Anticancer Effect of Hypophyllanthin, Niranthin and Lintetralin From Phyllanthus amarus on HeLa Cells And NIH/3T3 Cells

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Abstract: ***Phyllanthus amarus which is locally known as Dukung Anak was one of the herb used in traditional medicine and research of P. amarus in Malaysia was not widely reported. This present research has isolated three chemical compounds and its anticancer effect of this species has been determined. Three lignans namely hypophyllanthin was afforded from hexane crude while niranthin and lintetralin were afforded from ethanol crude. Anticancer test against HeLa cells and NIH/3T3 cells by MTT assays was tested for its anticancer effect. The result shows that hypophyllanthin was considered to possess an active anticancer effect on HeLa cells than NIH/3T3 cells compared to niranthin and lintetralin.***

Index Terms*: *Anticancer, HeLa cells, Lignans, NIH/3T3 cells and Phyllanthus amarus

I. INTRODUCTION

Phyllanthus amarus belongs to the family of Euphorbiaceae. Euphorbiaceae is a large family of flowering plants with 300 genera and around 7,700 species that are distributed in all tropical regions of the world from Africa to Asia, South America and the West Indies. This plant grows well in moist, shady and sunny places. They can be monoecious or dioecious [1]- [4]. *P. amarus* is a small, erect and annual plant. It grows up to 10-60 cm tall with elliptic oblong to obvate, obtuse or apiculate at apex, obtuse or inequilateral at base leaves. The flowers have 5 white, yellow or grey sepals, auxilliary with bisexual cymules and its capsules are 1.8 mm in diameter, oblate and rounded. The seeds are about 0.9 mm long, pale brown and triangular with transverse striations on the back [5]-[10].

P. amarus has been described in Ayurveda by Sanskrit name called Bhoomyaamalakee, Taamalakee and Bhoodhatree. It is also known as Bahupatra and Bhuiamla in Central and Southern India respectively which grows mostly on uncultivated land [11]. It is also known as Chanca piedra in Spanish which means stone breaker or shatter stone [12]. *P. amarus* is bitter, astringent, diuretic, stomachic, febrifuge and antiseptic. This plant is considered to be diuretic when boiled and can be used in treatment of diabetes, hepatitis, menstrual disorders and skin disorders [13]-[16]. It is also traditionally used in the treatment of jaundice, asthma, kidney problems, urinary bladder, female problems such as leucorrhoea and mammary abscess, tumor and chronic dysentery [17]. All parts of *P. amarus* have been studied and are found to contain various chemical compounds such as lignans, alkaloids, phenols, terpenoids, flavonoids, tannins and gallotanoids [18]-[19]. Phyllanthus lignans are reported to have a potential action as multidrug resistance reversing agent, hepatoprotective agent, antiviral agent [20]-[23].

However, *P. amarus* have not been widely investigated so far in Malaysia. This present study is to isolate chemical compounds from hexane and ethanol crude extract. This lead to isolation of compounds hypophyllanthin (**1**), niranthin (**2**), and lintetralin (**3**) and their structure are elucidated by NMR (^{1}H, ^{13}C and DEPT, COSY, HMQC, and HMBC). All the spectroscopic data are similar with the previous reported literature values [24]-[25].

II. MATERIALS AND METHODOLOGY

A. Plant Collection

The sample of *P. amarus* was collected from Melor, Kelantan. The whole part of the plants was air dried at room temperature and then was kept in a light-resistant container at the Herbarium of Organic Lab, Chemistry Department, UPSI, Perak, Malaysia.

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B. Extraction and Isolation

Dried *P. amarus* (2.01 kg) were ground into powder. It was continuously extracted with ethanol for 3 days and repeated for three times at room temperature and then it was filtered. The resulted ethanol crude extract (95.8 g) was then used to produce hexane, ethyl acetate and methanol crude extracts by using solvent-solvent partitioning method. All crude extracts were obtained by removing the solvent under reduced pressure and the yield was the dry weight of the extracts.

The hexane and ethanol crude extract were tested for the chemical constituent contents using TLC and spotting with sulphuric acid. Then the crude extracts were subjected to column chromatography over silica gel using mixtures of hexane, hexane/dichloromethane, dichloromethane and dichloromethane/methanol as eluents. All three compounds were obtained by recycling preparative high-performance liquid chromatography (HPLC). Compound **1** was obtained from hexane crude while compounds 2 and 3 were obtained from ethanol crude. The chromatographic separation was performed on a reverse-phase of JAIGEL-ODS-AP Liquid Chromatography (Japan). ODS-AP is a general reverse phase column, packed with highly pure silica gel bonded ODS and end-capped. The detection wavelength used was 230 nm. Before the injection, the column was saturated with acetonitrile as mobile phase.

