Document heading doi: 10.21276/apjhs.2020.7.1.13 **Original Research Article**

Cytotoxicity activity of Liverwort (*Marchantia Polymorpha* L.)Methanolic Extract to HeLa cell Line

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ABSTRACT

Background: Liverwort (*Marchantia polymorpha* L) is one of plant that has high active compound such as flavonoid, terpenoid, phenolic that has potential as anticancer. This research aimed to determine the effect of liverwort extract to viability and apoptosis induction. **Method**: Liverwort's leave were extracted using methanol solvent. HeLa cell line is used as model of treatment. Cell were incubated 72 hours in RPMI-1640 and then treated with various concentration of liverwort's extract. Active compound of liverwort assay using Thin layer Chromatography (TLC) method, viability/citotoxicity Hela cell using WST-1 method, apoptosis cell using fluoresen microscop. Analyz data used kruskawallis. **Result**s:Liverwort active compound concist of flavonoid, terpenoid and phenolic. Concentration 150, 250, 500, 1000 µg / mL extract methanolic marchantia polymorpha inhibit 40 %, 67 %, 67 % and 79 % growth of Hela cell line. Cell apoptosis induction begins at a concentration of 50 µg / mL, whereas at a concentration of 250 µg / mL cell apoptotic phase of almost 100%. **Conclusion:** Methanolic extract of liverworts (M. polymorpha L.) inhibits growth of Hela cell and induced cell apoptotic.

Key words: liverwort (*Marchantia polymorpha* L), citotoxicity, HeLa cell line

INTRODUCTION

Cervical cancer is a third-order type of cancer that causes death of women in the world.[1] Most cervical cancers are caused by infection with the human papilloma virus (HPV). Various ways to overcome this cancer have been done through surgery, chemotherapy, radiotherapy and chemoradiotherapy.[2] Conventional chemotherapy can cause multidrug resistance which decreases the sensitivity of cancer cells to drugs.[3]Moss is one of the low-level plants that have potential as anticancer and antioxidants.[4,5] Among the many species of mosses that are suspected of potential anticancer are liverworts (M. polymorpha L.). This moss can easily be found on river cliffs, well walls, wetlands, and wet rocks. With the abundance of mosses, there should be many benefits that can be developed.

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Previous research on the types of moss in the same genus, Marchantia convulata in China, found that many potential compounds contained in these plants are flavonoids, terpenoids and triterpenoids. Flavonoid compounds that are mostly contained are quercetin, luteolin and c-glycosides. These compounds are proven to contain anti-hepatitis substances.[5] According to Widiyati (2006) and Patil (2009). [6,7], alkaloids, flavonoids and terpenoids are able to increase caspase-3 activity to induce apoptosis of cancer cells. Until now, the potential of M. polymorpha L. mosses is only known to have antioxidant content in the form of phenolic acids such as galat, vanylate, chlorogenic, cinnamic, kumarat and ferulate[4], while important aspects of whether the moss extract has the potential as an anticancer is unknown . Therefore, specific research in this direction is important

MATERIALS AND METHODS

Marchantia polymorpha was collected in Mt. Merapi area, Yogyakarta, Indonesia on September, 2014.

Plant material and extraction

Marchantia polymorpha were washed with running water. Thallus (leaves) were separated from rhizoid. Thallus allowed to air-dry in shade for a week. During the drying process, the thallus wrapped with paper. The plant material was subsequently homogenised in electrical blender. About 350 g of powdered sample was extracted until exhaustion by maceration with 3 L of methanol 80% for 2×24 h. The extracts were filtered and re-extracted in same way three to four times until the colour of extract seen transparent. The filtrate were combined and evaporated using a rotary evaporator. Crude extract dissolved in dimethyl sulphoxide (DMSO).[8]

Cell culture

Hela cell line was cultured in Roswell Park Memorial Institute(RPMI)-1640 medium with a supplement of 10% FBS, 100 µg / mL penicillin, 100 µg / mL streptomycin, and 1 ml fungizone. Cells were cultured in flask culture at 37 ° C with 95% air humidity and 5% CO_2 until confluent.

Marchantia Methanolic Extract Assay

To detect the active compound of methanolic extract of liverworts, detection was done using thin layer chromatography (TLC) method. The active compounds detected in methanolic extracts of liverworts consist of flavonoid, alkaloid, terpenoid and phenol compunds.

