RESEARCH ARTICLE

Anti-Proliferative Effects of Methanol and Water Extracts of *Pyrrosia piloselloides* on the Hela Human Cervical Carcinoma Cell Line

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Abstract

Background: Cervical cancer is one of the most commonly diagnosed neoplasms and a leading cause of cancer death among females worldwide. Limitations with conventional medical treatments have driven researchers to search for alternative approaches using natural products. This study aimed to detemine potential anti-proliferative effects of methanol and water extracts of Pyrrosia piloselloides (P. piloselloides) on the HeLa cell line. **Methods:** 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were performed to determine IC50 concentrations and apoptosis analysis was by flow cytometry. To identify chemical compounds in the extracts, gas chromatography-mass spectrometry (GC-MS) was employed. **Results:** *P. piloselloides* methanol extracts (PPME) showed antiproliferative effects on HeL awith an IC50 of 16.25μg/mL while the *P. piloselloides* water extract (PPWE) was without influence. Neither extract showed any significant effects on apoptosis. GC-MS analysis, revealed 5-hydroxymethylfurfural (23.1%), allopurinol (8.66%) and 3, 5-dihydroxy-6-methyl-2,3-dihydropyran-4-one (7.41%) as major components in the PPME, while sulfolan-3-ol (10.1%), linoleic acid (9.06%) and β-sitosterol acetate (7.98%) predominated in the PPWE case. Conclusion: This first study of *P. piloselloides* showed PPME to exert potent anti-proliferative effect on HeLa cell lines. Further research now needs to be performed to establish the mechanisms of inhibition.

Keywords: *Pyrrosia piloselloides*- antiproliferative- HeLa cells

Introduction

Cancer is a generic term for a large group of diseases characterized by the growth of abnormal cells beyond their usual boundaries which invade adjoining parts of the body and/or spread to the other organs. Most of this abnormal growth are irreversible and cause numerous alterations that can distract the body systems. Cancer also can be described as malignant tumor or neoplasm, while the process of normal cell turning into cancer cells is called carcinogenesis. The disease can affect almost any part of the body and has many anatomies and molecular subtypes that each required specific management strategies to avoid or inhibit it. According to 2014 World Health Organisation (WHO) report, cancer is the second leading cause of death globally accounted for 8.8 million deaths in the year. There are more than 200 different types of cancer detected but the world most common cancer affected among men are lung, prostate, colorectal, stomach and liver cancers (Torre et al., 2015). While breast, cervix, colorectal, lung and stomach cancers are the most common diagnosed among women (Torre et al., 2015). Although some of the cancers are been said preventable yet it seems still becoming as one of the

leading causes of death to human for example the CxCa.

CxCa is a cancer that occurs at a part of female reproductive system called cervix; the entrance to uterus from vagina. Most CxCa cases are caused by an infection of a virus called Human Papilloma virus (HPV) on the surface of the cervix which led to cervical dysplasia or abnormal growth of the cervical cells (Bruni et al., 2016). Based on the statistics of World Health Organization (WHO), approximately 270,000 women died from CxCa in 2016 where more than 85% of the deaths occur in low and middle-income countries (Bruni et al., 2016). Hence, the highest incidence rates can been found in such countries such as in Central America, East Africa, Southeast Asia and Western Pacific (International Agency for Research on Cancer; GLOBOCAN 2012 v1.0 2013). Today, this illness has become one the gravest threat to women's lives worldwide. Though the number of CxCa cases illustrate a decrease in most of developed regions over the past three decades, but it keeps increasing or remaining unchanged in almost all developing countries including in the Asia Pacific's (International Agency for Research on Cancer; GLOBOCAN 2012 v1.0 2013). Likewise, the disease has also turn to be one of the most

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common cancers and death causal among Malaysian females (Health Ministry of Malaysia, 2009). According to Malaysian National Cancer Registry Report in 2016, the risk of Malaysian women to get CxCa is one over 116 where almost 60% of cases were detected at mild and moderate stage. Most of the patients are from the beginning of reproductive age group, 25-45 years old, and those who reached the menopause stage (International Agency for Research on Cancer; GLOBOCAN 2012 v1.0, 2013; Malaysian National Cancer Registry Report, 2016). Current estimates indicate that 2,145 women are diagnosed with CxCa and 621 dies from the disease annually.