C. Cell Line Culture

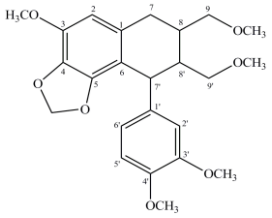
Human cervical cancer HeLa and normal mouse fibroblast NIH/3T3 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Those cells were grown in media containing RPMI-1640 media (Gibco, USA), supplemented with 10 % fetal bovine serum (Gibco, USA) and antibiotics (10,000 units/mL penicillin, 10 mg/mL streptomycin and 0.025 mg/mL amphotericin B (Biological Industries, Israel) which then maintained in an incubator at 37°C and at 5% CO_2 in a humidified atmosphere. The semi confluent cells from these two adherent cells were treated with trypsin-like enzyme with phenol red (Gibco, USA) for 5 minutes and then were resuspended in medium with serum and transferred into 3 new flasks. After trypsinization, the cells were count and the cell viability was tested by trypan blue using a hemocytometer. Cell viability above 95 % was used for this study.

D. MTT Cytotoxicity Assay

A volume of 50 µL of complete growth medium and 100 µL of 1.2 x 105 cells/ml HeLa and NIH/3T3 cells were seeded into the 96-wells flat bottom microtiter plate (Nunclon, USA). The plate was then incubated for 24 hours. Four samples treatments of 100 µL were added into wells in triplicate and serially diluted and then were incubated for 72 hours in 5 % CO_2 incubator. After 72 hours, 20 µL of 5 mg/mL MTT solution was added into each well and incubated for 3 hours. The culture medium was removed from each well after 3 hours and 100 µL of 100 % DMSO were added to each well. The plate then was read by microplate reader at 570 nm with reference of 630 nm wavelength (BioTek, USA). The graph of percentage of cell viability versus concentration of sample treatment was plotted.

III. RESULTS

Isolation process of this study from Recycling Preparative HPLC method gave lignans of hypophyllanthin (**1**) from hexane crude while niranthin (**2**) and lintetralin (**3**) from ethanol crude. From the MTT assay results, hypophyllanthin possess an active cytotoxic activity towards HeLa cells compared to lintetralin and niranthin.



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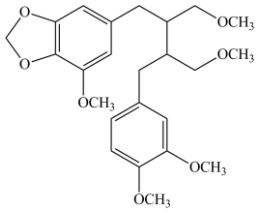

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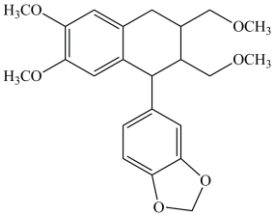

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A. Spectral Data of Isolated Compound

Hypophyllanthin (**1**): 10.5mg (29.9 %). ^{1}H NMR (500 MHz, $CDCl_3$), δ: 6.33 (1H, *s*, H-2), 3.87 (3H, *s*, 3-OCH_3), 5.73 (2H, *d*, *J*=1.70 Hz, O-CH_2-O), 5.65 (2H, *d*, *J*=1.75 Hz, -OCH_2O-), 2.80 (2H, *dd*, *J*=4.55 Hz, 15.45 Hz, H-7), 2.76 (2H, *t*, *J*=10.85 Hz, H-7), 1.98 (1H, *m*, H-8), 3.42 (2H, *dd*, *J*=4.00 Hz, 9.15 Hz, H-9), 3.36 (2H, *m*, H-9), 3.31 (3H, *s*, 9-OCH_3), 6.74 (1H, *d*, *J*=8.60 Hz, H-2'), 3.80 (3H, *s*, 3'-OCH_3), 3.85 (3H, *s*, 4'-OCH_3), 6.64 (1H, *dd*, *J*=1.70 Hz, 8.00 Hz, H-5'), 6.66 (1H, *d*, *J*=1.75 Hz, H-6'), 4.09 (1H, *d*, *J*=8.05 Hz, H-7'), 1.89 (1H, *m*, H-8'), 3.33 (2H, *s*, H-9'), 3.24 (2H, *dd*, *J*=3.40 Hz, 9.15 Hz, H-9'), 3.29 (3H, *s*, 9'-OCH_3). ^{13}C NMR (125 MHz, $CDCl_3$), δ: 131.8 (C-1), 106.4 (C-2), 142.1 (C-3), 55.9 (3-OCH_3), 133.3 (C-4), 101.2 (-OCH_2O-), 147.1 (C-5), 115.1 (C-6), 33.4 (C-7), 36.7 (C-8), 75.5 (C-9), 59.0 (9-OCH_3), 138.0 (C-1'), 110.6 (C-2'), 148.5 (C-3'), 55.8 (3'-OCH_3), 147.1 (C-4'), 56.4 (4'-OCH_3), 120.5 (C-5'), 111.7 (C-6'), 42.0 (C-7'), 45.5 (C-8'), 71.7 (C-9'), 59.0 (9'-OCH_3).

