

Biological activities of *in vitro* liverwort *Marchantia polymorpha* L. extracts

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Abstract

To overcome the problems in liverwort collecting such as small size and easily mixed with other species in the wild, we have successfully cultivated *Marchantia polymorpha* L. under *in vitro* conditions in the previous study. The aim of this study is to evaluate the biological activities of this *in vitro* biomass as a confirmation of the sufficient protocol in cultivation this species. Cultured biomass was dried at a temperature of 45-50 °C to constant weight and ground into a fine powder. The coarse powder was extracted with organic solvents of increasing polarization including n-hexane, chloroform, ethyl acetate, and ethanol using the maceration technique. Four extracts were investigated antioxidant (iron reduction power, DPPH), antibacterial (agar diffusion), tyrosinase inhibitory activity, anti-proliferation on MCF-7 cells. Additionally, the presence of natural metabolite groups of the extracts was detected by using specific reagents. For antioxidant activity, ethyl acetate fraction extract had the highest iron reducing power and DPPH free radical scavenging ability with $IC_{50} = 439.31 \mu\text{g ml}^{-1}$. All three n-hexane, chloroform, and ethyl acetate extracts possessed resistance to the bacterial strain tested. At a concentration of 2 mg ml⁻¹, n-hexane and chloroform extracts had the highest percentage of tyrosinase inhibition (69.54 and 69.10%, respectively). The n-hexane extract is a potent extract that inhibits the proliferation of MCF-7 cells with the lowest IC_{50} of 38.15 $\mu\text{g ml}^{-1}$. A preliminary chemical composition survey showed that the cultured biomass liverwort contains many bioactive compounds, particularly the compounds of range of non- and less-polarized fractions.

Keywords: biological activities; *in vitro* biomass; liverwort; *Marchantia polymorpha* L.

Introduction

Marchantia polymorpha L. is a common liverwort species, widely distributed in many parts of the world (Asakawa *et al.*, 2000; Shimamura, 2015). Their habitat may range from humid tropical rain forests to dense urban areas (Shimamura, 2015). Currently, this non-vascular plant species is manipulated as a model plant in

molecular biology experiments related to the adaptation of terrestrial plants (Bowmann *et al.*, 2007). Because this plant can maintain under laboratory conditions, in addition, the single haploid chromosome makes molecular biology approaches easier to perform, and the phenotypic results are easy to evaluate because several genotypes control the phenotype (Tanaka *et al.*, 2016).

In terms of medicine and pharmaceutical, *Marchantia polymorpha* has long been used as the folk medicinal experience of some countries such as China and India to help diuretic activity, hepatitis, open wounds and burns, insects, and snake bites, etc (Asakawa *et al.*, 2013; Chandra *et al.*, 2016). Studies on liverworts and *Marchantia polymorpha* as a member show that these plants a fascinating group because they can produce a wide range of bioactive compounds (Asakawa and Ludwiczuk, 2013; Asakawa *et al.*, 2013). Besides, the small, fragile body structure and the absence of a vascular system require the effectiveness of these compounds to help these plants adapt to attack from outside agents and survive under challenging conditions (Asakawa *et al.*, 2013). The secondary metabolites found in *Marchantia polymorpha* are volatile compounds that belong to the terpenoids (for example, thujopsene, β -chamigrene, etc.) and aromatic compounds, including bibenzyls and bisbibenzyls (Asakawa *et al.*, 2013; Tanaka *et al.*, 2016). Biological activity investigations show the potential of some bisbibenzyl structures such as marchantin A, C, perrottetin E, etc., to exhibit many activities including antibacterial, antifungal, antiviral, anti-inflammatory, anti-proliferation of cancer cell lines, etc (Asakawa *et al.*, 2000; Asakawa *et al.*, 2013).

The most significant difficulty when approaching this group of plants in the process of studying natural compounds and biological activity is biomass (Asakawa *et al.*, 2013; Hans *et al.*, 1991). Bryophytes collection in the natural habitats endures many obstacles because these species are small in size, fragile, and mixed between many species, causing the biomass impure (Hans *et al.*, 1991). For instance, Southeast Asia regions, with its humid climate, is a suitable place for many types of liverworts to grow, resulting in the collection of samples often mixed with many different species of leafy liverworts family-Lejeuneaceae (Asakawa and Ludwiczuk, 2017). Therefore, axenic culture can be considered as an independent and proactive method of biomass collection to overcome the problems mentioned above (Hans *et al.*, 1991).

