INVESTIGATION OF PHYTOCONSTITUENTS AND SOME BIOACTIVITIES OF LEAVES AND BARKS OF *HOLOPTELEA INTEGRIFOLIA* R. (PHYAUK-SEIK)

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Abstract

Due to the increasing resistance of pathogens to the antibiotics, the plant kingdom is more focused than ever because the most parts of the plants have the ability to synthesize a wide variety of chemical compounds that possess important biological functions. The selected Myanmar medicinal plant, *Holoptelea integrifolia* R. (Phyauk-seik), belonging to family Ulmaceae, is known to have many bioactivities. This research deals with phytochemical and medico-chemical investigations of leaves and barks of *H. integrifolia*. The preliminary phytochemical investigation revealed the presence of many valuable phytochemicals in both samples. Based on DPPH assay method, the 70% EtOH extracts of leaves and barks of *H. integrifolia* showed the mild antioxidant activity by comparing with the standard ascorbic acid. The antimicrobial activity of PE, EtOAc and 70 % EtOH extracts of leaves and barks were screened on six microorganisms, namely *B. substilis, S. aureus, P. fluorescens, A. flavus, C. albicans* and *E. coli* by paper disc diffusion assay. All of the tested extracts, except PE extract of barks exhibited the antimicrobial activity with inhibition zone diameters ranged between 8 mm ~ 18 mm for leaves and 10 mm ~ 22 mm for barks, while the PE extract of barks did not show the activity. The antiproliferative activity of PE, EtOAc and 70 % EtOH extracts of leaves and barks of *H. integrifolia* were determined by MTT assay on two human cancer cell lines: A 549 (lung) and HeLa (cervix). All of the tested extracts of leaves exhibited mild activities on two tested cancer cell lines with IC_{50} values > 100 µg/mL. And, PE and 70 % EtOH extracts of barks exhibited significant activities on two tested cell lines with IC_{50} values < 20 µg/mL, except the EtOAc extract, while the EtOAc extract of barks showed mild activity on cervix cancer cell line with IC_{50} value > 100 µg/mL.

Keywords: *Holoptelea integrifolia*, Phyauk-seik, antioxidant activity, antimicrobial activity, antiproliferative activity, MTT assay

Introduction

Myanmar is a fortunate country with a large number of medicinal plants. Most of the plants have been used as the traditional medicines from ancient times. They are widely used in Myanmar by the majority of the population either as an alternate or as a supplement to modern medicines. In this research, one of the Myanmar medicinal plant *Holoptelea integrifolia* R., Phyauk-seik, commonly called as India Elm tree have been selected for chemical analysis.

Plant **Leaves** **Barks**

Figure 1 Photographs of plant, leaves and barks of *H. integrifolia* (phyauk-seik)

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It is widely distributed all over tropical and temperate regions of Northern Hemisphere and the family Ulmaceae consists of 15 genera and 200 species (Sandhar *et al*., 2011). *H. integrifolia* is a roadside tree possessing wide range of biological activities. Traditionally it used for the treatment of inflammation, wound healing, leprosy, diabetes, hemorrhoids, rheumatism and intestinal cancers in many countries (Danthala *et al*., 2017).

It is a large, spreading, glabrous and deciduous tree as illustrated in Figure 1. Barks are 6-8 mm thick, grey, pustular and exfoliating in somewhat corky scales. Leaves are elliptic-ovate, acuminate, base rounded or sub-cordate. Flowers are greenish-yellow, polygamous in short racemes or fascicles on the leafless branches. Fruit is sub-orbicular samara with two membranous wings. Seed is one and flat. And, unpleasant odour appears on cutting the barks and crushing the leaves. The wood is light yellow, lustrous, interlocked-grained, medium and even-textured, moderately heavy and strong. Flowering occurs in February-March and fruiting March onwards (Shastri, 2007).