Cell viability assay

Treatment of marchantia extract are 11 treatments with negative control and concentrations of liverwort methanolic extract: 10; 15; 30; 45; 60 90; 120; 150; 250; 500 and 1000 µg. Cell survival was accessed by cleavage of the tetrazolium salt WST-1 to formazan by cellular enzymes. An expansion in the number of viable cells results in an increase in the overall activity of mitochondrial dehydrogenases in the sample. Quantification of the formazan dye produce by metabolically active cells by scanning multiwell spectrophotometer. Briefly, 10^3 cells in 100 µl of media to each well were seeded into 96-well cell culture plate and incubated for 72 h. The medium was then replaced with 100 ml fresh medium contained marchantia extract was added in the next day appropriate to its concentrations for 24 h. Then after, the medium was replaced with 100 µl fresh medium and 10 µl cell proliferation reagent WST-1 (4-[3-(Iodophenyl) -2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) (Roche). Incubate the cells for 1 h in a humidified atmosphere. Shake thoroughly for 1 min on a shaker. Measure the absorbance of samples againts a background control as blank using microplate (ELISA) reader at 450 nm. Cell viability is calculated by the formula:

$$\text{Cell viability} = \frac{\text{Aborbance of sample}}{\text{Absorbance of control}} \times 100$$

Cell Apoptotic Assay

The apoptotic treatment consisted of six treatments, are negative control without treatment and positive control giving doxorubicin 7 µg / mL and marchantia extract with concentration of 50; 100; 150 and 250 µg / mL. For treatment, insert cover slip into 24 micro-plate wells then cells were seeded into a microplate well with a density of 5×10^4 cell / well. Cell was incubated for 36 hours. After confluent, remove medium then added 200 µl of marchantia extract that has been dissolved in the medium into each plate well and incubated for 24 hours. Remove medium and take the cover slip.Added 10 µl acrydin orange to glass object. Place the cover slip on the glass object, then observe it using a fluorescent microscope with a wavelength of 515-565 nm (FITC).Counting the normal cell and apoptosis cell minimum 200 cells in each treatment.

Statistical analysis: Statistically analysis was performed using one-way ANOVA and Duncan for further analysis ($p<0.05$). Statistical data analysis was performed with SPSS software ver. 20. Densitometric of HSP70 bands was analysis using description method

RESULTS

Marchantia Methanolic Extract Assay

Qualitative analysis is done by thin layer chromatography (TLC) because it is relatively simple, inexpensive and quite accurate. The group of compounds identified are flavonoids, alkaloids, terpenoids and phenolics. The results of testing the active compound content of liverworts can be seen in [Table 1].

Table 1: Result of thin layer chromatograpy (TLC) of Marchantia polymorpha compound

S.No	Compound	Result	Rf Value
1	Terpenoid	Positive	0.15 0.24 0.82 0.97
2	Alkaloid	Negative	-
3	Flavonoid	Positive	0.8
4	Phenolic	Positive	0.53

[Table 1] showed, metholic extracts of liverworts detected consist of active compounds. They are terpenoids, flavonoids and phenolics. Alkaloids were not detected in the test. The Rf value is defined as the distance traveled by the compound divided by the distance traveled by the mobile phase. The number of spots with different Rf numbers can describe the number of types of compounds contained in the test extract. In the rays appear yellow flavonoid patches, red violet terpenoids, phenolic blue. In Table 1 it can be seen that terpenoids have the highest Rf values up to 0.97 of other detected compounds. Phenolic has the lowest Rf with an Rf value of 0.53, while the flavonoids have an Rf of 0.8. Alkaloids do not have Rf because the compound was not detected in the test.

Hela cell viability

Table 2: Hela cell viability

S. No	Marchantia concentration (µg/mL)	Cell viability (%)±SD
1	0	100±0
2	M10	98.95±1.01
3	M15	96.75±0.66
4	M30	95.34±1.02
5	M45	91.03±2.85
6	M60	90.26±5.10
7	M90	88.91±2.74
8	M120	74.74±3.91
9	M150	59.70±1.80
10	M250	33.33± 4.51
11	M500	32.95±7.06
12	M1000	20.98±1.40

