



## ***In vitro* Antioxidant and Anticancer Properties of Active Compounds from Methanolic Extract of *Pteris multifida* Poir. Leaves**

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### **Authors' contributions**

*This work was carried out in collaboration between all authors. Authors HLS and THPT designed the study. Author HLS managed the literature searches, wrote the protocol, and the first draft of the manuscript, read and approved the final manuscript. Author THPT prepared and isolated pure compounds from the leaf extract, performed the biological tests and statistical analysis.*

**Original Research Article**

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### **ABSTRACT**

**Aims:** To isolate pure compounds from the methanolic fraction obtained from successive fractionation of defatted ethanolic extract and evaluate *in vitro* antioxidant and anticancer activity of the crude ethanolic extract, methanolic fraction and pure compounds isolated from methanolic fraction from leaves of *Pteris multifida* Poir.

**Study Design:** Isolation and identification of the compounds, evaluation of antioxidant and anticancer activity on cervical cancer cell line (HeLa), lung carcinoma cell line (NCI-H460) and breast carcinoma cell line (MCF-7).

**Place and Duration of Study:** Vietnam Academy of Science and Technology of Ho Chi Minh City and School of Biotechnology, International University, Vietnam National University, Ho Chi Minh City, between December 2012 and September 2013.

**Methodology:** The crude ethanolic leaf extract and methanolic fraction obtained from successive fractionation of defatted ethanolic extract from *Pteris multifida* leaves were prepared. The isolated compounds from methanolic fraction were identified using different spectroscopic techniques. Antioxidant activity of the samples was evaluated by using the stable free radical 2, 2- diphenyl picrylhydrazyl (DPPH). Sulforhodamine B (SRB) assay was exploited for determination of anticancer activity against three selected human

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cancer cell lines: HeLa, NCI-H460 and MCF-7.

**Results:** Two main compounds were isolated from methanolic fraction obtained from successive fractionation of defatted ethanolic extract: rutin (1) and apigenin-7-O- $\beta$ -D-glucopyranoside (2). The crude ethanolic leaf extract showed weak antioxidant activity ( $IC_{50}$  = 89.84  $\mu$ g/mL) whereas the methanolic fraction expressed quite strong antioxidant activity ( $IC_{50}$  = 21.9  $\mu$ g/mL). Rutin (1) showed a good ingredient of antioxidant activity with  $IC_{50}$  value of  $37.70 \pm 0.03$   $\mu$ g/mL. Crude ethanolic leaf extract had cytotoxic activity against HeLa and NCI-H460 cell lines while the methanolic fraction had cytotoxic activity against HeLa, NCI-H460 and MCF-7 cell lines. Apigenin-7-O- $\beta$ -D-glucopyranoside (2) had strong anticancer activity against MCF-7 cell line with  $IC_{50}$  =  $22.62 \pm 0.59$   $\mu$ g/mL.

**Conclusion:** The crude ethanolic leaf extract and its methanolic fraction of *P. multifida* showed the potential activity in antioxidant and anticancer activity. Rutin had a potent antioxidant activity while apigenin-7-O- $\beta$ -D-glucopyranoside had a strong anticancer activity against the human breast adenocarcinoma cell line MCF-7.

**Keywords:** *Pteris multifida*; rutin; apigenin-7-O- $\beta$ -D-glucopyranoside; antioxidant; anticancer.

## 1. INTRODUCTION

From early until now, natural products, especially plants, have been widely used for the discovery of new therapeutic agents. These natural products used in pharmaceutical preparation can either be crude extracts, fractions, pure compounds or analogous compounds from highly active isolated compounds. Drug discovery from plant-derived substances plays an important role in the treatment of various diseases. Numerous types of bioactive compounds have been isolated from plant sources. Several of them are currently in clinical trials or preclinical trials or undergoing further investigation [1]. In comparison to synthetic agents, plant-based products have more beneficial characteristics such as inexpensive and safety. Thus, plant species are always regarded as an important source of starting material for drug discovery.