While local governing agencies are pushing the Papanicolaou smear (Pap smear) and HPV vaccine as the attempts to curb future CxCa rates, the effort of looking for cancer preventive and therapeutic drugs originating from the natural sources such as plants have grown in popularity of researchers. The scientific investigations on various herbs consisting bioactive compounds as natural chemopreventive and chemotherapeutics proved a promising future in combating CxCa. Some of the advantages that derived from this alternative therapeutic method include lower side effects and toxicity, higher accessibility and availability and lower cost consumption. Recently, a number of studies have demonstrated in vitro and/or in vivo that some plants can be new and potential anticancer agents (Kwon et al., 2006; Li et al., 2007; Kwon et al., 2007; Kim et al., 2010). Therefore, many researchers believe that chances of discovering more effective drugs to forbid or treat the life-threatening diseases such as CxCa is still wide open since the tropical Southeast Asian rainforest like those dominates in Malaysia is very rich with various diversities of plants and herbs (Wan Ghazali et al., 2016; Alternative Medicine, 2002).

P. piloselloides is one of the most common epiphytic fern originated from family of Polypodiaceae which naturally grows small in size but abundant and knows as "pokok duit-duit (money plant)" in Malaysia. The *P. piloselloides* plant usually appears creeping on the trunks of old trees at the roadside or in small jungle that have balanced moist and warm conditions to expand. Literally, this fern can be extensively found in Tropical Asia regions. The rhizomes are only approximately 1mm in diameter but extremely long and strong covered with small, almost round or heart-shaped scales, the leaves. It is reported that its leaves are used by aboriginal Negritos and Southern Thais as pain and fever relievers (Neamsuvan et al., 2014; Lin, 2005). In Indonesia, it is an old medicinal practice to use the leaves to treat breast cancer disease (Wulandari et al., 2013). Local folks also use it to soothe gallstone and as a skin rashes poultice. *P. piloselloides* leaves will either be crushed for external applications or decocted for oral consumptions (Lin, 2005). Previous study has reported that the plant contains flavonoids, tannins, steroid or triterpenoid, essential oils and glycosides which are highly potential to act as anticancer agent (Sahid et al., 2013). Thus, this present study aims to evaluate the antiproliferative attribution of *P. piloselloides* methanol and water extracts on cervical carcinoma cell line, HeLa as human in vitro model system. Following the treatments to the cell line, the concentration 50% inhibition in the cells

population (IC50) will be decided. The IC50 value will be used to examine the apoptosis induction of treatments using flow cytometer analysis. Later, the extracts will be analyzed by GC-MS to identify the probable bioactive compounds in the *P. piloselloides*.

Materials and Methods

Sample collection and preparation

Fresh *P. piloselloides* plants were collected from Pasir Hor, Kelantan, Malaysia. The authentication of the plant was made at Herbarium Kulliyyah of Pharmacy, International Islamic University Malaysia by the botanist. The identification of *P. piloselloides* specimen was deposited with a voucher number PIIUM 0305. To prepare *P. piloselloides* dry sample, the leaves were washed, oven-dried at 50°C for several days in incubator (Binder, Germany) and ground using a heavy duty grinder (Golden Bull, Malaysia). The powder then was kept and sealed in Duran bottles at room temperature until further used.

Preparation of methanol and water extracts

The *P. piloselloides* methanol extract (PPME) was prepared by soaking the 50g *P. piloselloides* powder in 500ml of absolute methanol with 1:10 (w/v) ratio and was shaken overnight at 1500 rpm using shaker incubator (Protech, Malaysia) at room temperature. The mixture was filtered using 150mm Whatman filter paper and concentrated using rotary evaporator (Heidolph, South Korea) followed by evaporating it in the fume hood for several days for complete solvent evaporation. The filtered methanol residue was dried out before further used in *P. piloselloides* water extraction. For *P. piloselloides* water extract (PPWE), all previous extraction steps were repeated using distilled water. The filtered extract was froze dried using freeze dryer (ILshin Lab, South Korea) till the PPWE would turn into powder form. Concentrated PPME and powdered PPWE were sealed separately in universal bottles and stored at 4°C for future usage.