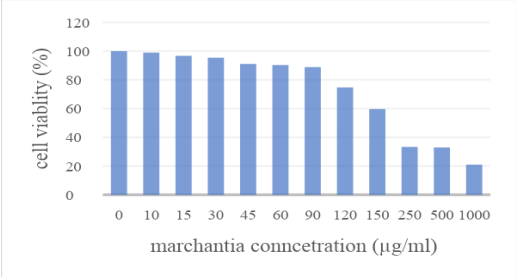

Figure 1: Graphic of Hela cell line viabilty

Hela cell cytotoxicity testing is a preliminary test to determine the potential of metholic extracts of liverworts as anticancer and to determine the IC50 of the extract against HeLa cells. The test results, in Figureure 1, showed decreasing of viability Hela cell line in each treatment. Concentration 150, 250, 500, 1000 µg / mL extract methanolic marchantia polymorpha inhibit 40 %, 67 %, 67 % and 79 % growth of Hela cell line[Table 2].

Apoptotic Cell Assay

The percentage of apoptosis is determined based on the comparison between red cells (apoptosis) and green cells (healthy cells and initial apoptosis). In Figure 2 observed increasing cell apoptosis is directly proportional based on increase extract concentration. At concentrations (50,100,150 µg / mL) Apoptosis cell reached 50%, whereas at concentrations of 250 µg / mL apoptosis cell reach 85.67% [Table 3]

Table 3: Apoptosis Precentage induced *Marchantia polymorpha* extract

S.No	Concentration(µg)	Apoptosis cell (%) ± SD
1	control	0±0
2	Doxo 7	26.33±5.13
3	M50	3.3±2.3
4	M100	19.33±6.66
5	M150	31±5.56
6	M250	85.67±10.69

Morphology of Hela cell (Figure 2) showed methanolic extract of liverworts induced cell apoptosis. The apoptotic phase that occurs starts from the initial phase to the end of apoptosis. Cell apoptosis induction begins at a concentration of 50 µg / mL, whereas at a concentration of 250 µg / mL cell apoptotic phase of almost 100%.