In Vietnam, we also noted the presence of *Marchantia polymorpha*, but there have been limited published claims regarding the use of this liverwort in treating some folk diseases or the natural compounds (Asakawa *et al.*, 2000). Along with the difficulties in the sample collection, we have successfully researched and cultivated this species under *in vitro* conditions in order to collect pure materials proactively (Tan *et al.*, 2020). This source should be estimated for biological activities and secondary metabolites. Because several studies around the world have shown that *in vitro* cultivating bryophytes are more active with wild-type specimens, as concluded by Aneta *et al.* when studying the antifungal activity of some mosses such as *Atrichum undulatum*, *Physcomitrella patens* (Aneta *et al.*, 2011). Or the study of Sabovljevic and colleagues on *Marchantia polymorpha* subsp. *ruderalis* found that *in vitro* culture materials could produce new bisbibenzyls which are not available in a natural source (Sabovljevic *et al.*, 2016).

In this study, the *in vitro* biomass was evaluated through investigations of some biological activities, thereby providing reference information for this liverwort species in Vietnam, which is not fully documented for both folk experience and scientific evidence.

Materials and Methods

Materials

Liverwort *Marchantia polymorpha* L. was obtained from the improved culture process of Tan *et al.* (2020), in which, the liverwort was cultured on liquid half-strength Gamborg B5 medium, supplemented with 20 g l⁻¹ sucrose, 1.5 g l⁻¹ tryptone, pH adjusted to 5.5-5.6, illuminated by blue LED light (455 nm) and light

intensity of 2300 lux, culture substrate is a cotton swab layer. The cultivate medium was autoclaved in 20 minutes, 1 atm and 121 °C. After 7 weeks of culture, the liverwort biomass was harvested, washed under running tap water and dried at a temperature of 50 °C.

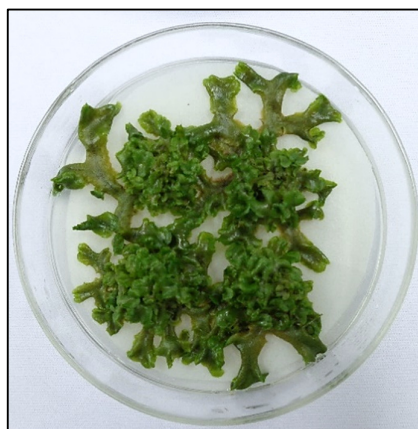


Figure 1. *Marchantia polymorpha* L. in improved cultured process (bar: 1 cm)

Bacterium strains including Gram-positive (*Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228) and Gram-negative (*Vibrio parahaemolyticus* ATCC 17802, *Salmonella typhimurium* ATCC 14028, *Pseudomonas aeruginosa* ATCC 27853) was stored and provided by Department of Plant Biotechnology and Biotransformation, Faculty of Biology and Biotechnology, University of Science, VNU-HCMC, Viet Nam. The Luria-Bertani Broth (LB) medium (yeast extract 5 g l⁻¹, tryptone 10 g l⁻¹, NaCl 10 g l⁻¹) was used to grow bacteria supplemented with 15 g l⁻¹ agar and adjusted pH to 7.0, autoclaved in 20 min, 1 atm and 121 °C.

Preparation of fraction extracts

After drying at 50 °C to constant mass, the liverwort sample was ground into a powder. This powder was used to prepared fraction extracts based on maceration method reference from Nada *et al.*, (2016). First, the powder was soaked with n-hexane at the ratio of 1 g powder: 10 ml solvent, the mixture was filtered, and the extract was collected using a vacuum rotary evaporator (HS-2005S-N, Hahnshin Scientific Co.). This process was carried out repeatedly until exhaustion. Plant powder after being extracted with n-hexane will be replaced successively with the other solvents of increasing polarization, including chloroform, then ethyl acetate and ethanol as final.

Evaluating reducing power

Liverwort extracts were evaluated for their reduction capacity through participation in the conversion of Fe³⁺/ K³[Fe(CN)₆] to Fe²⁺/ K⁴[Fe(CN)₆] as reported by Manmohan *et al.* (2014) with some minor modifications. Firstly, 1 ml of the extract (2 mg.ml⁻¹ in ethanol or DMSO) in a test tube is mixed with 2.5 ml of sodium phosphate buffer (0.2 M and pH 6.6) and 2.5 ml of 1% K₃[Fe(CN)₆]. The mixture is incubated at 50 °C for 20 min. Continue adding 2.5 ml of 1% trichloroacetic acid to the mix and chill. Centrifuge the solution at 2000 rpm for 10 min to remove the precipitate. 1 ml of the supernatant is added with 2 ml of distilled water, 0.5 ml of 1% FeCl₃, and mix. The mixture absorbance is detected at 700 nm after 5 min.