Niranthin (**2**): 5.6mg (30.8 %). ^{1}H NMR (500 MHz, $CDCl_3$), δ: 6.30 (1H, *d*, *J*=1.15 Hz, H-2), 5.93 (2H, *s*, -OCH_2O-), 3.83 (3H, *s*, 5-OCH_3), 6.25 (1H, *d*, *J*=1.15 Hz. H-6), 2.59 (2H, *t*, *J*=5.15, H-7), 2.63 (2H, *t*, *J*=8.05, H-7), 2.02 (1H, *m*, H-8), 3.30 (2H, *s*, H-9), 3.30 (3H, *s*, 9-OCH_3), 6.62 (1H, *d*, *J*=1.70 Hz, H-2'), 3.86 (3H, *s*, 3'-OCH_3), 3.82 (3H, *s*, 4'-OCH_3), 6.76 (1H, *d*, *J*=8.00 Hz, H-5'), 6.65 (1H, *dd*, *J*=2.30 Hz, 8.05 Hz, H-6'), 2.66 (2H, *dd*, *J*=2.85 Hz, 6.85 Hz, H-7'), 2.67 (2H, *t*, *J*=6.90 Hz, H-7'), 2.02 (1H, *m*, H-8'), 3.30 (2H, *s*, H-9'), 3.30 (3H, *s*, 9'-OCH_3). ^{13}C NMR

(125 MHz, $CDCl_3$), δ: 135.7 (C-1), 103.2 (C-2), 148.6 (C-3), 133.6 (C-4), 101.3 (-OCH_2O-), 143.4 (C-5) 56.5 (5-OCH_3), 108.0 (C-6), 35.5(C-7), 40.9 (C-8), 72.6 (C-9), 59.0 (9-OCH_3O), 133.2 (C-1'), 112.1 (C-2'), 147.1 (C-3'), 55.9 (3'-OCH_3), 148.7 (C-4'), 55.8 (4'-OCH_3), 110.9 (C-5'), 121.1 (C-6'), 35.0 (C-7'), 40.8 (C-8'), 72.5 (C-9'), 58.9 (9'-OCH_3).

Lintetralin (**3**): 3.0mg (9.4 %). ^{1}H NMR (500 MHz, $CDCl_3$), δ: 6.59 (1H, *s*, H-2), 3.61 (3H, *s*, 3-OCH_3), 3.84 (1H, *m*, 4-OCH_3), 5.94 (2H, *d*, *J*=4.00 Hz, -OCH_2O-), 6.23 (1H, *s*, H-5), 2.82 (2H, *d*, *J*=8.05 Hz, H-7), 2.14 (1H, *m*, H-8), 3.48 (2H, *dd*, *J*=2.90, 9.20 Hz, H-9), 3.43 (2H, *dd*, *J*=6.30, 9.15 Hz, H-9), 3.36 (3H, *s*, 9-OCH_3), 6.57 (1H, *d*, *J*=1.75 Hz, H-2'), 6.74 (1H, *d*, *J*=8.00 Hz, H-5'), 6.64 (1H, *dd*, *J*=1.75, 8.05 Hz, H-6'), 3.99 (1H, *d*, *J*=10.35 Hz, H-7'), 1.79 (1H, *m*, H-8'), 3.38 (2H, *d*, *J*=2.85 Hz, H-9'), 3.11 (2H, *dd*, *J*=3.40, 9.70 Hz, H-9'), 3.27 (3H, *s*, 9'-OCH_3).^{13}C NMR (500 MHz, $CDCl_3$), δ:129.0 (C-1), 111.0 (C-2), 147.0 (C-3), 55.8 (3-OCH_3), 147.1 (C-4), 55.9 (4-OCH_3), 100.9 (-OCH_2O-), 112.8 (C-5), 131.9 (C-6), 33.3 (C-7), 36.3 (C-8), 75.3 (C-9), 59.1 (9-OCH_3), 139.8 (C-1'), 109.4 (C-2'), 147.7 (C-3'), 145.9 (C-4'), 107.9 (C-5'), 122.8 (C-6'), 47.3 (C-7'), 45.1 (C-8'), 71.1 (C-9'), 59.0 (9'-OCH_3).