Cytotoxicity evaluation by MTT assay

HeLa and L929 cells (American Type Culture Collection Company, USA) with 80-90% confluent were plated into 96 wells microliters plate (5×10^4 cells/ mL). The cells were maintained at 37°C in a humidified incubator with an air mixture containing 5% (v/v) CO_2 for 24 hours. The adherent cells were trypsinised to detach the cells. The media then were replaced by 200μL of fresh complete media in each well. Cells were treated with 2μL of a serial dilution of PPME or PPWE from 0.39μg/mL to 100.0μg/mL. Two microliter of dimethyl sulfoxide (DMSO) and Tamoxifen (Sigma Chemical Co., USA), in same dilutions were used as negative and positive controls respectively. The cells then were incubated for 72 hours. The cell viability was subjected using MTT assay kit based on the guidelines from manufacturer and Tian and Zang (2015) method with slight modifications. The optical densities (OD) were measured through spectrophotometric plate reader (Thermo Electron Co., USA) at 570 nm. The viability percentage was calculated using below formula:

Viability percentage (%) = (OD sample-treated cells) x 100%

OD untreated cells

Dose-response curves were constructed to obtain the IC50 values. A graph of cell viability determined from MTT Assay was expressed and compared. Extract with lowest IC50 on HeLa cells would be further preceded for apoptosis assessment.

Apoptosis assessment by flow cytometer analysis

T1x105 of HeLa cell line was seeded in six well-plates. After the incubation at 37°C in 5% CO_2 and a humidified atmosphere for 24 hours, PPME was added into the HeLa cells at the concentration of IC50 value. After 24, 48 and 72 hours treatments, the PPME-treated and untreated HeLa cells were collected and assessed using Vybrant TM Apoptosis Assay Kit 3 (Molecular Probes, USA). Briefly, the collected cells were centrifuged and the supernatants were discarded. Then, cells were washed and centrifuged twice with cold PBS before resuspending them with 1x binding buffer at $1x10^6$ cells/mL. The cells were transferred to 5mL FACs tubes (Becton-Dickinson, USA) with 5μL of FITC Annexin V and 1μL of Propidium Iodide (PI). The mixture was gently vortexed and incubated at dark room temperature for 15-30 minutes. Lastly, 400μL of 1X Binding Buffer was added to each tube within one hour prior to flow cytometry analysis. For staining controls, one tube of each unstained, FITC Annexin V and PI were prepared and analyzed. Detected cell populations were grouped and labeled into four quadrants; Q1 - dead or debris cells which only permeable to PI and excluded from analysis, Q2 - cells in late apoptosis or necrosis stage which would be stained by Annexin V and PI, Q3 - viable cells and unstained by Annexin V and PI and Q4 - cells at early apoptosis and positive Annexin V and PI staining. The total percentage of apoptosis were summed up from periods of early apoptotic, Q4 to late apoptotic, Q2.

GC-MS Analysis

The GC-MS analysis was carried out on a GC Agilent Technologies 6890 Network System and gas chromatography interfaced to a mass spectrometer Agilent Technologies 5973 inert Mass Selective Detector. Ten micrograms of PPME or PPWE dissolved in 2mL methanol were transferred into GC-MS vial. This instrument employed the following condition: Column HP-MS (30 x 0.25mm ID x 0.25 film thickness), operating in electron impact mode at 70Ev, helium (99.999%) was used as carrier gas at a constant flow of 1.2mL/min, injection volume of 1μL was employed (splitless mode), injection temperature of 280°C. The oven temperature was programmed from 70°C (isothermal for 2 minutes) with an increase of 20°C/min, to 280°C for 30 min. The scan rate was 2.42s per scan. The solvent delay was 2 min and the total running time was 42.5 min. For the identification of the components, the interpretation on mass spectrum GC-MS was conducted using the database of National Institute Standard and Technologies (NIST) 2002. The spectrum of the unknown component was compared with the spectrum of the known components in the NIST

library. The name of the compounds, retention time and percentage area were ascertained in the end.

Statistical analysis

All measurements were carried out in triplicates from three independent experiments and the results were expressed as the mean ± SEM. The data were first tested for their normality using Kolmogorov-Smirnov test. The statistical significances of data obtained were calculated and regulated using two ways analysis of variance (ANOVA). For all the comparisons, the level of $p \leq 0.05$ was considered significant. All statistical analysis was performed using Graph Pad PRISM Version 6.0 by GraphPad Software Inc, California.