This plant has been reported to possess various chemical constituents. Different parts of the plant like stem bark, heartwood, leaves, seed, pollen and root are the major sources of various medicinally important phytochemicals. Two triterpenoids fatty acid esters holoptelin-A and B, 2-amino naphthaquinone, frieldelin, epifriedelinol, β-sitosterol and its β-D-glucose, β-amyrin, betulin and betulinic acid are derived from stem bark. And, hexacosanol, octacosanol, 1, 4-napthalenidone, β-sitosterol and β-amyrin are derived from leaves (Sharma, 2009; Mondal *et al.*, 2016). The aim of the present study is to screen the preliminary phytoconstituents and some bioactivities such as antioxidant, antimicrobial and antiproliferative activities of leaves and barks of *H. integrifolia.*

Materials and Methods

Collection and Preparation of Plants Materials

Holoptelea integrifolia (phyauk-seik) leaves and barks were collected from Nyaung Bin Seik Quarter, Mawlamyine Township in Mon State and identified at the Department of Botany, Mawlamyine University.

After collection, the leaves and barks of *H. integrifolia* were cleaned thoroughly with distilled water to remove any type of contamination. Then, the collected plant materials were shade dried to retain its vital phytoconstituents and subjected to size reduction. The powder of the samples were separately stored in air tight bottles and kept in a cool, dark and dry place until analyses were commenced.

Preliminary Phytochemical Tests

The preliminary phytochemical detection of leaves and barks of *H. integrifolia* were carried out with standard phytochemical methods (Evans *et al*, 2003; Harborne, 1984; Marini-Bettolo *et al*., 1981; M-Tin Wa, 1972; Robinson, 1983; Shriner *et al*., 1980; Trease *et al*., 1978).

Preparation of Plant Extracts for Biological Activity

The crude extracts of leaves and barks of *H. integrifolia* were prepared by extracting the sample with different solvents like pet ether (PE), ethyl acetate (EtOAc) and 70 % ethanol (EtOH) by successive maceration method at ambient temperature. All of these extracts were kept for the determination of antioxidant, antimicrobial and antiproliferative activities.

Determination of Antioxidant Activity

The antioxidant activity of 70 % EtOH extracts of leaves and barks of *H. integrifolia* and standard ascorbic acid were investigated by DPPH (2,2-diphenyl-1-picryl-hydrazyl) free radical scavenging assay (Ashokkumar and Ramaswamy, 2013). DPPH radical scavenging activity was determined by spectrophotometric method.

Preparation of sample and DPPH solutions

Each extract 2 mg and 10 mL of EtOH were thoroughly mixed by shaker. The mixture solution was filtered and the stock solution was obtained. The sample solutions (100, 50, 25, 12.5 and 6.25 μg/mL concentrations) were prepared from the stock solution by dilution with appropriate amounts of EtOH.

DPPH 2.364 mg was thoroughly dissolved in 100 mL of EtOH. This solution was freshly prepared in the brown coloured reagent bottle and stored in the fridge for no longer than 24 h.

Procedure for antioxidant activity

The control solution was prepared by mixing 1.5 mL of 60 μM DPPH solution and 1.5 mL of 95 % EtOH using shaker. And, the blank solution was prepared by mixing 1.5 mL of test sample solution and 1.5 mL of 95 % EtOH. The sample solution was also prepared by mixing thoroughly 1.5 mL of 60 μM DPPH solutions and 1.5 mL of test sample solution. The sample solution was allowed to stand at room temperature for 30 min. Then, the absorbance of these solutions was measured at 517 nm by using UV-Visible spectrophotometer. Absorbance measurements were done in triplicate for each concentration and then the mean values obtained were used to calculate % inhibition of oxidation by the following equation,

$$\%\,\text{Oxidative Inhibition} = \frac{A_c - (A - A_b)}{A_c} \times 100\,\%$$

Where, % oxidative inhibition = % oxidative inhibition of test sample

A_c = absorbance of the control (DPPH alone)

A_b = absorbance of the blank (EtOH + Test sample solution)

A = absorbance of test sample solution

Then IC_{50} (50 % inhibitory concentration) values were also calculated by linear regressive excel program (Brand-Williams *et al.,* 1995). The antioxidant activity is expressed as % radical scavenging activity (% RSA) and 50 % inhibition concentration (IC_{50}). When the concentrations of the samples were increased, the absorbance values decreased i.e. % inhibition or radical scavenging activities also increased.

