against MCF-7 cancer cells obtained IC50 results of 24.72 µg/mL; 22.70 µg/mL; 21.56 µg/mL; 23.49 µg/mL; and 50.08 µg/mL which is included in the strong cytotoxicity category, while the water extract is inactive.11 Statistical analysis t-test on the results of the IC50 value on the treatment of n-hexane extract, dichloromethane extract, chloroform extract, and ethyl acetate extract obtained a sig value of 0.356 (> 0.05), so that the treatment of the extract for the IC50 value showed no difference. Based on these data, several compounds were isolated from the n-hexane extract first. The isolation process was carried out by the chromatography method guided by the Bioactivity Guided Isolation method and the determination of Cite this article: Misgjati M, Widyawaruyanti A, Raharjo SJ, Sukardiman S. Ergosterol Isolated from Agaricus blazei Murill N-Hexane Extracts as Potential Anticancer MCF-7 Activity. Pharmacog J. 2021;13(2): 418-26.

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the elution of the structure resulting from the isolation. The activity of the isolates was carrying out on MCF-7 cancer cells. The purpose of this study was to elucidate the structure of isolates with UV spectrum, NMR spectrum, and MS results from the isolation of *Agaricus blazei* Murill n-hexane extract and determine the anticancer activity of MCF-7 cells and their mechanisms.

**MATERIAL AND METHODS**

**General**

The HPLC system consists of two LC-10AD pumps and an SCL-10A controller, Agilent RP-18 XDB 4.6x250mm column, eluted with methanol: acid water (9.95: 0.05) at 2 mL/min flow rate, UV-Vis Spectrum analyzed using UV-1900i UV-Vis Spectrophotometer, NMR spectrum recorded on JEOL ECS-400, using CDCl3 as solvent, Vacuum Column Chromatography (VLC) with silica Gel F254 (Merck, No. 1.05715.0001) and Thin Layer Chromatography (TLC) on Silica Gel 60 F254 (Merck, 1.05715.0001) and Silica RP-18 (Merck, No 1.15389.0001). TLC profile identification was carry out using the TLC Visualizer (Camag).

**Plant material**

*Agaricus blazei* Murill obtained from traditional medicine industry PT. ASIMAS Lawang Malang.

**Isolation and identification of active compounds**

Blend dry *Agaricus blazei* Murill. The resulting powder extracted by maceration. The hexane extract was fraction using Vacuum Column Chromatography (VLC) with a gradient of n-hexane and ethyl acetate (100% -25%). Based on the thin layer chromatography profile, several fractions of the same were combine to produce three fractions. Each fraction was identified with TLC Silica Gel 60 F254 (Merck, 1.05715.0001) and Silica RP-18 (Merck, No 1.15389.0001) and tested for the anticancer activity of MCF-7 cells. Activity testing the fraction that has the smallest activity is separate by dissolving it with methanol to produce two sub-fractions. Sub-fraction was carry out by the anticancer activity of MCF-7 cells. The sub-fraction which have the smallest activity is carried out semi preparative separation by the HPLC (High Liquid Pressure Chromatography) method consisting of two LC-10AD pumps and an SCL-10A controller, Agilent RP-18 XDB column 4.6x250mm, eluted with methanol:acid water (9.95: 0.05). Sub-fraction were eluted in a formic acid and methanol mixture (9.95: 0.05 v/v) with a flow rate of 1 mL/ min, obtained 5 isolates. The isolate profiles were analyst using TLC and HPLC.

Isolates were analyst using TLC, visualized at UV 254nm and 366 nm, and sprayed with anisaldehyde to see the presence of terpenoids. The isolates were test for the anticancer activity of MCF-7 cells. Isolates that are sufficient in number and have anticancer activity for MCF-7 cells are determined for their chemical structure. The chemical structure was determined using Nuclear Magnetic Resonance (JEOL, ECS-400) with CDCl3 as the solvent. The mass spectrum was identification by the LCMSMS system with Coloum: ACQUITY UPLC @ BEH C18 1.7 μm.