Results

Cell proliferation - MTT assay

Based on the Table 1, the IC50 value of PPME on HeLa cell was 16.25±1.96μg/mL while the extract seemed to have no growth inhibition on L929 cell when the graph exhibits linear trend in all concentrations (Figure 2). Meanwhile, PPWE experienced the same style when tested on HeLa cell but showed a slight emergence on cell viability when its concentration have been increased. Therefore, it can be described PPWE has no antiproliferative effect either on carcinoma CxCa or normal cell lines. As a result, based on the Figures 1 and 2, the IC50 values of PPME on L929 cell and PPWE on both treated cell lines were higher than 100μg/mL. On the hand, as the positive control drug, tamoxifen appeared to have greatest value of IC50, 1.87±1.32μg/mL, to the cancer cell line, HeLa. However, the IC50 value of tamoxifen in the L929 cell was more eminent than the IC50 value on the HeLa cell with 1.09±0.59μg/mL.

Apoptosis assessment by flow cytometry analysis

In order to detect apoptosis and effect of PPME in cell cycle progression of HeLa, flow cytometry analysis using PI and Annexin V was carried out. However, in comparison to the untreated HeLa cells, there were no significant difference was found in total apoptosis of PPME-treated HeLa cells after 24, 48 and 72 hours observation as shown in Figures 3 and 4.

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

The list of phytochemical compounds in PPME and PPWE resulting from GC-MS analysis are reported in Tables 2 and 3 respectively. Their qualitative and

Table 1. Cytotoxicity Activities of PPME, PPWE and Tamoxifen on HeLa and L929 Cell Lines

	Cell growth inhibition, IC_{50} (μg/mL)	
	HeLa cell	L929 cell
PPME	16.25±1.96	>100
PPWE	>100	>100
Tamoxifen	1.87±1.32	1.09±0.59

IC_{50} values are presented as means±SEM; PPWE, P. piloselloides methanol extract; PPWE, P. piloselloides water extract
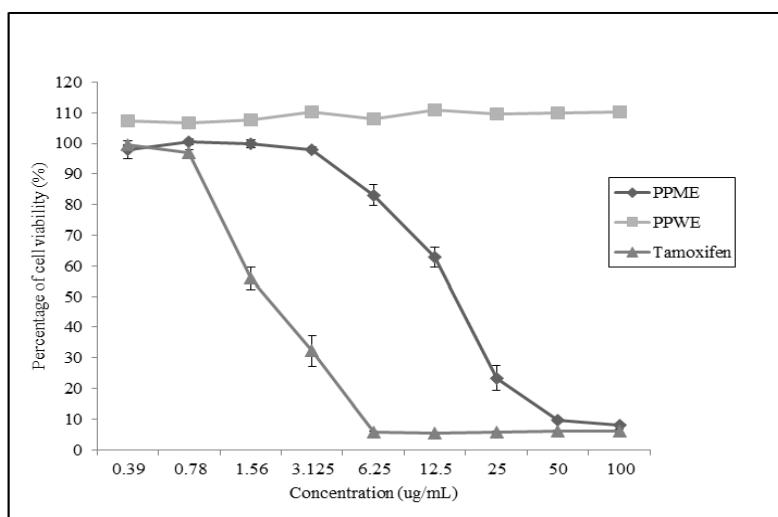
Figure 1. The Percentage of Cells Viability of HeLa Cell at Different Concentrations of PPME, PPWE and Tamoxifen
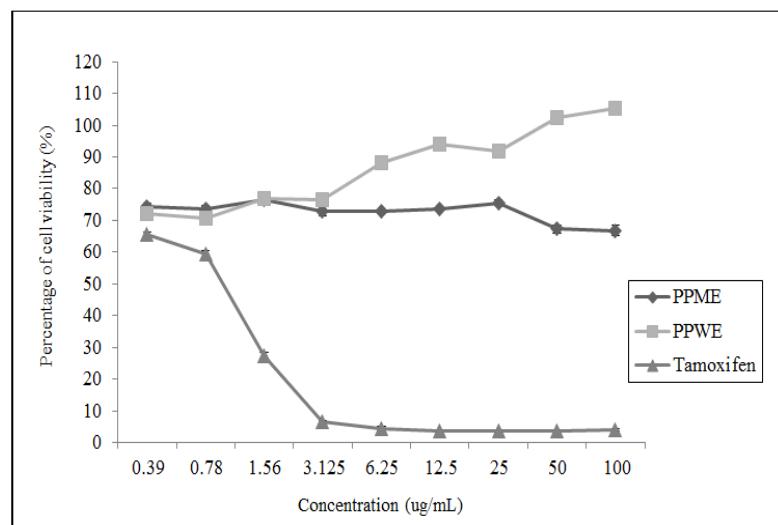