Screening of Antimicrobial Activity

An antimicrobial is a substance that destroys microorganisms or inhibits their growth. The antimicrobial activity of different crude extracts such as PE, EtOAc and 70 % EtOH extracts of leaves and barks of *H. integrifolia* were tested with six microorganisms such as *Bacillus substilis, Staphylococcus aureus, Pseudomonas fluorescens, Aspergillus flavus, Candida albicans* and *Escherichia coli* species by using paper disc diffusion assay. These tests were performed at Department of Botany, University of Yangon.

Preparation of broth medium

Isolated bacterial strains grown on nutrient agar were inoculated into 50 mL conical flasks containing 10 mL of sterile growth medium. Then, they were incubated at 30 ºC for 72 h on a reciprocal shaker at 200 rpm.

Procedure for paper disc diffusion assay

Test organisms 0.3 mL was added to assay medium, then poured into plates. After solidification, paper discs impregnated with broth samples of PE, EtOAc and 70 % EtOH extracts of leaves and barks of *H. integrifolia* were applied on the test plates and these plates were incubated for 24-36 h at 30 ºC. After incubation, clear zones (inhibitory zones) surrounding the test discs indicate the presence of bioactive compounds which inhibit the growth of test organisms.

Determination of Antiproliferative Activity

In vitro antiproliferative activities of PE, EtOAc and 70 % EtOH extracts of leaves and barks of *H. integrifolia* were determined with two human cancer cell lines A 549 (lung cancer) and HeLa (cervix cancer). The antiproliferative activity was measured by MTT (3- (4, 5-dimethylthiazol-2-yl) - 2, 5- diphenyltetrazolium bromide) assay (Win *et al*., 2015). These tests were done at Department of Natural Products Chemistry, Institute of Natural Medicine, and University of Toyama, Japan. This assay detects the reduction of MTT by mitochondrial dehydrogenase to blue formazan product, which reflects the normal function of mitochondria and cell viability.

Preparation of sample and control solutions by serial dilution method

Each 1 mg of the PE, EtOAc and 70 % EtOH extracts of leaves and barks of *H. integrifolia* was dissolved in 100 µL of dimethyl sulfoxide (DMSO) solution to get 10000 µg/mL sample solution. It is necessary to mix with a vibrator. And the two eppendorf tubes for each sample were used for serial dilution.

The fresh medium 686 µL was added in the first eppendorf tube and then another fresh medium 540 µL was put into the second tube. The stock sample solution 14 µL was added to the 686 µL fresh medium with first eppendorf tube and vibrated well for solubility. And then 60 µL from the first eppendorf tube was added to the 540 µL fresh medium with second eppendorf tube and slowly pipetted up and down 2 to 3 times. Finally, 200 and 20 µg/mL of serial solutions were obtained and kept in the refrigerator.

The control solutions were serially prepared as described above procedure. Instead of sample extract, 5-fluorouracil (5 FU) was used for the positive control. Only DMSO was used for negative control.

Preparation of cell growth

The cell was taken from the stock and transferred into a 15 mL centrifuge tube followed by addition of 5 mL of respective medium. The suspension cell was centrifuged in the refrigerated centrifuge machine 1000 rpm for 3 min. After the centrifugation, the supernatant was carefully removed without disturbing the cell pellet. And then fresh medium 2 mL was gently added to the side of the tube and slowly pipetted up and down 2 to 3 times to re-suspend the cell pellet. The suspension cell was centrifuged for 3 min. The supernatant was carefully removed without disturbing the cell pellet. The cell suspension was diluted with 6 mL of medium. Finally, the cells were transferred to the desired sterile container and the cells were incubated until 70 -100 % cell confluences for 7 days at an incubator.