IV. DISCUSSION

Hypophyllanthin (**1**) was isolated as white amorphous. The UV spectrum showed maxima absorption at 280 nm while the IR spectrum showed absorption at 1591 cm^{-1} for C=C of aromatic ring groups. The molecular formula of this compound **1** was $C_{24}H_{30}O_7$ correspond with protonated ion peak spectrum 430.10 [M^+].

The ^{1}H NMR spectrum of **1** showed one singlet signals in the aromatic region at δ 6.33 which assigned for C-2. Aromatic protons were showed by two doublet signals at δ 6.74 (*d*, *J* =8.60 Hz), δ 6.66 (*d*, *J*=1.75 Hz) and a doublet of doublet signals at δ 6.64 (*dd*, *J*=1.70 Hz and 8.00 Hz) which corresponding to proton at position H-2', H-6', and H-5', respectively. Another five singlet signals which belongs to five methoxy protons appeared at δ 3.87, δ 3.80, δ 3.85, δ 3.31 and δ 3.29 were attached to C-3, C-3', C-4', C-9 and C-9', respectively. Peak of methylenedioxy proton were showed as doublet signal at δ 5.73 (*d*, *J*=1.70 Hz) and δ 5.65 (*d*, *J*=1.75 Hz). Besides that, the methylene protons were showed by signal of δ 2.80 (*dd*, *J*=4.55 Hz and 15.45 Hz) and δ 2.76 (*t*, *J*=10.85 Hz) at position H-7, δ 3.42 (*dd*, *J*=4.00 Hz and 9.15 Hz) and multiplet signal at δ 3.36 at position H-9 while singlet signals at δ 3.33 and 3.24 (*dd*, *J*=3.40 Hz and 9.15 Hz) were corresponding to H-9'.

The ^{13}C NMR spectrum of **1** showed a total of 24 carbon atoms. Among the 24 carbons, there were eight quaternary carbons: δ 131.8 (C-1), δ 142.1 (C-3), δ 133.3 (C-4), δ 147.1 (C-5), δ 115.1 (C-6), δ 138.0 (C-1'), δ 148.5 (C-3'), δ 147.1 (C-4'); seven methine carbons: δ 106.4 (C-2), δ 36.7 (C-8), δ 110.6 (C-2'), δ 120.5 (C-5'), δ 111.7 (C-6'), δ 42.0 (C-7'), δ 45.5 (C-8'); five methoxy carbons: δ 55.9 (3-OCH3), δ 59.0 (9-OCH_3), δ 55.8 (3'-OCH_3), δ 56.4 (4'-OCH_3), δ 59.0 (9'-OCH_3); and three methylene carbons: δ 33.4 (C-7), δ 75.5 (C-9), δ 71.7 (C-9'). In addition, one methylenedioxy carbon signal at δ 101.2 was also observed.

The position of these carbon resonances were further supported by ^{1}H-^{1}H COSY which provided the proton-proton coupling relations between H7-H8, H8-H9 and H7'-H8'. Further correlation signals in the HMBC spectrum essential for elucidation of the structure of **1** were observed for H-2 to C-3, C-4, C-6 and C-7; H-7 to C-1, C-2, C-6, C-8, C-8' and C-9; H-9 to C-8 and C-8'; H-2' to C-1' and C-3'; H-5' to C-4' and C-6'; and H-6' to C-1', C-4' and C-7'.

The complete assignments for the proton and carbon signals shown have confirmed the compound **1** as hypophyllanthin as all its NMR data were identical to those reported in the literature for hypophyllanthin [25].

Compound **2** was obtained as a brownish amorphous. The UV spectrum showed absorbance band at 280 nm. The IR spectrum showed absorption at 16591 cm^{-1} for C=C of aromatic ring groups. The molecular formula of **2** was $C_{24}H_{31}O_7$. The mass spectrum revealed a protonated molecule ion peak at 432.21 [M^+]. This compound was isolated from ethanol crude using 100 % of acetonitrile by Recycling Preparative HPLC.