Figure 2. The Percentage of Cells Viability of L929 Cell at Different Concentrations of PPME, PPWE and Tamoxifen

quantitative results are recorded based on the retention time (rt), compound mass and percentage of peak area of detected compounds. From the analyses, 31 and 28 bioactive compounds were identified in *P. piloselloides* of methanol and water extracts. 2-Furancarboxaldehyde,

5-(hydroxymethyl) (23.13%), allopurinol (8.66), 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-(7.41%), n-hexadecanoic acid (5.98%) and 1,3,5-Triazine-2,4,6-triamine (5.34%) were discovered to be the most discrimant phytochemical compounds in PPME.
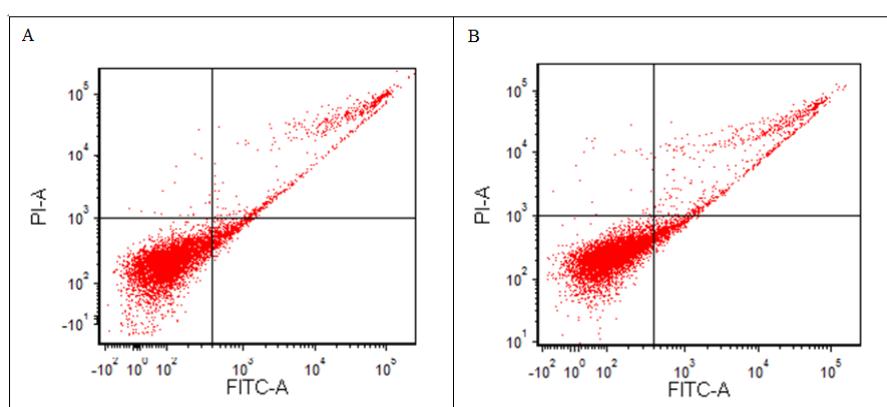

Figure 3. Scatter Plot Diagram of Apoptosis Rate Through Flow Cytometry Analysis of HeLa Cell after 72 Hours PPME Treatment at IC_{50} Value. Apparently, no decipherable changes have exhibited within the 24, 48 and 72 hours of observations. The figure is representing the PPME treatment on HeLa cell within periods. (A) Untreated HeLa cell; (B) Treated HeLa cell, (Q3) Live cell, (Q4) Early apoptosis, (Q2) Late apoptosis, (Q1) Necrotic/dead cell.