Cancer is a main public health trouble in both developed and developing countries. The environmental, physical, chemical, metabolic and genetic factors play a direct and/or indirect role in the induction and deterioration of cancers. The limited success of clinical therapies including radiation, chemotherapy, immunomodulation and surgery in treating cancer, as evident by the high morbidity and mortality rates, indicates that there is an imperative need of new cancer management [2]. Medicinal plants maintain the health and vitality of individual and also cure various diseases including cancer without causing toxicity. Natural products discovered from medicinal plants have played an important role in treatment of cancer [3]. Thus, search in drug discovery should involve in finding new safe compounds from medicinal plants that are capable of killing cancer cells.

*Pteris multifida* Poir. (*P. multifida*) is a medicinal plant belonging to genus of *Pteris* of Pteridaceae family. *P. multifida* contains various flavonoids and possesses antipyretic, detoxified, antibiotic, anti-inflammototy and antimutagenic activities [4]. However, flavonoids extracted from *P. multifida* leaves have not yet been evaluated for antioxidant and anticancer activity. The purpose of this present contribution was to isolate target compounds from the methanolic fraction from the leaf extract of *P. multifida* and assay for antioxidant and anticancer activity.

## 2. MATERIALS AND METHODS

### 2.1 Reagents and Equipment

Medium Pressure Liquid Chromatography was performed on CombiFlash Rf machine (USA) using silica gel (E. Merck, 230-400 mesh) column (RediSep cartridge, USA). Precoated silica gel F254 plates (Germany) were used for thin layer chromatography (TLC). Sulforhodamine B (SRB) solution, DPPH, ascorbic acid and various solvents were purchased from Merck (Germany). All reagents and solvents were of analytical grade.

The cervical cancer HeLa, lung cancer NCI-H460, and breast cancer MCF-7 cell lines were supplied from the National Cancer Institute of the United States (NCI - Frederick, MD, USA). The cells were cultured in E'MEM environment including L-glutamine (200 mM), HEPES (1M), amphotericin B (0.1%), penicillin-streptomycin 200X and 10% (v/v) FBS and incubated at 37°C with 5% CO<sub>2</sub>.

The <sup>1</sup>H-NMR and <sup>13</sup>C-NMR spectra (500 MHz for <sup>1</sup>H-NMR and 125 MHz for <sup>13</sup>C-NMR) were recorded using DMSO as solvent. The structures of pure compounds were elucidated based on the distortionless enhancement by polarization transfer (DEPT), correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC) and heteronuclear multiple-quantum correlation (HMQ).

### 2.2 Collection and Preparation of the Plant Materials

Fresh leaves of *P. multifida* were collected from Nghe An Province in November of Vietnam. The plant was identified by Associate Prof. Dr. Tran Van Minh of the Institute of Tropical Biology, Viet Nam. The specimen was deposited in the herbarium of Applied Biochemistry laboratory, Department of Applied Chemistry, School of Biotechnology, International University, Viet Nam National University Ho Chi Minh City, Viet Nam with voucher No. of HB-BIO-08-11-12. The leaves were first rinsed thoroughly with tap water, shaded dried for 5 days. The dried plant materials were then ground into uniform powder using an electric grinder prior to the extraction.

### 2.3 Preparation of Leaf Extract and Isolation of Compounds

Plant extraction was done by cold maceration of 1.5 kg of dried leaf in 96% ethanol for one week at room temperature with frequent stirring. The filtrate obtained was concentrated using rotator evaporator to obtain crude ethanol extract. The crude ethanolic extract was then defatted by using hexane to remove the chlorophyll out of the extract. The residues were successively partitioned with pure methanol several times and removed organic solvent using rotator evaporator affording known weight of methanolic fraction. The extracts and fraction were stored at 4°C for further analysis.

Thirty grams of methanolic extracts was chromatographed on silica gel column (250 g) with chloroform-methanol as the gradient eluent and then purified by preparative reverse-phase high-performance liquid chromatography (RP-HPLC) with water-methanol as the gradient eluent for isolation of compounds. Compound (1) rutin was isolated from fraction 5 (5:5) and (2) apigenin-7-O-β-D-glucopyranoside was isolated from fraction 7 (3:7).