The ^{1}H NMR spectrum of compound **2** showed aromatic hydrogen region of δ 6.25 (*d*, *J*=1.15 Hz), 6.30 (*d*, *J*=1.15 Hz), 6.62 (*d*, *J*=1.70 Hz), 6.65 (*dd*, *J*=2.30 Hz, 8.05 Hz) and 6.76 (*d*, *J*=8.00 Hz) that attached at H-6, H-2, H-2', H-6' and H-5', respectively. Singlet signal of δ 5.93 showed the presence of methylenedioxy, -OCH_2O-. It have five methoxy groups which were singlet signals of δ 3.82, 3.83 and 3.86 attached at 4'-OCH_3, 5-OCH_3 and 3'-OCH_3, respectively. Besides, this compound also has two methoxy groups bonded to the alkyl side chain which are singlet signals of δ 3.30 at both 9-OCH_3 and 9'-OCH_3. Methine groups (CH) were showed as multiplet signals at H-8 and H-8' (δ 2.02). The methylene groups appeared at H-7 as triplet signals of δ 2.59 (*J*=5.15 Hz) and δ 2.63 (*J*=8.05 Hz), H-7' appeared as doublet of doublet signal of δ 2.66 (*J*=2.85 Hz, 6.85 Hz) and triplet signal of δ 2.67 (*J*=6.90 Hz) whereas a singlet signals of δ 3.30 appeared at H-9 and H-9'. The COSY spectrum showed proton correlation between H-5' with H-6' and H-7' with H-8'.

According to the ^{13}C and DEPT NMR spectrum, there were seven quarternary carbons that appeared at δ 133.2, 133.6, 135.7, 143.4, 147.1, 148.6 and 148.7 corresponding to C-1', C-4, C-1, C-5, C-3', C-3 and C-4'. Methylenedioxy carbon showed a signal at δ 101.3 and methoxy group that attached to aromatic ring showed signals at δ 55.8, 55.9 and 56.5 corresponding to 4'-OCH_3, 3'-OCH_3 and 5-OCH_3, respectively. Meanwhile, the methoxy carbon that attached to alkyl chain showed signals of δ 58.9 (9'-OCH_3) and δ 59.0 (9-OCH_3). The other signals were four methylene carbons of C-7', C-7, C-9' and C-9 at δ 35.0, 35.5, 72.5 and 72.6 and two methine carbons of C-8' and C-8 at δ 40.8 and 40.9. The five aromatic carbons were showed at δ 103.2 (C-2), 108.0 (C-6), 110.9 (C-5'), 112.1 (C-2') and 121.1 (C-6'). On the basis of spectral data analysis and by comparison of these assignments with those of structurally related, the chemical structure of compound wasdeduced as niranthin [24].

Analysis of ^{1}H and ^{13}C spectra along with COSY,

HMQC and HMBC spectrum suggested that compound **3** was lintetralin which obtained as brownish oil with molecular formula $C_{23}H_{28}O_6$. Mass spectrum revealed a protonated molecule ion peak at 400.19 [M^+] and IR spectrum data suggested the following information for the structural elucidation of compound **3**

was 1591 cm^{-1} indicate the C=C for aromatic ring groups while the maximum absorption of UV spectrum was 282 nm for the aromatic ring groups.

The ^{1}H NMR spectrum showed four methoxyl proton at δ 3.61 (*s*), 3.84 (*m*), 3.36 (*s*) and 3.27 (*s*) which were assigned to proton at 3-OCH_3, 4-OCH_3, 9-OCH_3 and 9'-OCH_3, respectively. There were also five aromatic proton signals at δ 6.59 (*s*), 6.23 (*s*), 6.57 (*d*, *J*=1.75 Hz), 6.74 (*d*, *J*=8.00 Hz) and 6.64 (*dd*, *J*=1.75 and 8.05 Hz) that belongs to proton at position H-2, H-5, H-2', H-5' and H-6', respectively. The methylene proton at H-7 was showed by doublet signals of δ 2.82 (*J*=8.05 Hz), H-9 were showed by two doublet of doublet signals at δ 3.48 (*J*=2.90 and 9.20 Hz) and 3.43 (*J*=6.30 and 9.15 Hz) and at H-9' also showed doublet signal at δ 3.38 (*J*=2.85 Hz) and a doublet of doublet signals at δ 3.11 (*J*=3.40 and 9.70). There were also three signals of methine protons at H-7', H-8 and H-8' of doublet signals at δ 3.99 (*J*=10.35 Hz) and two multiplet signals at δ 2.14 and δ 1.79, respectively. Besides that, dioxycyclopentane proton showed doublet signals of δ 5.94 with coupling of 4.00.