Table 2. Bioactive Components Identified in PPME by GC-MS

No	Rt	% Peak area	Name of compound	Molecular formula
1	3.27	1.24	N,N-Dimethyltrimethylsilamine	C5H15NSi
2	4.26	3.67	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	C6H8O4
3	4.83	1.21	2H-Imidazole-2-thione, 1,3-dihydro -1-methyl-	C3H4N2S
4	4.92	0.48	1,3,6-Trioxocane, 2-methyl	C6H12O3
5	5.22	5.34	1,3,5-Triazine-2,4,6-triamine	C3H6N6
6	5.28	1.08	2-Furancarboxylic acid, hydrazide	C5H6N2O2
7	5.73	0.53	2-Propanamine, N-methyl-N-nitroso-	C4H10N2O
8	5.81	7.41	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	C6H8O4
9	5.99	3.17	Benzoic Acid	C7H6O2
10	6.15	0.85	4H-Pyran-4-one, 3,5-dihydroxy-2-methyl-	C6H6O4
11	6.33	1.94	Butanedioic acid	C4H6O4
12	6.38	2.57	Benzofuran, 2,3-dihydro-	C8H8O
13	6.51	23.13	2-Furancarboxaldehyde, 5-(hydroxymethyl)	C6H6O3
14	6.64	2.85	1,2,3-Propanetriol, diacetate	C7H12O5
15	7.15	1.41	2-Methoxy-4-vinylphenol	C9H10O2
16	7.19	1.03	2-Naphthalenol	C10H8O
17	7.72	1.62	Benzene, 1-chloro-2-methoxy-	C7H7ClO
18	8.02	8.66	Allopurinol	C5H4N4O
19	8.19	1.52	6-Hydroxymethaqualone	C16H14N2O2
20	8.42	2.19	3,4-Altrosan	C6H10O5
21	9.24	5.45	Benzenepropanoic acid, 4-hydroxy-	C9H10O3
22	10.28	0.37	1-Pentadecene	C15H30
23	10.9	5.98	n-Hexadecanoic acid	C16H32O2
24	11.66	2.31	Phytol	C20H40O
25	11.75	1.85	9,12-Octadecadienoic acid (Z,Z)-	C18H32O2
26	11.78	4.73	9,12,15-Octadecatrien-1-ol, (Z,Z,Z)-	C18H32O
27	12.52	0.38	Benzene, 6-heptynyl-	C13H20
28	14.75	0.5	2,6,10,14,18,22-Tetracosahexaene	C30H50
29	17.91	1.28	Ergost-8(14)-en-3-ol, (3.beta.)-	C28H48O
30	18.22	1.03	Stigmasterol	C29H48O
31	18.85	4.23	Stigmast-7-en-3-ol, (3.beta.,5.alpha.,24S)-	C29H50O

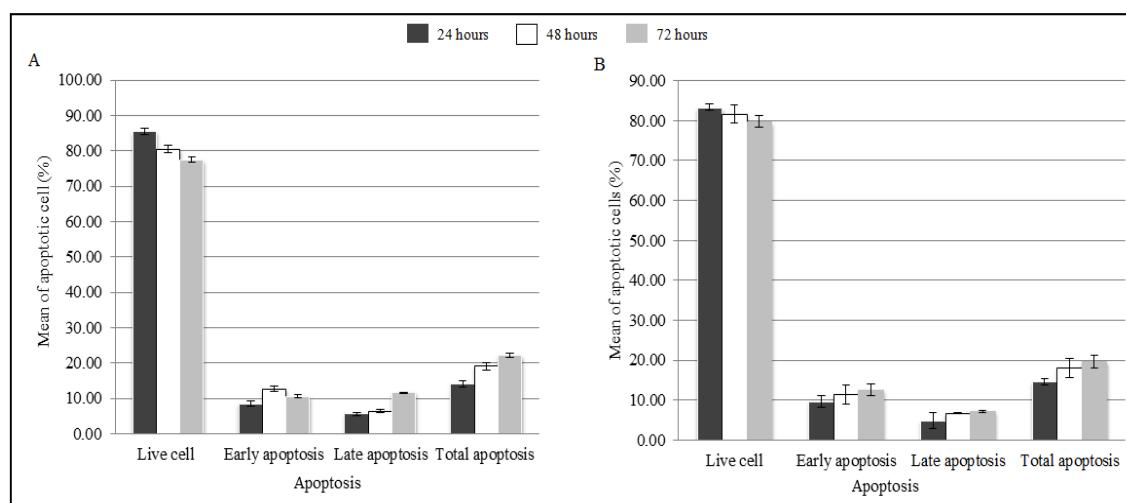Rt, retention time; PPME, P. piloselloides methanol extract

Figure 4. Graph Summary of Apoptosis Assessment the Cell Growth of HeLa Cell after 24, 48 and 72 hours. A, Untreated HeLa cell lines; B, Treated HeLa cells lines.