Procedure for antiproliferative activity

After the cell growth, the 70-100 % cell in the medium was aspirated with aspirator. The cell was washed with 5 mL of phosphate buffer saline (PBS) 2 times. The cells are trypsinased with 4 mL of trypsin and incubated for 2–3 min. And then the medium 1 mL was added to stop trypsinization. The cell suspension was transferred to 15 mL centrifuge tube. The tube (cell suspension) was centrifuged in the refrigerated centrifuge machine 3000 rpm with the same centrifuge tube for 3 min. After the centrifugation, the supernatant was carefully removed without disturbing the cell pellet and the cell was found at the bottom of the centrifuge tube. The cell in the centrifuge tube was added with 3 mL of fresh medium gently to the side of the tube and slowly pipetted up and down 2 to 3 time to re-suspend the cell pellet. The number of cell was counted with Haemocytometer.

The cell solution 10 µL was mixed in the 40 µL of Tryphan blue. The chamber and the covered slip were cleaned with alcohol (70 % EtOH). The chamber was dried and the over slip was fixed in position. The cell was harvested and the 10 µL of the cell was added to the Haemocytometer (do not overfill). And then the chamber was placed in the inverted microscope under a 10 × objective and phase contrast was used to distinguish the cell. The cell was counted in the large, central gridded square (1 mm^2). The gridded square was circled and multiplied by 10^4 to estimate the number of cells per millimeter. The number of cells was counted by the following equation,

No. of cells in stock = counted cell/4 × 10^4 × dilution factor × volume of stock cell solution

After the cell counting, the cell was added with 50 mL (500 µL) of medium for 12 plates. 10 mL (100 µL) medium of cell was filled in 96 well plates. The cell in 96 well plates was incubated in an incubator for 24 h. After the incubation, the medium was removed by absorption machine (very carefully) and washed with 100 µL PBS solution. And then 100 µL each of the different concentrations of sample and control solution was added in the 96 well plates. The sample solutions in 96 well plates with cells were incubated in an incubator for 72 h.

The sample solution with cell and medium was added with 100 µL MTT reagent. And then the 96 well plates were incubated in an incubator for 3 h. After the incubation, cells in the medium were aspirated with aspirator. The cell was washed with 5 mL of PBS for 2 times. Then, DMSO was added about 100 µL per well and the 96 well plates were placed in the dark for 15 min. And then, the absorbance of each solution was measured at 570 nm by using UV-visible spectrophotometer. The percent cell viability activity was calculated by the following equation.

% Cell viability = [(Abs (test sample) −Abs (blank)) / (Abs (control) −Abs (blank))] × 100

Where, $Abs_{(test\ sample)}$ = absorbance of test sample solution
$Abs_{(control)}$ = absorbance of DMSO solution
$Abs_{(blank)}$ = absorbance of MTT reagent

IC_{50} (50 % inhibitory concentration) values were calculated by linear regressive excel program. The standard deviation was also calculated by the following equation.

$$\text{Standard Deviation (SD)} = \sqrt{\frac{(\bar{x}-x_1)^2+(\bar{x}-x_2)^2+\cdots(\bar{x}-x_n)^2}{(n-1)}}$$

Where, $\bar{X}$ = average % inhibition
$x_1, x_2, \ldots, x_n$ = % cell inhibition of test sample solution
n = number of times

Results and Discussion

According to the preliminary phytochemical analysis of *H. integrifolia* showed the presence of alkaloids, α-amino acids, carbohydrates, flavonoids, glycosides, organic acids, phenolic compounds, reducing sugars, saponins, starch, steroids, tannins and terpenoids in leaves, while α-amino acids, carbohydrates, flavonoids, glycosides, organic acids, phenolic compounds, reducing sugars, saponins, steroids, tannins and terpenoids were found to be present in the barks. However, cyanogenic glycosides were not found in these samples. The main constituents such phenolic compounds, flavonoids, terpenoids and steroids, present in *H. integrifolia* may contribute to bioactivities such as antimicrobial, antioxidant and anticancer properties. Test reagents, observations and inferences for the analyses are summarized in Table 1.