## 2.4 Biological Studies

The crude ethanolic leaf extract, methanolic fraction and pure compounds were *in vitro* tested for their antioxidant and anticancer activities.

### 2.4.1 Antioxidant activity

It is essential to select and employ a stable and rapid method to assay antioxidant activity, because the determination of many samples is time consuming. In this work, 2,2-diphenyl picrylhydrazyl (DPPH) assay was used for the evaluation of antioxidant activity of crude ethanolic extract, methanolic fraction and pure compounds.

The DPPH assay was performed as described by Ghawi et al. [5] with minor modification. This method depends on the reduction of purple DPPH radicals to a pale yellow colored diphenyl picrylhydrazine and the remaining DPPH radicals which showed maximum absorption at 517 nm was measured.

Fifty micro liters of various concentrations of each sample was added to 150  $\mu$ L of a 250  $\mu$ M solution of DPPH in methanol. Controls with 50  $\mu$ L of DMSO without the extract were also prepared under the same condition for all experiments. Ascorbic acid was served as positive control with concentration between 5 - 100  $\mu$ g/mL. After 30 minute incubation in the dark at room temperature, the absorbance was measured at 517 nm. The experiment was performed in triplicates. The percentage of inhibition (%) of DPPH radical was calculated according to the following equation:

$$\% \text{ Inhibition} = [(Abs \text{ of control} - Abs \text{ of sample}) / Abs \text{ of control}] \times 100\%$$

The IC<sub>50</sub> was determined as the concentration of the sample required to inhibit the free radical by 50%.

### 2.4.2 Anticancer activity

Crude ethanolic extract, methanolic fraction and pure compounds were investigated *in vitro* towards three kinds of human cancer cell lines: cervical cancer cell line (HeLa), lung carcinoma cell line (NCI-H460) and breast carcinoma cell line (MCF-7) using method described by Sivakumar et al. [6] with minor modification. This is a colorimetric assay estimates cell number indirectly by staining total cellular protein with the dye sulforhodamine-B (SRB). This dye is a bright pink aminoxanthrene dye with two sulfonic groups. It is a protein stain that binds to the amino groups of intracellular proteins under mildly acidic conditions to provide a sensitive index of cellular protein content. Cells were cultured in the media to the sub-culture of fourth generation and seeded in 96-well microplates at a density of  $1 \times 10^4$  cells/well (HeLa and MCF-7 cell line) and  $7.5 \times 10^3$  cells/well (NCI-H460 cell line). The plates were incubated at 37°C for 24 h in 5% CO<sub>2</sub> atmosphere and the duplicate concentration cultured-media containing test samples was then added in the plates without removing the initial media. After 48 h incubation period at 37°C in 5% CO<sub>2</sub> atmosphere, the cells were fixed by adding trichloroacetic acid. The plates were incubated at 4°C for 1-3 h. The plates were gently washed five times with tap water (200  $\mu$ L/well) to discard the supernatant and were then air-dried for 12-24 h. The air-dried plates were stained with 0.2% SRB solution for 20 min at room temperature. The unbound dye was then removed by gently washing the plates five times with 1% acetic acid. The plates were then air-dried at room temperature for 12-24 h. 200  $\mu$ L of 10 mM trizma base was then added to each well. The

plates were then shaken on a gyratory shaker for 15-20 min to completely dissolve the protein-bound dye. The optical density (O.D) of each well was measured spectrophotometrically with an ELIZA microplate reader at 492 and 620 nm. The experiment was repeated 3 times for each cell line. The percentage of growth inhibition was calculated based on the following equation:

$$OD = OD_{492} - OD_{620} \quad (1)$$

$$OD_{492} \text{ (or } OD_{620}) = OD_{\text{cell}} - OD_{\text{control}} \quad (2)$$

$$\% \text{ Growth Inhibition} = [1 - (OD_{\text{TN}} - OD_{\text{C}})] \times 100\%$$

$OD_{\text{cell}}$  : OD values of wells containing cells

$OD_{\text{control}}$  : OD values of wells without cells

$OD_{\text{TN}}$  : OD values of sample calculated from (1) and (2)

$OD_{\text{C}}$  : OD values of control calculated from (1) and (2).