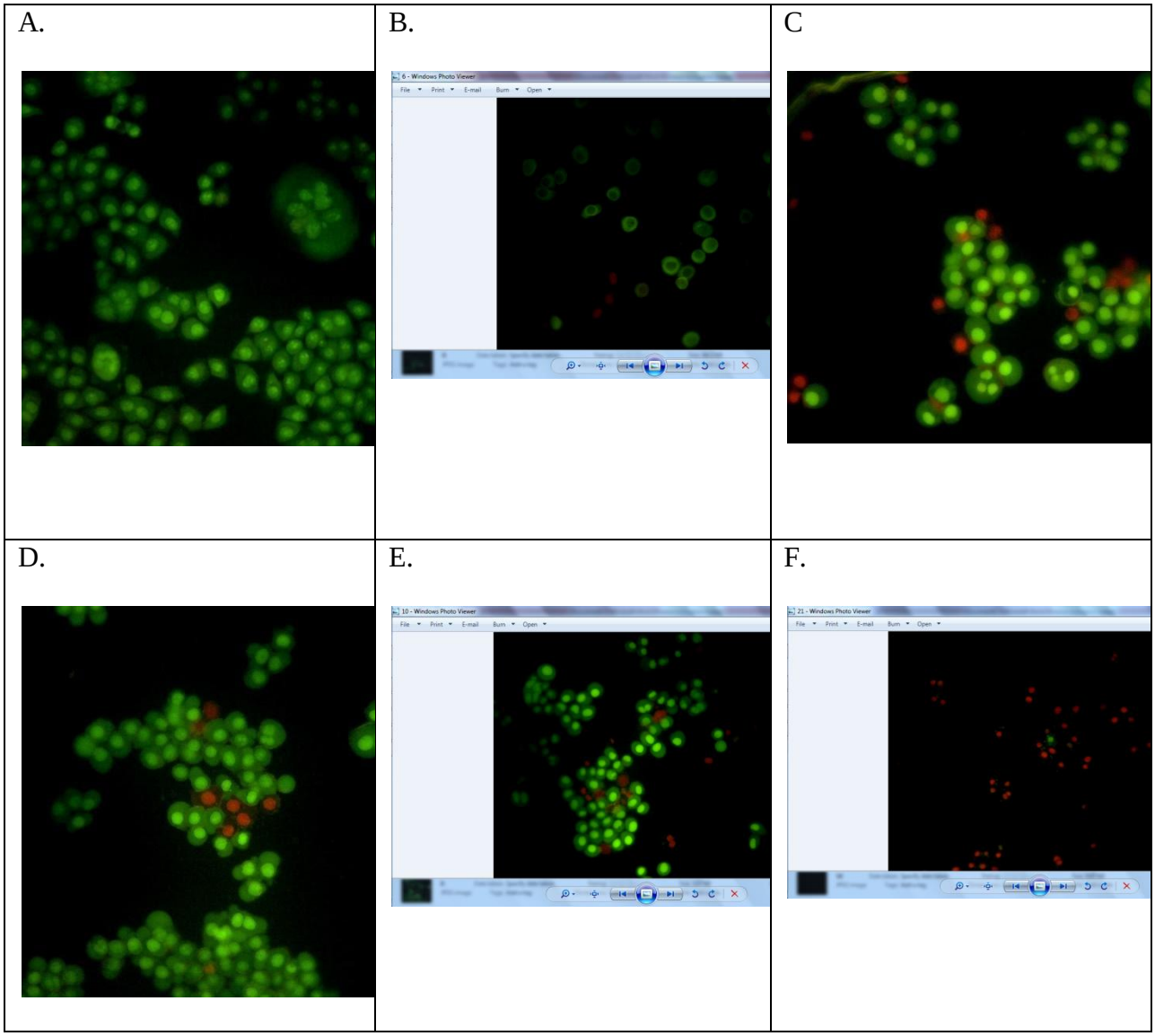

Figure 2:Morphology of Hela cell line using fluorescent microscope., zoom in 10x4, colouring using *ethidium bromide-acrydin orange*, a) control treatment; b) doxorubicin 7 µg/mL; c) 50 µg/mL; d) 100 µg/mL; e) 150 µg/mL; f) 200 µg/mL; g) 250 µg/mL; h) 300 µg/mL; g) normal cell; h) early apoptotic; i) apoptotic cell

DISCUSSION

Krishnan and Murugan (2013) detected compounds of liverworts and found that liverworts extract contained active compounds in the form of flavonoids, phenols, terpenoids and glycosides and also detected alkaloids.[4] The undetectable alkaloids in this study may be influenced by environmental factors and the location of the moss sampling, so that there are differences in the class of compounds detected by the research of Khrishnan and Murugan. Ammasavate *et al*., (2010) used the standard of a cytotoxic material if the IC50 value <100 µg / ml.[9] Based on Ampasavate *et al* (2010) methanolic extracts of liverworts are not cytotoxic to HeLa cells.[9] Decreased cell viability due

to the content of active compounds from methanolic extracts of liverworts that induce cell death (apoptosis) based on the results of TLC tests the content of active compounds of liverworts in the form of flavonoids, terpenoids and phenolics. The same study was also carried out by Jian-Bo (2006), Macrchantia convulata flavonoids were treated with cell line.A dose of 40 µg / ml 75% inhibits cell growth.[5] The molecular mechanism of how flavonoids induce apoptosis is not yet known. Several mechanisms may be involved, including inhibiting the activity of DNA topoisomerase I / II, regulation of heat shock expression of protein, release of cytochrome c by activation of caspase-9 and caspase-3, decreasing the regulation of Bcl2 and Bcl-X expression but increasing the expression of Bax and Bak, transcription factors kappaB (NF-kappaB), endonuclear activation and suppress Mcl-1 protein[10]. Essential oils and tritepenoids are one of the terpenoid groups which are also potential as anticancer. The methanolic extract of liverworts contains terpenoids which indicate that liverworts also have potential as anticancer. Terpenoids induce apoptosis through increased p-53 activity. [6,7]Healthy cells are permeable to acrydin-orange. Healthy cells have cell membranes that are impermeable to ethidium bromide, so the normal cell nucleus appears green under the observation of fluorescent microscopy. Cells that are in the early apoptotic phase still have a membrane that is impermeable to ethidium bromide, so the cell nucleus is green but the cell nucleus is condensed. Late apoptosis phase, the cell membrane is permeable to ethidium bromide so that the cell nucleus is orange. In this study, 31 % cell apoptotic induced by concentration 150 µg / mL.

CONCLUSION

1. The content of the active compound of metholic extract of liverworts (*M. polymorpha* L.) in the form of flavonoids, terpenoids and phenolics.
2. Methanolic extract of liverworts (M. polymorpha L.) inhibits 40% of cell growth at concentration 150 µg / ml.
3. Extracts of liverworts induce cell apoptosis.

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