DPPH scavenging assay

The fractional extract was dissolved in an ethanol solution containing 10% DMSO (v/v) and diluted in concentrations gradually reduced from 5 to 0 mg ml⁻¹. 0.5 ml of the test extract solution was mixed with 3 ml

of absolute ethanol. The mixture was then added with an additional 0.5 ml of DPPH 0.6 mM (prepared in absolute ethanol) (Ghasemi *et al.*, 2009). The mixture was shaken well and incubated in the dark for 30 min at 28-30 °C. The solutions were then measured with a 517 nm wavelength absorbance. The formula calculated the percentage of free radical scavenging:

$$\% \text{ Inhibition} = 1 - \frac{A_t}{A_c} \times 100 \quad (1)$$

In which, A_t and A_c is the absorbance value of the test sample and the negative control (ethanol containing 10% DMSO), respectively. Based on the corresponding percentage value of each decreasing concentration, the IC_{50} value was calculated.

Agar diffusion for anti-bacterial investigation

Anti-bacterial activity was investigated by agar diffusion method (Balouiri *et al.*, 2016). First, the bacteria are cultured in liquid LB medium to reach OD 625 nm ≥ 0.5 , then the bacterial solution is adjusted to OD 625 nm in the range of 0.1 to 0.15 and bring 100 μ l to spread on Petri dish containing LB agar medium. A 7 mm diameter aluminum tube is used to make the agar wells, and each well was added 50 μ l of test solution. The test solutions include a 0.4 mg ml⁻¹ chloramphenicol as positive control, 60 mg ml⁻¹ test extracts and a negative control-aqueous solution containing 10% DMSO. After incubation of the petri dishes in the dark at 37 °C for 24 h, the diffusion zones from the agar wells were recorded in diameter and calculated by the formula:

$$\text{Inhibitory zone (mm)} = \text{diameter of the diffusion zone of the test solution (mm)} - \text{diameter of the diffusion zone of negative control (mm)}. \quad (2)$$

Tyrosinase inhibitory activity assay

The inhibitory activity of tyrosinase was conducted according to the procedure of Hanh *et al.*, (2017) with some minor modifications. The experiment was conducted with a 96-well plate, each initially containing 70 μ l of the liverwort extract made in potassium phosphate buffer (50 mM and pH 6.8) containing 5% DMSO, 30 μ l mushroom tyrosinase solution (Sigma T3824-25KU) 124.61 U ml⁻¹. The mixture was incubated at 32-34 °C for 15 min. Then, continue to add 110 μ l of L-tyrosine substrate 2 mM and incubate for another 20 min. The product of the reaction was measured for absorbance at 490 nm. The blank sample is enzyme-free and the negative control is a non-extracts sample. Fraction extracts were prepared at 2 mg ml⁻¹ and positive control was kojic acid at 200 μ g ml⁻¹.

The percentage of inhibition is calculated by the formula:

$$\% \text{ Inhibition} = 1 - \frac{(A_s - A_{sb})}{(A_c - A_{cb})} \times 100 \quad (3)$$

With:

A_s : mixture absorbance of test sample

A_{sb} : mixture absorbance of test blank sample

A_c : mixture absorbance of negative control

A_{cb} : mixture absorbance of negative blank control

Anti-proliferation of MCF-7 using SRB method

The experiment was conducted according to the process of My-Nuong and Thuy-Duong, (2016) with some minor modifications. MCF-7 breast cancer cells are grown and spread on a plate of 96-wells so that each well contains 100 μ l medium and 10,000 cells. The cells were investigated with extract at a concentration of 100 μ g ml⁻¹, parallel to the negative control DMSO 0.25% and positive control-camptothecin 0.05 μ g ml⁻¹. After 48 hours of induction, the cells were washed and fixed with 50% cold trichloroacetic acid, continued to stain sulforhodamine B (SRB) 0.2% for 20 min, washed 4 times with acetic acid 1%, allowed to dry for 12 to 24 hours and dissolve the SRB completely with Tris 10 mM in 10 min. The absorbance value (measured with

an ELISA reader) was calculated by the 492 nm wavelength absorbance minus 620 nm absorbance value. The percentage of cell proliferation inhibition is calculated by the formula:

$$\% \text{ Inhibition} = 1 - \frac{A_t}{A_c} \times 100 \quad (4)$$

In which, A_t and A_c are the absorbance value of the extract sample and negative control, respectively.

Detection of some secondary metabolites by using specific reagents

The experiment was conducted in consultation with Harborne, (1984) and Jayaveera *et al.*, (2010) to determine the presence of typical secondary metabolites in plant extracts. Metabolite groups (with corresponding reagents) include: phenolics (FeCl_3 5%), quinone and coumarin (KOH 5%/methanol), tannins (saline gelatin), alkaloids (Wagner reagent), flavonoids (concentration sulfuric acid, lead acetate 10%, NaOH 1%/ethanol, Cyanidin test), terpenoid (Rosenheim for tri-terpen and Salkowski reaction for steroid, saponin foaming reaction), glycoside derivatives (Molisch reagent) and lactone ring compounds (Baljet reagent). The experiment was carried out simultaneously between liverwort extract samples at 2 mg ml⁻¹ and ethanol containing 10% DMSO as negative control.