## 2.5 Statistical Analysis

All determinations for bioassay were carried out triplicates. Data were presented as the mean  $\pm$  SD analyzed by Skott-Knott test ( $p < 0.05$ ) to examine the significant differences between experimental data. A  $p < 0.05$  was considered significant. All statistical tests were carried out using SPSS package (version 16.0).

## 3. RESULTS AND DISCUSSION

### 3.1 Chemical Investigation of Methanolic Fraction

Chemical investigation of methanolic fraction results in the isolation of two compounds, rutin (1) and apigenin-7-O- $\beta$ -D-glucopyranoside (2). The structures of these compounds (Fig. 1) were unambiguously determined by their chromatographic behaviors as well as spectroscopic analysis via  $^1\text{H-NMR}$  and  $^{13}\text{C-NMR}$ .

#### 3.1.1 Identification

##### *Compound 1 (rutin)*

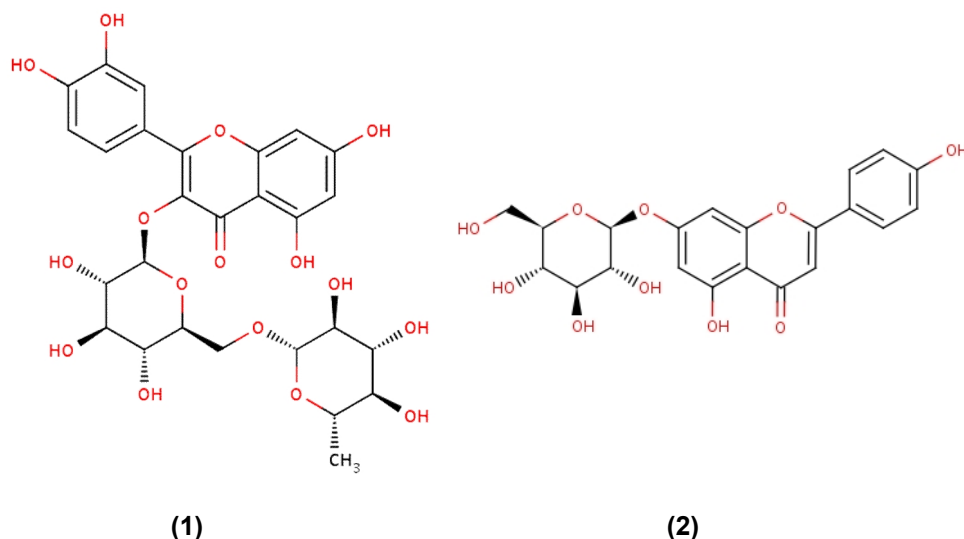
A pale yellow powder, mp 190-191°C.  $^1\text{H-NMR}$  (500 MHz,  $\text{CD}_3\text{OD-d}_4$ ):  $\delta$  7.65 (1H, dd,  $J = 8, 5$  Hz,  $\text{H}_6'$ ), 6.88 (1H, d,  $J = 8, 5$  Hz,  $\text{H}_5'$ ), 7.68 (1H, d,  $J = 2, 5$  Hz,  $\text{H}_2'$ ), 6.22 (1H, d,  $J = 2$  Hz,  $\text{H}_6$ ), 6.41 (1H, d,  $J = 2$  Hz,  $\text{H}_8$ ), 4.54 (1H, d,  $J = 1$  Hz,  $\text{H}_1'''$ ), 5.12 (1H, d,  $J = 7, 5$  Hz,  $\text{H}_1''$ ), 1.13 (3H, d,  $J = 6, 5$ ,  $\text{H}_6''$ ), 3.45-3.49 (1H, m,  $\text{H}_6''_{\text{a}}$ ), 3.82 (1H, d,  $J = 10$  Hz).  $^{13}\text{C-NMR}$  (125 MHz,  $\text{CD}_3\text{OD-d}_4$ ):  $\delta$ : 17.9 (C-6'''), 68.6 (C-6''), 75.8 (C-2''), 77.3 (C-3''), 71.4 (C-4''), 78.2 (C-5''), 72.1 (C-2'''), 72.3 (C-3'''), 73.9 (C-4'''), 69.7 (C-5'''); 99.9 (C-6), 94.9 (C-8), 117.7 (C-2'), 116.1 (C-5'), 123.6 (C-6'), 104.7 (C-1''), 102.4 (C-1'''), 105.7 (C-10), 123.2 (C-1'), 159.4 (C-2), 135.7 (C-3), 163.0 (C-5), 158.5 (C-7), 166.0 (C-9), 145.9 (C-3'), 149.8 (C-8'), 179.5 (C-4).