**MTT cytotoxic test**

According to the Cancer Chemoprevention Research Center (2013), the cytotoxic test of the MTT method was carry out by implanting MCF-7 cells planted in 5000 cells/ wells 96 microplate and incubated for 48 hours to get good growth. The MEM medium was replace with new samples added at various concentrations (3.375 μg/mL, 6.75 μg/mL, 12.5 μg/mL, 25 μg/mL, 50 μg/mL, cell control, solvent control) with DMSO co-solvent and incubated at 37°C in a 5% CO2 incubator for 48 hours. At the end of incubation, MEM media and extract were discard and the cells were wash with PBS. To each well, 10 μL of MTT reaction 5 mg/mL was add cell were incubated for 4-6 hours in a 5% CO2 incubator at 37°C. The MTT reaction was stopped with isopropanol acid reagent (HCl 4N and isopropanol; 1: 4), shaken on a shaker for 10 minutes. The absorption was reading with an ELISA reader at a wavelength of 550 nm. Data collection for the cytotoxic test is a) The absorbance data obtained from the cytotoxic test is converted into percent of living cells, b) The percentage of living cells is calculated using the formula:

\[
\text{Absorbance of cells with treatment - Absorbance of cell media control} \\
\times 100\%
\]

**Testing of isolates against apoptosis induction by flowcytometry**

Cells as many as 5x10^6 cells / well were planting in 6-well plates then the cells were incubating until normal. Cells were treating with DMSO solvent (0.25%) and active isolate at IC_{50}. The cells were then incubating again for 24 hours. At the end of the incubation time, the media taken and transferred into a centrifuge tube and centrifuged (2000 rpm, 3 minutes) then the supernatant was discarded. In wells that have been taken, the media, added with PBS, and PBS are transferred to the same micro tube from one treatment, and then centrifuged and then the supernatant is discarded. This stage is repeating once more then the cells are harvesting with trypsin. Cells were transferring into the same micro tube and then centrifuged (2000 rpm, 3 minutes). The remaining harvested cells in the well are rinsed with PBS and centrifuged again, then PBS is discarded. The precipitate was adding with the PI-Annexin V reagent carefully and immediately homogenized. Micro tubes containing cell suspension were wrapping in aluminum foil and incubated in a 37°C water bath for 5 minutes. The cell suspension homogenized again and transferred into a flowcytometer tube, using a nylon filter, and then it was ready to be analysis using a flow cyrometer.

**RESULTS**

**Isolation and identification of active substances**

4 gram n-hexane extract was fractionated with VLC using a gradient mobile phase of n-hexane and ethyl acetate (100% -25%) to produce three fractions (Fractions 1-3, 45 mg; 247,6mg; and 632,4 mg). Identification using TLC showed that the third fraction contained a bluish purple color indicating the presence of terpenoids. The third fraction is separate by dissolving with methanol, to separate the residue and filtrate. The residue was separate by semi preparative HPLC with methanol: acid water (9.95: 0.05) to produce five isolates (isolates 1-5, 0 mg; 4 mg; 1mg; 0 mg and 0,7mg).

In vitro testing of isolates on MCF-7 cells with an IC_{50} value of 43.51 µg/mL. This isolate was analyze by HPLC (Figure 1 [A]) at a concentration of 0.5 mg / mL, using methanol: acid (9.95: 0.05) as a solvent, indicating a major peak with a retention time of 24.39 minutes. The results of the UV spectrum of these compounds showed the absorption at 262, 271, 282, and 293 nm (Figure 1 [B]), which had the same absorption values as the ergosterol standards, namely 262, 271, 282, and 293 nm². Based on the 1H NMR spectrum, (i) there is an overlapping aliphatic proton signal in the 0.50-2.50 bpj region which indicates the characteristic of terpenoids, (ii) there is six methyl signals at δ 0.93 (3H, s, H-18) shift; 0.62 (3H, s, H-19); 1.03 (3H, d, J = 6.5 Hz, H-21); 0.92 (3H, d, J = 6.5Hz, H-28); 0.84 (3H, d, J = 6.6 Hz, H-26); 0.82 (3H, d, J = 6.6 Hz, H-27).
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Figure 1: [A] HPLC Chromatogram of A. blazei isolate profile from hexane extract using a 4.6x250 mm RP shimpack column, carried out with methanol solvent: acid water (99.5: 0.5), injection volume 0.04 mL, flow rate 0.7 mL/minute. [B] UV-Vis Spectra of isolate A. blazei from n-hexane extract by 1900i UV-Vis Spectrophotometer.

J = 6.6Hz, H-27) bpj, (iii) there is a characteristic signal in the chemical shift of 3.62 bpj which appears as a multiplet which is characteristic of the presence of -OH bound to C-3, and (iv) there is a typical signal in the chemical shift of 5.38-5.57 bpj which appears as a multiplet indicating the presence of a double bond, as showed Table 1. The spectrum profile is in accordance with the literature search of ergosterol. Based on the $^{13}$CNMR spectrum: (i) there are 28 carbon signals, (ii) one oxygenated carbon signal at 70.5 bpj which correlates with the typical multiplet signal at 3.62 bpj chemical shift ($^1$HNMR) which also shows the presence of -OH bound to carbon which is oxygenated, and (iii) there is a chemical shift at 116.4 - 141.5 ppm which indicates the presence of a double bond at positions C-6, C-7, C-21 and C-22. From the $^1$HNMR and $^{13}$CNMR data, it is known that the compound is an ergosterol structure with 28 carbon atoms, 44 proton atoms, and 1 atom oxygen, which is in accordance with the mass spectrophotometer data showing the results of m/z 397.23 [M·-H] which is consistent with the molecular formula C$_{28}$H$_{44}$O (Figure 2).