Statistical analysis

Each experiment was repeated three times to obtain results except the preparation phase of liverwort fraction extracts. All data was processed by SPSS 16.0 (IBM SPSS Inc.) and Microsoft Excel 2010 (Microsoft Software). Results were presented as means and standard deviations (Mean \pm SD). The Duncan test is used to evaluate statistical differences at level of $p < 0.05$.

Results and Discussion

Preparation of fraction extracts

The extraction efficiency compared to the dry mass (24.20 g) of the cultured plant sample (409.28 g) was recorded in Table 1.

Table 1. Fraction extracts' mass (g) and efficiency (%) of extracts compared to dried weight (24.20 g) of *in vitro* cultured liverwort *Marchantia polymorpha* L

Fraction extracts	Extract's mass (g)	Efficiency (%)
n-Hexane	0.32	1.32
Chloroform	0.43	1.78
Ethyl acetate	0.32	1.32
Ethanol	1.27	5.26

The ethanol fraction had the largest mass and extraction efficiency (1.27 g and 5.26%, respectively), followed by chloroform, the two extracts had the same lowest extraction efficiency of n-hexane and ethyl acetate with 1.32%. As can be seen that *in vitro* cultured biomass of *Marchantia polymorpha* contained more compounds with strong polarity, better solubility in ethanol than weak and medium polar compounds.

Evaluating reducing power

The reducing capacity of the test substance is expressed through the 700 nm wavelength absorbance, the higher the absorbance value, the greater the reduction capacity. Absorbance values of 700 nm wavelength for positive samples and ascorbic acid are shown in Table 1. The concentration of ascorbic acid is 0.5 mg ml⁻¹ and the extracts are 2 mg ml⁻¹.

The ethyl acetate extract had the highest absorbance compared to the rest, which means that the reducing power of this extract is the greatest among the four fractions (Figure 2). The remaining extracts had

reducing capacity in descending order of chloroform, hexane, and finally ethanol. Although different in test concentration, the extracts did not stand out when compared to the positive ascorbic acid (absorbance nearly six times higher than the absorbance of ethyl acetate).

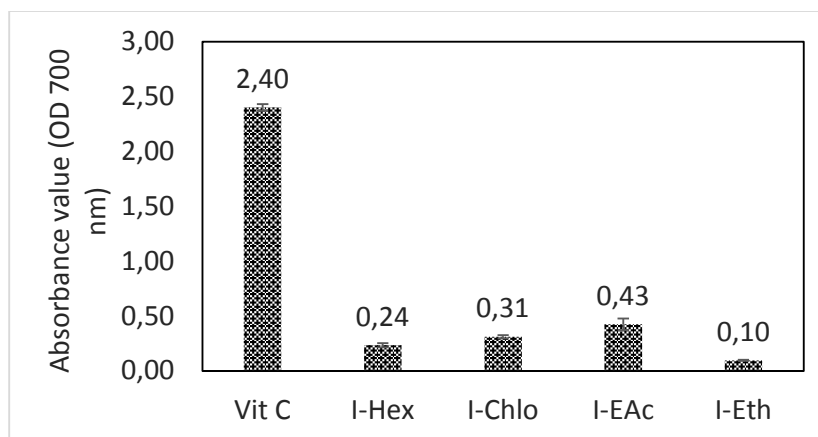


Figure 2. Absorption value at the wavelength of 700nm of fractional extract samples and positive vitamin C. Note: Vit C-Ascorbic acid; I-Hex, -Chlo, -EAc and -Eth correspond to n-hexane, chloroform, ethyl acetate, and ethanol extracts

DPPH scavenging activity

In addition to the reduction capacity, the extracts were also assessed for their ability to scavenge free radicals. In this model, the IC₅₀ (half-maximal inhibition concentration) value will show the DPPH's free radical scavenging capacity of a substance or a test sample, notably the lower the IC₅₀ cost, the stronger the antioxidant capacity is. IC₅₀ values of extracts and ascorbic acid are presented in Table 2.

Ethyl acetate extract had the most excellent ability in free radical scavenging with the lowest IC₅₀ of the four extracts (439.31 µg ml⁻¹). The next is the chloroform fraction (487.14 µg ml⁻¹), and the position of the other two extracts is similar to that of the reduction power test. In particular, the IC₅₀ value of ethanol is very high, six times higher than the ethyl acetate extract, which shows the lightest scavenging ability of this fraction.

Table 1. Half-maximal inhibition concentration (IC₅₀) values of four extracts and ascorbic acid in DPPH scavenging ability

Test sample	IC ₅₀ (µg ml ⁻¹)
Ascorbic acid (Positive control)	17.76 ^a ± 3.36
n-Hexane	1,409.50 ^d ± 57.37
Chloroform	487.14 ^c ± 5.98
Ethyl acetate	439.31^b ± 2.86
Ethanol	2,759.40 ^e ± 43.80

The IC₅₀ results of DPPH free radicals scavenging are compared in columns, each different letter (a, b, c, etc.) is corresponding to each mean showing a statistical difference with p < 0.05.