Table 1 Phytochemical Test Results of Leaves and Barks of *Holoptelea integrifolia* (Phyauk-seik)

Sr.No.	Tests	Extracts	Test Reagent	Observation	Leaves	Barks
1	Alkaloids	1% HCl	(i) Dragendorff's reagent	Orange ppt	+	–
			(ii) Sodium picrate solution	Yellow ppt	+	–
			(iii) Wagner's reagent	Brown ppt	+	–
			(iv) Mayer's reagent	White ppt	+	–
2	α-amino acids	H_2O	Ninhydrin reagent	Purple colour	+	+
3	Carbohydrates	H_2O	10% ∝-naphthol, conc:H_2SO_4	Red ring	+	+
4	Cyanogenic glycosides	H_2O	Sodium picrate	No brick red colour	–	–
5	Flavonoids	EtOH	Mg turnings, conc : H_2SO_4	Pink colour	+	+
6	Glycosides	H_2O	10% lead acetate	White ppt	+	+
7	Organic acids	H_2O	Bromocresol green	Blue colour	+	+
8	Phenolic compounds	H_2O	10% $FeCl_3$	Deep blue	+	+
9	Reducing sugars	H_2O	Benedict's solution	Brick-red ppt	+	+
10	Saponins	H_2O	Distilled water	Frothing	+	+
11	Starch	H_2O	I_2 solution	Deep blue	+	–
12	Steroids	PE	Acetic anhydride, conc:H_2SO_4	Green colour	+	+
13	Tannins	H_2O	$FeSO_4$	Deep blue	+	+
14	Terpenoids	$CHCl_3$	Acetic anhydride, conc:H_2SO_4	Pink colour	+	+

(+) present (-) absent (ppt) precipitate

Antioxidant activity of Leaves and Barks of *Holoptelea integrifolia* (Phyauk-seik)

The antioxidant activity of 70 % EtOH extract of leaves and barks of *H. integrifolia* was investigated with five different concentrations (100, 50, 25, 12.5 and 6.25 μg/mL) by DPPH free radical scavenging assay. Their results are shown in Table 2 and Figure 2. The smaller IC_{50} value indicates the higher the free radical scavenging activity. Here, the IC_{50} values of 70 % EtOH extract of leaves and barks were found to be 32.75 and 77.97 μg/mL. Therefore, 70 % EtOH extract of

leaves was more potent activity than barks. But, the 70 % EtOH extracts of both samples showed mild antioxidant activities by comparing with the IC_{50} value 6.56 µg/mL of standard ascorbic acid.

Table 2 % Radical Scavenger Activity and IC_{50} Values of Crude Extracts of Leaves and Barks of *Holoptelea integrifolia* (Phyauk-seik)

Test Samples	% RSA ± SD of Different Concentration (µg/mL)					IC_{50} (µg/mL)
	6.25	12.5	25	50	100	
70 % EtOH (Leaves)	28.57 ±0.49	40.24 ±0.74	49.83 ±0.99	67.42 ±0.74	81.53 ±0.49	32.75
70 % EtOH (Barks)	26.12 ±0.49	27.88 ±0.99	33.45 ±0.49	41.82 ±0.49	56.45 ±0.99	77.97
Ascorbic Acid (Standard)	47.91 ±1.93	87.91 ±0.66	91.84 ±0.33	95.12 ±0.33	95.44 ±0.99	6.56