Anticancer activity of extracts, fractions, subfractions, and isolates (Ergosterol)

The activity was determined based on the MTT method that was guide by Bioactivity Guided Isolation of ergosterol isolate on MCF-7 cells having an IC$_{50}$ value of 43.10 µg /mL. This value indicates that the compound has strong cytotoxicity anticancer activity.
Table 1: Data on Proton and Carbon Shifting of compound 1 in the \textsuperscript{1}HNMR and \textsuperscript{13}C-NMR Spectrums.

| C | Compound-1 \textsuperscript{1}HNMR | Library-1 (Alexandre dkk, 2017) | Library-2 (Li, X., 2015) | Compound-1 \textsuperscript{13}CNMR | Library-1 (Alexandre dkk, 2017) | Library-2 (Li, X., 2015) |
|---|---|---|---|---|---|---|
| 1 | - | - | - | 38.4 | 38.4 | 38.5 |
| 2 | - | - | - | 32.1 | 31.9 | 32.1 |
| 3 | 3.62 m (1H) | 3.64 m (1H) | 3.61 m (1H) | 70.5 | 70.5 | 70.5 |
| 4 | - | - | - | 40.9 | 40.8 | 40.9 |
| 5 | - | - | - | 139.8 | 139.8 | 139.8 |
| 6 | 5.57 dd (1H) | 5.58dd (5.53,0 Hz,1H) | 5.56dd (5.4,2.2 Hz,1H) | 119.7 | 119.6 | 119.7 |
| 7 | 5.38 m (1H) | 5.38dd (5.4,2.9 Hz,1H) | 5.38dd (5.4,2.5 Hz,1H) | 116.4 | 116.3 | 116.4 |
| 8 | - | - | - | 141.5 | 141.4 | 141.3 |
| 9 | - | - | - | 46.3 | 46.2 | 42.3 |
| 10 | - | - | - | 37.1 | 37.1 | 37.1 |
| 11 | - | - | - | 21.2 | 21.1 | 21.1 |
| 12 | - | - | - | 39.2 | 39.1 | 39.1 |
| 13 | -- | -- | -- | 42.9 | 42.9 | 42.9 |
| 14 | - | - | - | 54.6 | 54.6 | 54.6 |
| 15 | - | - | - | 23.0 | 22.9 | 23.1 |
| 16 | - | - | - | 28.4 | 28.3 | 28.3 |
| 17 | - | - | - | 55.8 | 55.7 | 55.8 |
| 18 | 0.93 s (3H) | 0.95s(3H) | 0.95 s(3H) | 12.1 | 12.1 | 12.1 |
| 19 | 0.62 s (3H) | 0.65 s (3H) | 0.63 s (3H) | 16.4 | 16.3 | 16.3 |
| 20 | - | - | - | 40.3 | 40.3 | 40.4 |
| 21 | 1.03 d (J=6.5, 3H) | 1.04 d (J=6.6Hz, 3H) | 1.00 d (J=6.6Hz, 3H) | 21.2 | 21.1 | 21.2 |
| 22 | 5.19 m (1H) | 5.20 m (1H) | 5.20 m (1H) | 135.7 | 135.6 | 135.6 |
| 23 | 5.21 m (1H) | 5.21 m (1H) | 5.20 m (1H) | 132.0 | 131.9 | 132.1 |
| 24 | - | - | - | 42.9 | 42.9 | 42.9 |
| 25 | - | - | - | 33.1 | 33.1 | 33.1 |
| 26 | 0.84 d (J=6.6 Hz, 3H) | 0.84 d (J=6.7Hz, 3H) | 0.84 d (J=6.7Hz, 3H) | 20.0 | 19.9 | 20 |
| 27 | 0.82 d (J=6.6Hz, 3H) | 0.82 d (J=6.7Hz, 3H) | 0.83 d (J=6.7Hz, 3H) | 19.7 | 19.7 | 19.7 |
| 28 | 0.92 d (J=6.5Hz, 3H) | 0.92 d (J=6.6Hz, 3H) | 0.95 d (3H) | 17.7 | 17.6 | 17.6 |

Figure 2: Ergosterol structure.
Results of MCF-7 cell cycle regulation due to ergosterol treatment

The treatment of ergosterol compounds at a dose of IC₅₀ 43.1 µg/mL on MCF-7 cell proliferation caused changes in the cell cycle with an increase in G2 phase accumulation 27.3%. Based on the results of statistical tests showed that the increase in G2-M phase accumulation was significantly different from control (p <0.001). Changes in the distribution of the cell cycle after treatment will direct the resting cells to a certain phase in the cell cycle, so that this causes the induction of cell death. These results lead to the induction of cell death. The results of the MCF-7 cell cycle analysis due to treatment of the ergosterol are presented in Figure 3.