The recent *M. polymorpha* L. antioxidant activity evaluation of Gokbulut *et al.* (2012) on ethyl acetate and methanol extracts showed that ethyl acetate fraction was better than methanol fraction. The similar results are obtained here with better antioxidant activity in ethyl acetate extract than ethanol extract.

Remya and Murugan, when investigating the antioxidant activity of the wild *Marchantia polymorpha*, reported that this species exhibits antioxidant ability through FRAP (ferric-reducing capacity), neutralizing free radicals' DPPH, ABTS, OH⁻ and protect DNA. Moreover, they found that this liverwort contains several compounds

such as gallate, protocatechol, etc. with coumarins, glycoside derivatives act as reducing agents (Remya and Murugan, 2013).

Anti-bacterial activity by generating inhibited zone

Antimicrobial activity was assessed through a diameter (mm) of the diffusion zone from the agar well. Results of the width of the antibacterial region of 4 extracts (3 mg per well) and chloramphenicol- positive control are presented in Table 3.

Table 2. Diameter of inhibited zones of four extracts and chloramphenicol on six bacterial strains

Bacteria strains	Diameter of inhibited zones (mm)				
	Chloramphenicol	n-Hexane	Chloroform	Ethyl acetate	Ethanol
<i>V. parahaemolyticus</i>	21.00 ^a ± 1.00	9.00 ^b ± 3.61	10.33 ^b ± 2.89	9.00 ^b ± 3.46	-
<i>S. typhimurium</i>	19.33 ± 0.58	-	-	-	-
<i>P. aeruginosa</i>	11.33 ^a ± 0.58	-	-	4.67 ^b ± 1.15	-
<i>E. faecalis</i>	9.67 ^a ± 1.15	4.00 ^b ± 1.00	4.67 ^b ± 1.15	5.67 ^b ± 1.15	-
<i>S. aureus</i>	27.67 ^a ± 0.58	9.67 ^{bc} ± 3.05	11.33 ^b ± 3.21	5.67 ^{cd} ± 2.89	-
<i>S. epidermidis</i>	20.67 ^a ± 2.52	7.33 ^{bc} ± 1.53	7.00 ^{bc} ± 1.00	5.33 ^c ± 0.58	-

The diameter results are compared in each row, each different letter (a, b, c, etc.) is corresponding to each mean showing a statistical difference with $p < 0.05$. (-): Not detected

Results of inhibited zone diameters showed that all six bacteria strains were sensitive to chloramphenicol with a concentration of 25 µg per well, for *P. aeruginosa*, the concentration was increased to 500 µg per well. For gram-negative strains, three fractions from n-hexane to ethyl acetate all inhibited *V. parahaemolyticus*, while no extracts inhibited *S. typhimurium*. Exceptionally, only ethyl acetate fraction can inhibit *P. aeruginosa*, which is a remarkable record because there are currently very few substances that can hinder this bacterium. For gram-negative strains, three fractions such as n-hexane, chloroform, and ethyl acetate all inhibited well with all strains, almost no statistically significant difference concerning the diameter of antibacterial zones in *E. faecalis*. For the two strains of *Staphylococcus*, the n-hexane and chloroform extracts were slightly higher than the ethyl acetate fraction. At least, the ethanol fraction did not inhibit all bacteria strains examined.

Studies on the natural *Marchantia polymorpha* samples also noted that extracts from weakly polar solvents such as chloroform had better activity than highly polarized solvents such as ethanol or methanol (Gahtori and Chaturvedi, 2011; Singh *et al.*, 2011). Besides, Gahtori and Chaturvedi's research also showed that the chloroform and methanol extracts of *M. polymorpha* have no inhibitory activity against *Salmonella enterica* (Gahtori and Chaturvedi, 2011). In present study, *in vitro* cultured liverwort, chloroform, ethyl acetate, and even n-hexane fraction exhibited more outstanding antibacterial activity than ethanol fraction. Besides, the antimicrobial compounds from *Marchantia polymorpha* are mainly in the bisbibenzyl group (Asakawa *et al.*, 2013; Tanaka *et al.*, 2016; Asakawa and Ludwiczuk, 2017), which predicts that the cultured material may also produce similar compounds and that they could be distributed into from n-hexane to ethyl acetate.