##### *Compound 2 (apigenin-7-O- $\beta$ -D-glucopyranoside)*

Pale yellow powder, mp 241-242°C.  $^1\text{H-NMR}$  (500 MHz,  $\text{DMSO-d}_6$ ):  $\delta$ : 6.67 (1H, s, H-3), 6.50 (1H, d,  $J = 2.5$ , H-6), 6.81 (1H, d,  $J = 2.5$ , H-8), 7.89 (2H, d,  $J = 8.0$ , H-2', H-6'), 6.94 (2H, d,  $J = 8.0$ , H-3', H-5'), 5.09 (1H, d,  $J = 7.0$ , H-1''), 3.49-3.53 (2H, m, H-2'', H-5''), 3.55-3.59 (1H, m,

H-3"), 3.41-3.45 (1H, m, H-4"), 3.94-3.98 (1H, m, H-6"<sub>a</sub>), 3.74 (1H, dd, J = 2, 10, H-6"<sub>b</sub>). <sup>13</sup>C-NMR (125 MHz, DMSO-d<sub>6</sub>) δ : 166.5 (C-2), 104.2 (C-3), 183.9 (C-4), 158.8 (C-5), 101.1 (C-6), 164.7 (C-7), 96.1 (C-8), 158.8 (C-9), 107.0 (C-10), 123.0 (C-1'), 129.6 (C-2', C-6'), 117.0 (C-3', C-5'), 101.6 (C-1"), 74.7 (C-2"), 78.4 (C-3"), 71.2 (C-4"), 77.8 (C-5"), 62.4 (C-6").

The spectra data obtained were in agreement with those reported in literature [7,8,9].



**Fig. 1. Compounds isolated from methanolic fraction obtained from successive fractionation of defatted ethanolic leaf extract of *P. multifida***

## 3.2 Biological Activities

### 3.2.1 DDPH free radical scavenging activity

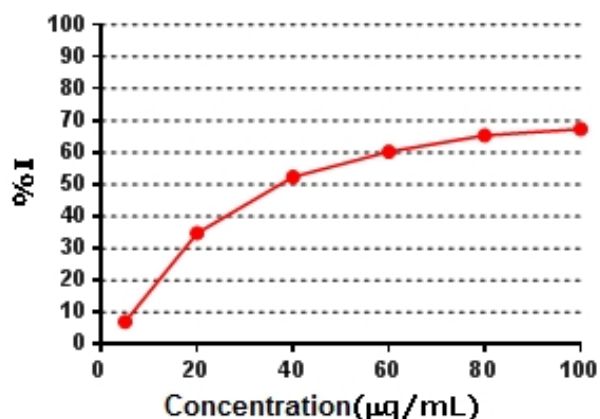
The model of scavenging of the stable DPPH radicals is a widely method to evaluate the antioxidant activity of the investigated sample in relatively short time compared with the other methods. The DPPH is a commercially available, stable organic nitrogen free radical with a deep purple color. In this assay, the purple chromogen (DPPH) radical is reduced by antioxidant/reducing compounds to the corresponding pale yellow hydrazine. The reducing ability of antioxidants towards DPPH can be measured at 517 nm (the maximum absorption of a stable DPPH radical).