The result of the mechanism of action of Ergosterol on the induction of apoptosis

The results of the analysis of cell apoptosis caused by ergosterol treatment (isolates) on MCF-7 cells showed that the cells experienced an increase in early apoptosis 30.4%, late apoptosis 16.4%, and a decrease in necrosis 7.4%. Based on the results of statistical tests showed that the increase in apoptosis induction was significantly different from the control (p <0.001). The increase in cell apoptosis also correlates with the increase in cell accumulation in the G2/M phase. The results of the apoptosis induction analysis of MCF-7 cells due to treatment of ergosterol compounds showed in Figure 4.

DISCUSSION

This research has identified active anticancer compounds from n-hexane extract of Agaricus blazei Murill. The terpenoid compound in the form of ergosterol has been identified, based on the results of HR-MS analysis revealed the ion peak (M⁺H⁻) at 397, 23 and MS analysis is consistent with the molecular formula C₂₈H₄₄O₂. Agaricus blazei Murill has been widely reported to have anticancer activity of several cancer cells, including leukemia cells¹, prostate cells²⁷, human lung LU99 and stomach KATO III cancer line²⁶, pancreatic cells¹⁶, while for MCF-7 cells it has not been reported. Ergosterol isolated from Pleurotus salmonostramineus on Trypanosoma cruzi activity², isolation in Amauroderma rude to the activity of murine cancer cell line B16.
and breast cancer cells MDA-MB-231. Isolation of *Gonaderma sp* on dipeptidyl peptidase-4 inhibitory activity, can also inhibit human tumor cells and HUVECs in vitro. Ergosterol from the isolation of *Agaricus blazei* Murill, which has activity against cells of MCF-7 cancer, have not reported. The results of the isolation of *Agaricus blazei* Murill, namely ergosterol, including having strong cytotoxicity activity with an IC₅₀ value of 43.10 µg/mL. In this mechanism, cancer cells will die naturally (programmed), so as not to cause a systemic inflammatory response that causes serious side effects even to death. Ergosterol is a terpenoid compound, a white powder, in terms of cell regulation, it shows that ergosterol treatment on MCF-7 cells causes cell cycle inhibition in the G2/M phase, which is characterized by an increase in the number of cells in the G2/M phase by 9.9%. This increase in the G2/M phase has an effect on the induction of apoptosis of cancer cells.

Ergosterol isolated from *Amauroderma rude* can suppress the growth of MDA-MB-231 breast cancer cells through the apoptotic pathway by increasing FOXO3 expression. FOXOs can induce apoptosis via the mitochondrial pathway as well as via the Fas ligand (FasL). Myochondrial pathways through the proapoptotic Bcl-2 family, such...
as Bim and bNIP3, cause mitochondrial permeability, which activates caspase9, caspase3 and apoptosis. The Fas ligand pathway is to activate FasL and TRAIL which will activate caspase 8, caspase 3 and finally increase the induction of apoptosis

CONCLUSION

Ergosterol isolated from n-hexane extract of Agaricus blazei Murill is a good candidate for the development of anti-cancer drugs, which are able to increase the apoptosis induction of MCF-7 cancer cells through cell cycle inhibition mechanisms in the G2 / M phase.

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**GRAPHICAL ABSTRACT**

- **EXTRACTION** (Maceration n-Hexan)
- **FRACTIONATION** (N-Hexan: Aethyi Acetate)
- **ISOLATION** (HPLC)
- ERGOSTEROL
- MTT cytotoxic test
- Apoptosis Induction Assay

**ABOUT AUTHORS**

**Sukardiman** is Professor Pharmacognosy on Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia. He has vast experience in the area of pharmacognosy, natural product and pharmacology. He has projects in developing product antidiabetic, anticancer from Indonesian herbal medicine, and herbal standardization. Guiding students for PhD and studies of various Universities. He has publication in National and International Journal.

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Sentot Joko Raharjo as Lecturer in Academic of Pharmacy and Food Analyst of Putra Indonesia Malang Indonesia. He has vast experience in the area of Pharmacy Analyst, Medical Chemistry, Natural Organic Chemistry, and Food Function. He has projects gen database, protein database and active compound database in UB Smonagen Group. He has publication in National and International Journal. He research is focuses in silico on drug discovery from bioactive natural product compounds for various diseases therapy.

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