Tyrosinase inhibitory activity

The enzyme inhibiting capacity of kojic acid was powerful, at concentrations ten times lower than the extract; the positive control inhibited almost 100% tyrosinase enzyme activity (Figure 3). In terms of *in vitro* liverwort extracts, the three n-hexane, chloroform, ethyl acetate extracts still had a much better attribute than the ethanol fraction. Of which, n-hexane and chloroform extracts were the two potential inhibiting extracts with no significant difference in the percentage of inhibition (69.54 and 69.10%, respectively). Reports on biological activities and metabolites on *M. polymorpha* did not mention much on tyrosinase inhibitory activity

(Asakawa *et al.*, 2013; Asakawa and Ludwiczuk, 2017). Recently, Gawel-Beben *et al.* (2019) announced that marchantin A, a weak polar bisbenzyl group, did not inhibit tyrosinase activity. Meanwhile, these *in vitro* *M. polymorpha* n-hexane and chloroform extracts may contained other weak polar potential bioactive compounds have not been investigated.

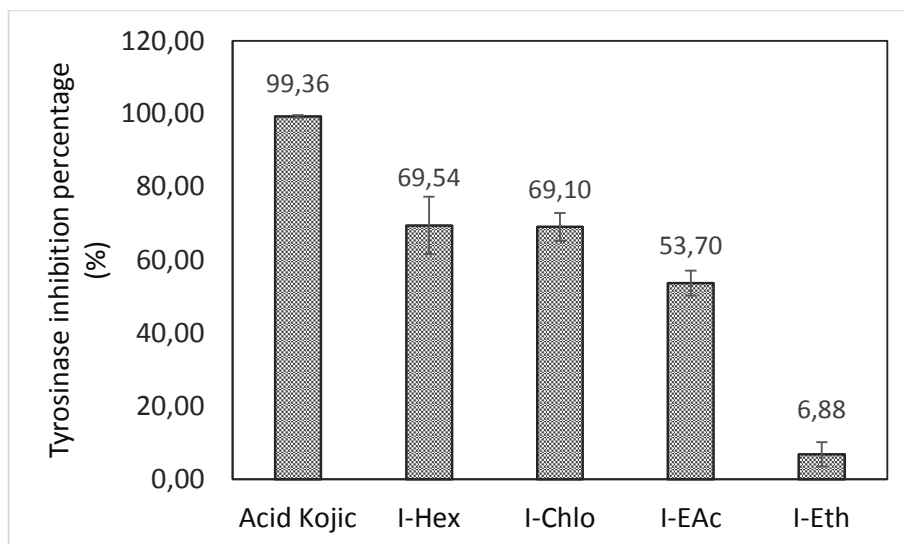


Figure 3. Tyrosinase inhibition percentage (%) of kojic acid as positive control and four extracts of cultured *Marchantia polymorpha*

Note: I-Hex, -Chlo, -EAc and -Eth correspond to n-hexane, chloroform, ethyl acetate, and ethanol extracts

Anti-proliferation activity on MCF-7 cell lines

At a concentration of $100 \mu\text{g ml}^{-1}$, three extracts of n-hexane, chloroform, ethyl acetate all inhibited the proliferation of MCF-7 cells higher than 60% (Table 4). Therefore, the IC_{50} index (a concentration that inhibits 50% of cell growth) was determined for these three extracts through a gradual reduction of the extract concentration from 100 to $0 \mu\text{g ml}^{-1}$. The n-hexane extract has the lowest IC_{50} value, which means that this extract inhibits the proliferation of MCF-7 cells more strongly than chloroform and ethyl acetate extracts.

MCF-7 cells morphology showed the difference after 48 h of induction of extracts and positive-camptothecin (Figure 4). In the negative control DMSO 0.25%, the cells grow into clusters, the cell morphology expands and adheres to the bottom of the feeding well. In the ethanol extraction sample, the cell membrane has a resolving phenomenon but obviously without affecting the cell development and clusters. In contrast to this, in the positive control and especially the extract from n-hexane, chloroform, and ethyl acetate, the cluster shrinkage and spread were significantly reduced, the cells were fragmented with cell membranes almost entirely resolved.

Table 3. The percentage of inhibited MCF-7 cell (%) at concentration $100 \mu\text{g.ml}^{-1}$ and IC_{50} of four extracts and camptothecin as the positive control

Extracts and Positive control	Percentage of inhibition (%)	IC_{50} ($\mu\text{g ml}^{-1}$)
Camptothecin	$65.14^b \pm 3.26$	$0.005^d \pm 0.001$
n-Hexane	$73.54^{ab} \pm 1.96$	$38.15^c \pm 1.58$
Chloroform	$75.77^a \pm 2.39$	$65.76^b \pm 3.67$
Ethyl acetate	$64.83^b \pm 0.45$	$77.73^a \pm 2.12$
Ethanol	$3.81^c \pm 9.32$	-

The results are compared in each row, each different letter (a, b, c, etc.) is corresponding to each mean showing a statistical difference with $p < 0.05$. (-): Not detected