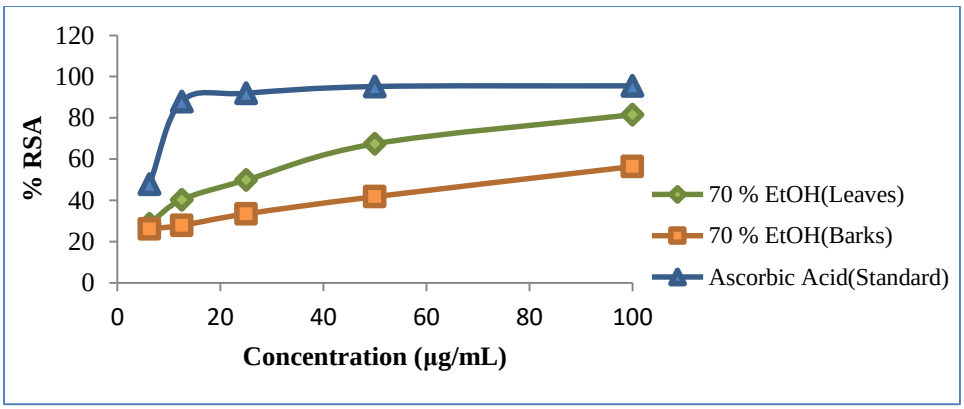

Figure 2 % RSA of 70 % EtOH extracts of leaves and barks of *H. integrifolia* (Phyauk-seik)

Antimicrobial activity of Leaves and Barks of *Holoptelea integrifolia* (Phyauk-seik)

In vitro antimicrobial activity of PE, EtOAc and 70 % EtOH extracts of leaves and barks of *H. integrifolia* was studied by paper disc diffusion assay. Tested six microorganisms were *B. substilis, S. aureus, P. fluorescens, A. flavus, C. albicans* and *E. coli*. It was carried out at Department of Botany, University of Yangon. The activities of the test extracts are expressed by measuring the zones (mm) of inhibition. Generally, the more susceptible the organism, the bigger is the zone of inhibition.

Among the extracts, the EtOAc and 70 % EtOH extracts of barks (10 ~ 22 mm) was found to be highly effective against six microorganisms than EtOAc and 70 % EtOH extracts of leaves (8 ~ 18 mm). And, the PE extract of leaves was observed to possess significant activity, but PE extract of barks did not show the activity against all tested microorganisms. The observed antimicrobial activity is expressed as the inhibition zone diameters as shown in Table 3 and Figure 3.

Table 3 Inhibition Zone Diameters of Crude Extracts of Leaves and Barks of *Holoptelea integrifolia* (Phyauk-seik)

Microorganisms	Inhibition Zone Diameters (mm)					
	Leaves			Barks		
	PE	EtOAc	70 % EtOH	PE	EtOAc	70 % EtOH
A. flavus	16 (+++)	10 (+)	10 (+)	-	14 (++)	16 (+++)
B. subtilis	14 (++)	8 (+)	10 (+)	-	16 (+++)	12 (++)
C. albicans	18 (+++)	14 (++)	12 (++)	-	16 (+++)	22 (+++)
E. coli	18 (+++)	8 (+)	14 (++)	-	14 (++)	16 (+++)
P. fluorescens	12 (++)	10 (+)	12 (++)	-	10 (+)	20 (+++)
S. aureus	14 (++)	14 (++)	14 (++)	-	10 (+)	18 (+++)

Agar well = 6 mm
(+) = 6 -10 mm (low activity)
(++) = 11-15 mm (moderate activity)
(+++) = 16 mm & above (high activity)
(-) = no zone of inhibition