Table 3. Bioactive Components Identified in PPWE by GC-MS

No	Rt	% Peak area	Name of compound	Molecular Formula
1	3.93	3.63	2-Cyclopenten-1-one, 2-hydroxy	C6H8O2
2	4.58	7.41	Cyclobutaneoctol	C4H8O8
3	5.14	4.22	2,5-Dimethyl-4-hydroxy-3(2H)-furanone (Furaneol)	C6H8O3
4	5.47	10.06	Thiophene-3-ol, tetrahydro-, 1,1-dioxide	C4H8O3S
5	6.37	6.43	2-Pentene, 2-methoxy	C6H12O
6	6.56	2.84	Benzofuran, 2,3-dihydro	C8H8O
7	6.78	3.66	Naphthalene, 2,6-bis(1,1-dimethylethyl)-	C18H24
8	7.2	5.77	2-Methoxy-4-vinylphenol	C9H10O2
9	7.33	1.29	1,3-Bis(trimethylsiloxy)benzene	C12H22O2Si2
10	7.47	1.76	2-Ethyl-2,3-dihydro-1H-indene	C11H14
11	7.63	1.21	Propenone, 3-(2-benzoxazolylthio)-1-phenyl-	C16H11NO2S
12	7.75	4.68	Tributylamine	C12H27N
13	7.94	1.55	Phenol, 2-(1,1-dimethylethyl)-4-(1-methyl-1-phenylethyl)-	C23H32O
14	8.06	1.02	Methylphosphonothiodifluoride	CH3F
15	8.1	4.37	1-Ethylcyclopropanol	C5H10O
16	8.21	1.22	7H-1-Benzopyran-7-one, 5-methoxy-6-methyl-2-phenyl	C17H14O3
17	8.69	1.1	4-Hydroxyphenylacetic acid, ethylester, tert-butyldimethylsilyl	C16H26O3Si
18	9.62	0.84	2-(p-(Dimethylamino)phenyl)benzimidazole	C15H15N3O2
19	10.46	0.76	Carbazole, pentamethyl-	C12H9N
20	10.74	2.35	Hexadecanoic acid, methyl ester	C17H34O2
21	10.92	5.14	Pentadecanoic acid	C15H30O2
22	11.81	9.06	Octadec-9-enoic acid	C18H34O2
23	12.35	1.05	Cyclononasiloxane, octadecamethyl-	C18H54O9Si9
24	12.98	1.42	Octadec-9-enoic acid	C18H34O2
25	13.35	1.77	Butane, 1-(benzyloxy)-2-[(benzyloxy)methyl]-	C5H12
26	18.3	4.33	Stigmastan-3,5,22-trien	C29H46
27	18.8	4.33	7-Dehydrodiosgenin 3-acetate	C29H42O4
28	19.1	7.98	.beta.-Sitosterol acetate	C31H52O2

Rt, retention time; PPWE, P piloselloides water extract

Whereas, the most major compounds existed in PPWE are thiophene-3-ol, tetrahydro-, 1,1-dioxide (10.06%), octadec-9-enoic acid (9.06%), beta-sitosterol acetate (7.98%), cyclobutaneoctol (7.41%) and 2-pentene, 2-methoxy (6.43%). Moreover, there are two similar compounds encountered present in both extracts when using methanol as mobile phase which are benzofuran, 2,3-dihydro (rt at 6.38 in PPME and 6.56 in PPWE) and 2-Methoxy-4-vinylphenol (rt at 7.15 in PPME and 7.2 in PPWE).

Discussion

Botanicals are gaining massive attention in therapeutics management for cancer due to their efficacy to inhibit critical processes of cancer growth, angiogenesis and metastasis with minimal side effects and domestic reachable too. Nowadays, more researchers are exploring and innovating the compounds from natural sources such herbs and plants for cancer prevention or treatment used. Due to that, almost two over third of new drugs for malignant treatments today are originated from the compounds of natural sources (Wang et al., 2013).

Recently, various studies have demonstrated in vitro and/or in vivo that a number of plants able to suppress the infected cell and potentially become an anticancer agents (Mishra and Das, 2015; God et al., 2010; Gomathinayagam et al., 2008). In fact, some of the plants have been reported to be a promising antiproliferative to CxCa cell for instances *Passiflora foetida* (Talik Sisin, 2016), *Lawsonia inermis* (Kumar et al., 2015), *Ophiocoma erinaceus* (Baharara et al., 2016) and so forth (Kwon et al., 2006; Li et al., 2007; Kwon et al., 2007; Kim et al., 2010).