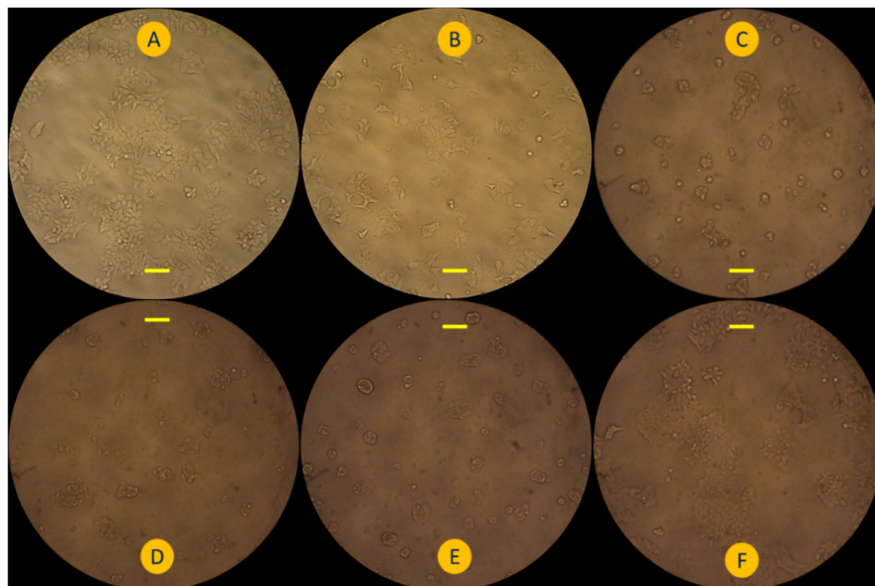


Figure 4. MCF-7 cell morphology after 48 h of induction with 0.25% DMSO (A), Camptothecin (B), n-Hexane (C), Chloroform (D), Ethyl acetate (E), and Ethanol (F) extracts. (Bar: 30 μ m)

Studies of the secondary metabolites of *Marchantia polymorpha* have shown that the bisbibenzyl group has excellent potential in inhibiting the growth of some cancer cell lines (Asakawa *et al.*, 2000; Asakawa *et al.*, 2013; Asakawa and Ludwiczuk, 2017). For example, Huang *et al.* reported that marchantin A induced the inhibition of MCF-7 cell growth via apoptosis through a caspase-dependent pathway (Huang *et al.*, 2010). Asakawa *et al.* found that other bisbibenzyl marchantin C, D, and bisbibenzyl derivatives also had cytotoxicity on the KB and P-388 cell lines (Asakawa *et al.*, 2013). In liverwort species, sesqui- and di-terpenoid compounds also contribute to the toxic activity on some cancer cell lines (Asakawa *et al.*, 2013). Diethyl ether extract of Tahitian *Frullania* sp. strongly inhibited HL-60 and KB cell lines with IC_{50} values of 1.6 μ g/ml and 11.2 μ g/ml, respectively (Asakawa *et al.*, 2013). The sesquiterpene lactones such as costunolide and tulipinolide, presenting as the significant components in diethyl ether extract, have been reported to have intense inhibitory activity on these two cell lines (Asakawa., 1995; Komala *et al.*, 2011; Asakawa *et al.*, 2013). In *M. polymorpha*, typical sesquiterpene compounds include *ent*-9-oxo- α -chamigrene, *ent*-thujopsan-7 β -ol, *ent*- α -cyperone... (Asakawa *et al.*, 1990).

Here, the results on the MCF-7 cell line show that n-hexane fraction, mainly weak polar compounds, might contain several bioactive that inhibit this cell line.

Preliminary survey of secondary metabolites

The qualitative results in Table 5 show that *Marchantia polymorpha in vitro* generally contains groups of phenolic compounds, quinones, coumarins, flavonoids, steroids, glycoside derivatives, and lactone ring compounds, and all these groups are present in chloroform and ethyl acetate extracts. Besides, the negative Cyanidin test showed that flavonoids include flavones, flavonols, chalcones, and aurones only. According to Markham and Porter, in *M. polymorpha* liverwort, luteolin and apigenin are the two main flavone flavonoids, besides their glycoside derivatives (Markham and Porter, 1974). In addition, the formation of these compounds is due to the activity of the enzyme chalcone synthase found in the *M. polymorpha* gametophyte tissue (Fischer *et al.*, 1995). Therefore, products on the synthesis pathway would have the presence of chalcone, aurone, and naringenin (Asakawa, 1995; Fischer *et al.*, 1995).

Identified bioactive compounds found liverwort species, particularly in *Marchantia polymorpha* L. are mono-, sesqui-, di-terpenes, steroids, phenanthrenes, aromatic (bibenzyl or bisbibenzyl), flavonoids and its glycoside derivatives (Asakawa *et al.*, 1990; Asakawa *et al.*, 2013; Tanaka *et al.*, 2016). Most of these compounds exist in the oil bodies of *M. polymorpha* and have non-polar to medium polarity (Asakawa *et al.*, 2013; Tanaka *et al.*, 2016), respectively, in the current study as n-hexane, chloroform, and ethyl acetate fraction.