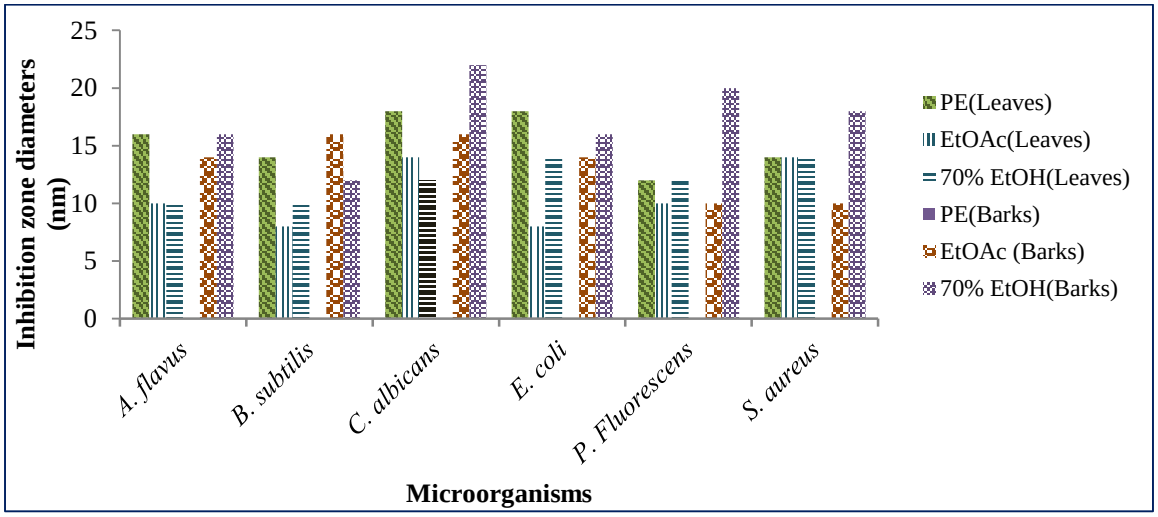

Figure 3 A bar graph of inhibition zone diameters of PE, EtOAc and 70% EtOH extracts of leaves and barks of *H. integrifolia* against six tested microorganisms

Antiproliferative activity of Leaves and Barks of *Holoptelea integrifolia* (Phyauk-seik)

In *in vitro* antiproliferative activity of PE, EtOAc and 70 % EtOH extracts of leaves and barks of *H. integrifolia* were determined by MTT assay with two human cancer cell lines such as A 549 (lung cancer) and HeLa (cervix cancer) cell lines. These tests were done at Department of Natural Products Chemistry, Institute of Natural Medicine, University of Toyama, Japan. The antiproliferative effect was expressed as IC_{50} values.

The antiproliferative activities of the tested samples are summarized in Table 4. Since, the lower the IC_{50} values exhibit the higher the antiproliferative activity. The PE and 70 % EtOH extracts of barks showed significant antiproliferative activity against A 549 and HeLa cell lines

with the IC_{50} values less than 20 µg/mL. And also, the EtOAc extract of barks less than 20 µg/mL showed potent activity against A 549 lung cancer cell line. They possessed higher activity than standard 5 FU (IC_{50} values is 19.06 ~ 35.84 µg/mL) against two tested cell lines. The remaining EtOAc extract of barks, PE, EtOAc and 70 % EtOH extracts of leaves exhibited mild antiproliferative activities against A 549 and HeLa cell lines because of their IC_{50} values were greater than 100 µg/mL (Figures 4 and 5).

Table 4 % Cell Viability IC_{50} Values of Crude Extracts of Leaves and Barks of *Holoptelea integrifolia* (Phyauk-seik) Against Two Human Cancer Cell Lines

Test Samples	50 % Inhibition (IC_{50} µg/mL)			
	Leaves		Barks	
	Lung A 549	Cervix HeLa	Lung A 549	Cervix HeLa
PE extract	>200.00	>200.00	<20.00	<20.00
EtOAc extract	118.75	131.43	<20.00	141.20
70 % EtOH extract	100.56	118.84	<20.00	<20.00
5-Fluorouracil (Positive Control)	19.06	35.84	19.06	35.84