According to National Cancer Institute, substance with the IC_{50} value lower than or equal to 20μg/mL has appropriate cytotoxic attribution and potential to be formulated as an anticancer agent. In the present study, PPME has exhibited the IC50 value on HeLa cell at 16.25μg/mL while no cell growth inhibition occurred in mammalian normal cell line, L292. The attitude of PPME considerably ideal to be a cervical anticancer agent as the extract has only reacted to the dysplasia cells but not to the normal's. In contrast, even though no significant proliferative activity was established, PPWE has been noticed to induce positive effect on normal cell growth but constantly inactive in HeLa cell in dose-dependent

manner (Table 1, Figures 2 and 3). Thus, it can be indicated that this epiphyte plant may contain the bioactive compounds which possess both antiproliferative effect and cell proliferative stimulator in cell-dependent manner. Moreover, from the MTT test result, it is proved that *P. piloselloides* leaves is a non-toxic part plant and can be safely consumed as a traditional/alternative medicine. However, further studies should be performed to solidify the aforementioned findings and isolate the potential compounds beforehand for new drugs development either for neoplasm defected cell inhibitor or as cell growth enhancer.

The methanol extract of *P. piloselloides* at IC50 value gained from the cell viability test, MTT, was further examined to flow cytometric analysis for analyzing the cell cycle and apoptosis of the HeLa cells using Annexin V/PI staining method. The apoptosis assessment of HeLa cell was studied at 24, 48 and 72 hours after the PPME treatments. While staining with Annexin V is typically useful for identification of early and late apoptosis cells. Viable cells with intact membrane are impermeable to PI stain, whereas the membranes of dead and damaged cells are permeable to it. Notably, there was no significant change in total apoptosis at particular hours after PPME treatment compared to the untreated HeLa cells as shown in Figures 3 and 4. This data states that PPME is able to inhibit the proliferation of CxCa cell in time- and dose-dependent manner, yet does not exhibit a significant effect on the mortality of cell in apoptosis cell cycle within the 72 days period. Same observations was also documented by Boulaaba et al., (2013), Chacko et al., (2012) and Wicaksono et al., (2009). In such case, previous researchers have come across that the apoptopic effect of PPME towards the cancer cell HeLa might have a little delayed and the effect will only can be seen if the observation period would be prolonged to more than 72 hours as reported in a study by (Konarikova et al., 2015). Another possible reason is, PPME might comprise with the element which work directly to cell cycle progress or direct hormonal link rather than through the apoptosis pathway in order to diminish cancer cell, HeLa as studied by Boulaaba et al., (2013) and Arditi et al., (2007). Nevertheless, more researches should be performed to affirm the hypothesis above.

Both extracts have been evaluated through GC-MS to specify the bioactive compounds present in the extracts. The GC-MS analysis of PPME revealed the presence of 2-Furancarboxaldehyde, 5-(hydroxymethyl) at the highest level, 23.13% and could be the main contributor to medicinal quality of *P. piloselloides* leaves. It was previously reported that, 2-Furancarboxaldehyde, 5-(hydroxymethyl) or also known as 5-HMF exhibits antioxidant and inhibitory effect on human melanoma A375 cell lines (Zhao et al., 2013). He has suggested that the compound could be developed as a novel natural antioxidant with potential applications in cancer chemoprevention. The highest compound identified in PPWE was thiophene-3-ol, tetrahydro-, 1,1-dioxide (also known as sulfolan-3-ol) with 10.06%. Tilstam (2012) stated that sulfolan-3-ol is a dipolar aprotic industrial solvent, usually used in oil refining and gas production.

However, till to date, no study has been published to relate the compound with any antiproliferative or anticancer activity. Pertaining to the present MTT result, PPWE consists of sulfolan-3-ol contributes to negative cytotoxicity effect to the HeLa and L292 normal cells. Further investigations needs to be done to define the association and mechanism of anti-proliferative effect among highest compounds in PPME and PPWE such as 5-HMF, allopurinol, DDMP-4-1 with CxCa and non-malignant cells.

To reiterate, *P. piloselloides* is a potent anticancer agent since it produced the IC50 value<20 μg/mL on HeLa cell line though no significant apoptosis induction was promoted within 72 hours observation. This might also be a good indication that the cytotoxic compounds in that extracts might have less toxicity and exhibit cytoselectivity towards mammalian normal cells with the IC50 value was more than 100 μg/mL. As a result, it is an encouraging evidence for the usage of *P. piloselloides* as cancer traditional/alternative medicine. To the best of our knowledge, this study is the first to conduct on the antiproliferative activity on cancer cell.

Statement conflict of Interest

All authors declare having no conflict of interest and are fully responsible for all experimental works and the content of this article.

Acknowledgements

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