The results of free radical scavenging properties of the crude ethanolic leaf extract, methanolic fraction and pure compounds compared to ascorbic acid are shown in Table 1. The crude ethanolic leaf extract expressed weak antioxidant activity with  $IC_{50} = 89.84 \pm 0.01$   $\mu$ g/mL whereas the methanolic fraction obtained from successive fractionation of defatted ethanolic leaf extract showed quite strong antioxidant activity with  $IC_{50} = 21.90 \pm 0.02$   $\mu$ g/mL as compared to that of ascorbic acid with  $IC_{50}$  of  $14.26 \pm 1.81$   $\mu$ g/mL. Rutin (**1**) isolated from methanolic fraction showed potent antioxidant activity in a concentration-dependent manner with  $IC_{50} = 37.70 \pm 0.03$   $\mu$ g/mL (Fig. 2.)

**Table 1. DPPH free radical scavenging activity ( $IC_{50}$ ) of crude ethanolic extract, methanolic fraction from successive fractionation of defatted ethanolic extract and rutin from *P. multifida* leaves**

Samples	$IC_{50}$ [ $\mu\text{g/mL}$ ]
Crude ethanolic extract	$89.84 \pm 0.01$
Methanolic fraction	$21.90 \pm 0.02$
Rutin	$37.70 \pm 0.03$
Apigenin-7-O- $\beta$ -D-glucopyranoside	-
Ascorbic acid	$14.26 \pm 1.81$

(-) No inhibition; Value of  $IC_{50}$  are expressed as mean  $\pm$  SD



**Fig. 2. Antioxidant activity of rutin (1) at different concentrations**

### **3.2.2 Anticancer activity**

Table 2 shows the results of *in vitro* cytotoxicity screening expressed in percentage of growth inhibition of crude ethanolic leaf extract and methanolic fraction of *P. multifida* against HeLa, NCI-H460 and MCF-7 cell lines at a single concentration (100 ppm) in accordance with the protocol of the Drug Evaluation Branch, National Cancer Institute [10] using the sulforhodamine B (SRB) method which has been widely used for drug-toxicity testing against different types of cancerous and non-cancerous cell lines [11].

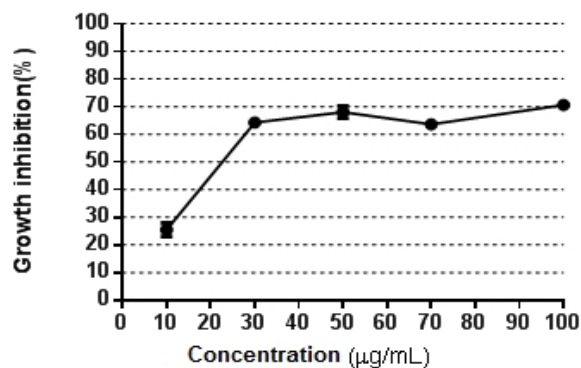
The crude ethanolic extract showed potent cytotoxic activity against HeLa ( $35.26 \pm 3.50\%$ ) and NCI-H460 ( $50.54 \pm 4.18\%$ ) whereas the methanolic fraction obtained from successive fractionation of defatted ethanolic extract exhibited cytotoxic activity against all three types of cancer cell lines: HeLa ( $36.97 \pm 2.15\%$ ), NCI-H460 ( $53.74 \pm 3.37\%$ ) and MCF-7 ( $68.21 \pm 2.47\%$ ).

Fig. 3 shows the cytotoxic activity of apigenin-7-O- $\beta$ -D-glucopyranoside (2) isolated from methanolic fraction against MCF-7 cell line at various concentrations and (2) was found to exhibit high cytotoxic activity against the MCF-7 cell line with  $IC_{50} = 22.62 \pm 0.59 \mu\text{g/mL}$ .