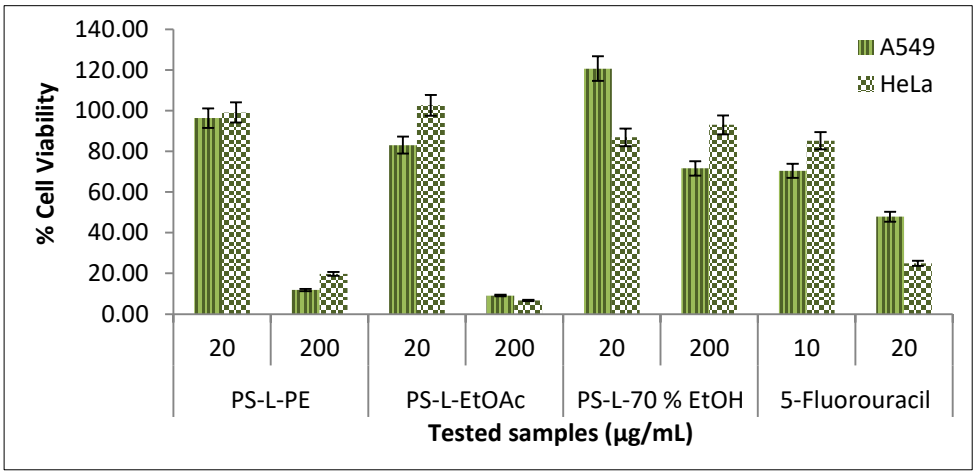

Figure 4 A bar graph diagram for antiproliferative activity of crude extracts of leaves of *H. integrifolia* against human lung (A549) and cervix (HeLa) cancer cell lines

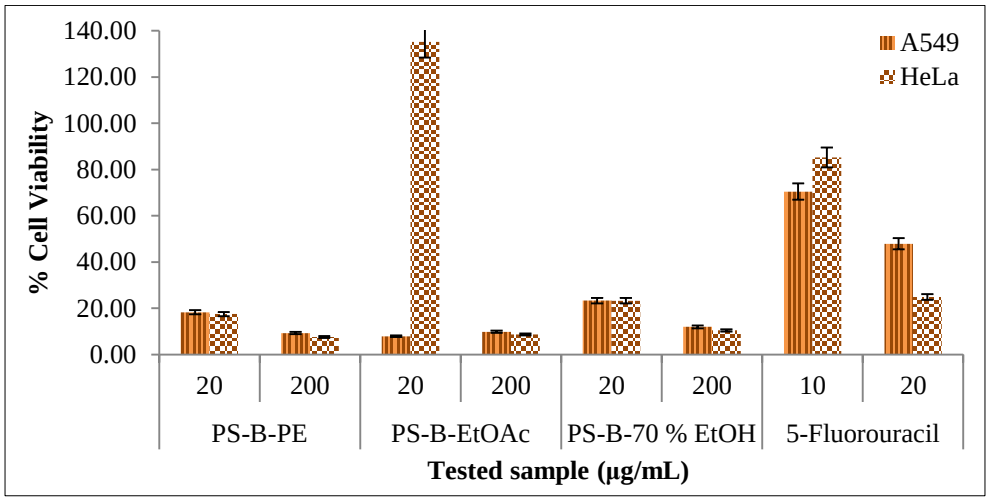

Figure 5 A bar graph diagram for antiproliferative activity of crude extracts of barks of *H. integrifolia* against human lung (A549) and cervix (HeLa) cancer cell lines

Conclusion

Based on preliminary phytochemical determination of leaves and barks of *H. integrifolia*, various types of bioactive organic constituents were found in both samples, except cyanogenic glycosides. The results of antioxidant activity suggested that the 70 % EtOH extracts of leaves and barks exhibited mild activity by comparing with the standard ascorbic acid. The antimicrobial activity results of EtOAc and 70 % EtOH extracts of both samples and PE extract of leaves showed significant antimicrobial activity on six tested microorganisms, except PE extract of barks. According to the antiproliferative activity determination, PE, EtOAc and 70 % EtOH extracts of barks were more potent activity than that of leaves extracts against two human cancer cell lines. According to the results, *H. integrifolia*, Phyauk-seik, leaves and barks contained many phytochemicals, mild antioxidant activities, and most of the extracts showed significant antimicrobial activities and had good news for cervix and lung cancer. However, this study is a preliminary step and further study is necessary to investigate the toxicology, detailed chemical characterization and other pharmacological profile of these plants constituents for the development of new drugs.

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