The ^{13}C and DEPT NMR data of **3** showed the presence of 23 carbon with seven quaternary carbons; 129.0 (C-1), 147.0 (C-3), 147.1 (C-4), 131.9 (C-6), 139.8 (C-1'), 147.7 (C-3') and 145.9 (C-4'), 4 methylene carbons; 33.3 (C-7), 75.3 (C-9), 71.1 (C-9') and 100.9 (-OCH_2O-), four methoxy carbons; 55.8 (3-OCH_3), 55.9 (4-OCH_3), 59.1 (9-OCH_3) and 59.0 (9'-OCH_3) and eight methine carbons; 111.0 (C-2), 112.8 (C-5), 36.3 (C-8), 109.4 (C-2'), 107.9 (C-5'), 122.8 (C-6'), 47.3 (C-7') and 45.1 (C-8').

A. Anticancer Activity of Isolated Compound

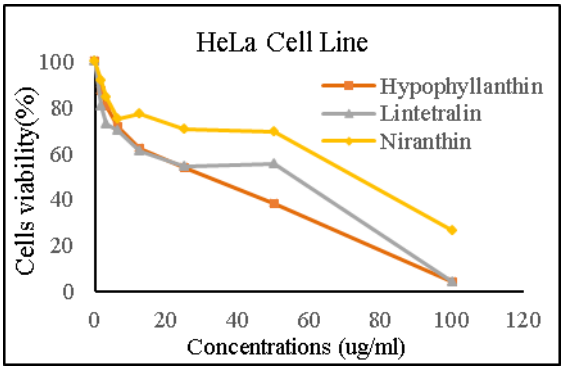

Fig. 1 Effects of Compounds on the Viability of HeLa cells

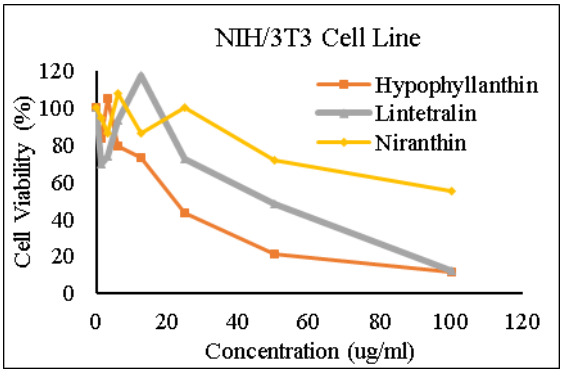

Fig. 2 Effects of Compounds on the Viability of NIH/3T3 cells

Hypophyllanthin, niranthin and lintetralin were tested for anticancer activity on human cervical cancer cell lines (HeLa) and normal mouse fibroblast cell lines (NIH/3T3) which evaluated by MTT assays. Those three compounds were tested with different concentration varying from 100 to 1.5625 µg/mL. From the MTT assay results, hypophyllanthin decreased the HeLa cell population to 50 % (IC_{50}) at 30.1µg/mL compared to niranthin and lintetralin at 70.4 µg/mL and 50.5 µg/mL, respectively as referred to Figure 1. However, the niranthin was inactive in cytotoxic activity towards normal mouse fibroblast cell line, NIH/3T3. Figure 2 showed no IC_{50} value when NIH/3T3 cells treated with niranthin. The cell viability of the cells was reduced to 75 % only even when treated with 50 µg/mL and slightly reduced to about 60 % when the concentration of niranthin was increased to 100 µg/mL. Hypophyllanthin showed the lowest IC_{50} values of 20.2 µg/mL when tested on NIH/3T3 cell lines. The viable cells of NIH/3T3 cells were decreased to 50 % (IC_{50}) when treated with 50 µg/mL of lintetralin which means that lintetralin was moderately active in cytotoxic activity to the NIH/3T3 cell lines. However, this result was very important for further study on the isolation of cytotoxic compounds from this *P. amarus*.

V. CONCLUSION

This research work has identified the presence of hypophyllanthin (**1**), niranthin (**2**) and lintetralin (**3**) from *Phyllanthus amarus*. The result from MTT assay showed that hypophyllanthin has a strong anticancer effect on HeLa cells and has potential to be used in cancer treatment compared to niranthin and lintetralin. However, all these three compounds were also found to be less cytotoxic towards NIH/3T3 cells.

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