**Table 2. Cytotoxic activity (%) of crude ethanolic extract and methanolic fraction from successive fractionation of defatted ethanolic extract of *P. multifida* at 100 ppm on HeLa, NCI-H460 and MCF-7**

Plant extract	HeLa	NCI-H460	MCF-7
Crude ethanolic extract	35.26 ± 3.50	50.54 ± 4.18	-
Methanolic fraction	36.97 ± 2.15	53.74 ± 3.37	68.21 ± 2.47

(-)No inhibition; Data are expressed as mean ± SD



**Fig. 3. Cytotoxic activity of apigenin-7-O-β-D-glucopyranoside (2) on MCF-7 cell line at various concentrations**

*P. multifida* is widely distributed in the north and central of Vietnam and has been traditionally used by local people to cure various diseases including cancer, so it is necessary to examine the pharmacological characteristics of this plant. The data obtained from this research would be helpful in scientifically validating the traditional use of this plant species. This is the first investigation of focusing on the elucidation of components from methanolic extract of *P. multifida* growing in Viet Nam and evaluation of biological activities of active compounds including antioxidant and anticancer activities. Two active compounds, rutin (1) and apigenin-7-O-β-D-glucopyranoside (2) were isolated from methanolic fraction obtained from successive fractionation of defatted ethanolic extract of *P. multifida* leaves. These compounds were subjected to the assay for antioxidant and anticancer activities.

Rutin (3,3',4',5,7-pentahydroxyflavone-3-rhamnoglucoside) has been evaluated for antioxidant activity by DPPH assay reported from many plant species and the IC<sub>50</sub> values were found to be different. Examples are *Cineraria abyssinica* with IC<sub>50</sub> = 3.53 μg/mL [12] and *Osmanthus fragrans* with IC<sub>50</sub> = 10.3 μg/mL [13]. Rutin was even used as a standard in evaluation of antioxidant potential of compounds from various medicinal plants and the IC<sub>50</sub> values were also varied from 5.44 ± 0.21 μg/mL [14] to 22.96 ± 1.99 μg/mL [15] and 480 μg/mL [16]. In this study, rutin was found with IC<sub>50</sub> value = 37.70 ± 0.03 μg/mL which indicates that our findings seem to be consistent with other previous reports as mentioned above. It should be noted, however, that the different values of IC<sub>50</sub> of rutin resulting from DPPH assay using different plant sources are due to the distribution of several factors such as differences in used solvents, concentration of DPPH working solutions, ratio between volumes of sample/reagent, duration of reaction, and equations for calculation of the results [17].



Rutin did not show any significant inhibitory activity against all three selected cancer cell lines, HeLa, NIC-H460, and MCF-7 whereas apigenin-7-O- $\beta$ -D-glucopyranoside exhibited strong cytotoxicity against MCF-7 with  $IC_{50} = 22.62 \pm 0.59 \mu\text{g/mL}$ . Presence of apigenin-7-O- $\beta$ -D-glucopyranoside has been reported from some plant species like *Gaillardia aristata* [18]; *Achillea millefolium* [19]; and *Platycodon grandiflorum* [20]. However, our findings are significantly different from previous reports of Salama et al., 2011 where apigenin-7-O- $\beta$ -D-glucopyranoside did not show any significant cytotoxicity against MCF-7. It is too early to conclude the potential of apigenin-7-O- $\beta$ -D-glucopyranoside in treatment of breast cancer; however, it is convincingly believed that the therapeutic effects of *P. multifida* should be considered in supporting the treatment of breast cancer.

#### 4. CONCLUSION

The results demonstrate that the crude ethanolic leaf extract of *P. multifida* has weak antioxidant activity and cytotoxic activity against two kinds of cancer cell lines: HeLa and NCI-H460 whereas methanolic fraction obtained from successive fractionation of defatted ethanolic extract exhibits quite strong antioxidant activity and cytotoxicity against three kinds of cancer cell lines: HeLa, NCI-H460 and MCF-7. Two purified compounds were isolated from methanolic fraction: Rutin (1) possess a potent antioxidant activity while apigenin-7-O- $\beta$ -D-glucopyranoside has a strong anticancer activity against MCF-7 cell line.

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#### CONSENT

Not applicable.

#### ETHICAL APPROVAL

Not applicable.

#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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