Table 4. Result of preliminary qualitative analysis of secondary metabolite groups of four extracts of cultured *Marchantia polymorpha*

Compounds	Reagents	I-Hex	I-Chlo	I-EAc	I-Eth
Phenol	FeCl ₃	+	+	+	-
Quinone, coumarine	KOH 5%	+	+	++	-
Tanin	Saline gelatin	-	-	-	-
Alkaloid	Wagner	-	-	-	-
Flavonoid	H ₂ SO ₄	-	+	+	+
	NaOH 1%	-	+	+	-
	Lead acetate 10%	-	+	+	-
	Cyanidin-Mg	-	-	-	-
Terpenoid-Steroid	Cyanidin-Zn	-	-	-	-
	Rosein hem	-	-	-	-
Saponin	Salkowshi	+	+	+	-
	Foaming	-	-	-	-
Glycoside	Molisch	+	+	+++	+
Lactone ring compounds	Baljet	+	+	++	-

Notes: (+) presence, (-) no. I-Hex, -Chlo, -EAc and -Eth correspond to n-hexane, chloroform, ethyl acetate, and ethanol extracts.

When comparing chloroform, ethyl acetate, and ethanol extract, that the number of compounds that contribute to the biological activity of chloroform and ethyl acetate fraction is more prominent than the ethanol fractions. The ethanol fraction had the highest extraction efficiency but mainly present flavonoids and glycoside compounds that made the biological activity of this fraction not stand out from the other remains. And other highly polar compounds in this fraction may not be involved in the biological activity examined. In some plant materials, ethanol is the right solvent to extract compounds that are highly polarized and have potent antioxidant activity (Abalaka *et al.*, 2011; Jahan *et al.*, 2014; Divya and Vijaya Anand., 2015; Amudha and Rani; 2016; Venkatesan *et al.*, 2019). However, for *in vitro M. polymorpha*, solvents of lower polarity are more suitable than ethanol for extracting compounds that have positive results in these investigated activities.

The chloroform fraction has a weak polarization; the secondary compound groups in this segment have similarities with the ethyl acetate segment. However, this segment's antioxidant activity is lower than ethyl acetate but much more durable than that of n-hexane and chloroform segments. In addition, this segment is between n-hexane (non-polar) and ethyl acetate, so the chloroform segment also contains secondary groups similar to n-hexane. Therefore, in tyrosinase inhibitory assay and anti-proliferation activity on MCF-7 cell lines, the chloroform fraction is better than ethyl acetate although the biological activity is lower than n-hexane.

The ethyl acetate fraction is the most pronounced presence of phenolic compounds and quinone, coumarin, glycoside derivatives when there was a product specificity during interaction with the reagent (expressed in numbers of (+) signs more than other fractions). Besides of the above compounds, bisbibenzyls may be compounds that make ethyl acetate fractions have more prominent antioxidant activity than others.

Compared with the above fractions, the n-hexane lacks the flavonoid group. However, this fraction has many excellent activities such as antibacterial, tyrosinase inhibition, MCF-7 cell growth inhibition, and even α -glucosidase inhibition (Tan *et al.*, 2020). Therefore, the n-hexane fraction is a potential target that needs further investigation both in terms of biological activity and natural compound. Also, the absence of too many

groups of natural compounds (compared with ethyl acetate extract and chloroform) is a strong point of the n-hexane extract, because this makes it easier to approach target compounds.

Conclusions

The *in vitro* *Marchantia polymorpha* is a potential source of materials with positive biological activities. Among four extracts, ethyl acetate had the best antioxidant activity in both reducing power test and DPPH scavenging models. All three n-hexane, chloroform, and ethyl acetate extracts have well antimicrobial activity and are relatively similar. The n-hexane extract is prominent in tyrosinase inhibition activity and inhibiting the growth of MCF-7 cells. In addition, this fraction seems to be potent, and further biological activity, as well as new target compounds from this extract, should be further investigated.

Authors' Contributions

Conceptualization: Tran Quoc Tan and Quach Ngo Diem Phuong; Formal analysis: Tran Quoc Tan; Investigation: Tran Quoc Tan and Quach Ngo Diem Phuong; Methodology: Tran Quoc Tan, Phan Ngo Hoang and Quach Ngo Diem Phuong; Project administration: Quach Ngo Diem Phuong; Resources: Phan Ngo Hoang; Supervision: Quach Ngo Diem Phuong; Validation: Bui Lan Anh; Visualization: Bui Lan Anh; Writing - original draft: Tran Quoc Tan; Writing - review and editing: Bui Lan Anh and Quach Ngo Diem Phuong. All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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