The Effect of *Phyllanthus taxodiifolius* Beille Extracts and its Triterpenoids Studying on Cellular Energetic Stage of Cancer Cells

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Abstract: Problem statement: The Multidrug Resistance phenomenon (MDR) is the cause of unsuccessful in cancer chemotherapy. The tradition plant is the population used as the alternative medicine in cancer therapy. Due to, *P. taxodiifolius* is medicinal Thai plant which is used as diuretic drug which has never been explored as the anticancer activities.

Approach: Air-dried powder of *P. taxodiifolius* leaves and twigs were serially extracted by hexane, ethyl acetate, acetone and methanol. These four extracts were tested the antiproliferative activity on four cancer cell lines. These results lead to successively purify two triterpenoids that are glochidone (1) and lupeol (2). Both pure compounds were tested the anticancer properties on same cancer cell lines and further investigate the cellular energetic state perturbation by measuring the mitochondrial membrane potential modification.

Results: Four crude extracts were extracted and two triterpenoids (glochidone and lupeol) were purified and identified from hexane extract. Our antiproliferative activity of both compounds respectively showed in the IC\(_{50}\) value of K562, K562/Adr, GLC4 and GLC4/Adr equal to 2.2 ± 0.6, 4.2 ± 1.5, 3.1 ± 1.0 and 3.2 ± 0.9 µg mL\(^{-1}\) for glochidone and 2.3 ± 0.6, 4.5 ± 1.7, 2.3 ± 0.5 and 2.6 ± 0.5 µg mL\(^{-1}\) for lupeol. The R value, which represents the multidrug resistance phenotype, is about 2 for P-glycoprotein overexpression in K562/Adr and 1 for MRP1 overexpression in GLC4/Adr. Conclusion: All crude extractions and two triterpenoids show the clear evidence of anticancer activity of both sensitive and resistance of erythroleukemic and Small cell lung cancer cell lines. Both compounds are not recognized by ABCB1 and ABCC1 proteins. Our results also indicated that lupeol initiate cell death by mitochondria membrane potential modification specially the sensitive cell line.

Key words: *Phyllanthus taxodiifolius* beille, triterpenoid, anticancer, multidrug resistance, mitochondria membrane potential, cellular energetic state, glochidone, lupeol, antiproliferative activity

INTRODUCTION

Cancer is one of the major death causes of disease. After the treatment, the Multidrug Resistance phenomenon (MDR) is a major obstacle to successful treatment outcome of many human malignancies cause by the reducing of intracellular drug target accumulation. MDR phenomena often associated with...
the over expression of protein transports, cellular energetic state and also cellular oxidative stress. This obstacle was wildly studied on its mechanism to find out the effective anticancer drug. New molecules either from the synthesis or pure compounds of plant extraction were explored (Monkodkaew et al., 2009; Nantapap et al., 2010; Tangjai et al., 2008; Haque et al., 2006). Recently, the tradition plant is the population used as the alternative medicine in cancer therapy.

The Phyllanthus genus belongs to the Eupohibae family. By the diversified species of Phyllanthus, these plants have been wildly studied in various activities such as anti-inflammatory, anti-diabetic, anti-diarrheal, antinoiceptive, hepatoprotective activity and anticancer (Chudapongse et al., 2010; Kumar et al., 2008; Naaz et al., 2007; Shanbhag et al., 2010). The phytochemical study showed that the Phyllanthus genus consist triterpenoids, resins, steroids, alkaloids and phenolic compounds (Khaatoon et al., 2006). The research on P. urinaria presented the apoptotic cell death in cancer cell lines. Anyway, P. taxodiifolius Beille which is used as diuretics drug in Thai traditional herbal medicine has not been study yet. In this work, we aim to study the anticancer properties by focusing its activity on mitochondrial.

MATERIALS AND METHODS

Plant extractions: The P. taxodiifolius Beille leaves and twigs were collected from Amnatcharoen, Thailand and identified by voucher specimen (BKF no. 127614). It has been deposited at the Forest Herbarium, Department of National Park, Wildlife and Plant Conservation, Ministry of Natural Resources and Environment, Bangkok, Thailand before using. These leaves and twings (5 kg) were air-dried and grinded to be fine powder before marinate sequentially from n-hexane, ethyl acetate, acetone and methanol. Fractions (500 mL each) were collected and combined on the basis of TLC behavior. Fraction 2 and 3 which eluted by 10% ethyl acetate-methanol, was obtained as two white needles which identified as glochidone (1) and lupeol (2), respectively.

Cell and cell culture: The bioactivity of compounds were performed in four cancer cell lines (the erythroleukemia sensitive (K562) and its Adriamycin resistance cell line (K562/Adr) and the small cell lung cancer sensitive (GLC4) and its Adriamycin resistance cell line (GLC4/Adr)). Cells were cultivated in RPMI-1640 medium with 10% fetal bovine serum albumin and 1% antibiotic at 37°C, 5% CO2 and 95% humidity incubator for 3 days before experiment. The K562/Adr and GLC4/Adr are characterized as multidrug resistance phenotype by the over expression of plasma membrane proteins that is P-glycoprotein (ABCB1) and MRP1 (ABCC1), respectively.

Cytotoxic assay: The toxicity of compounds on cell growth was tested by incubate each cell line (5000 cells) with various concentration of each compound from 0 up to 250 µg mL⁻¹ in RPMI-1640 containing 10% fetal bovine serum albumin and 1% antibiotic then cultured in 37°C for 72 h. Cell viability was determined by using MTT-colorimetric assay. In this assay MTT will be reduced to be formazan crystal which the cellular proliferation was inhibited to 50%.

Cell viability was calculated. The percentage of cell-growth inhibition (IC%) was obtained from the following equation:

\[ IC\% = \frac{C_{72} - S_{72}}{C_{72} - C_{0}} \]

When:

- \( C_{0} \) = OD at 560 nm value represent the initial cell amount of non treated cell (control)
- \( C_{72} \) = OD at 560 nm value represent the cell amount of control at 72 h
- \( S_{72} \) = OD at 560 nm value represent the cell amount of treated cell at 72 h
The Resistance factor (R value) represents the multidrug resistance phenotype of such compound can be calculated as the IC₅₀ value of resistance cell divide by IC₅₀ value of its corresponding sensitive cell line.

**Determination of the mitochondria membrane potential (ΔΨₘ):** The mitochondria membrane potential which reflects to cellular energetic state of each cell line was determined by using the kinetic uptake of rhodamine B. The rhodamine B is a fluorescence probe that can be uptake into cell and accumulated in mitochondria by the defect of its potential. The initial rate of rhodamine B fluorescence extinction could be determined and calculated to predict the ΔΨₘ (mVolt) value of intact cell as described by Reungpatthanaphong et al. (2003). The effect of 2 pure compounds on mitochondrial membrane potential modification was studied on four cancer cell lines. The 2×10⁶ cells of each cell were incubated with pure compound at the IC₅₀ concentration in 2 mL of Luckhoff Na⁺ pH 7.3 at 37°C for 30 min before mitochondria membrane potential determination.

**RESULTS**

The cytotoxic activity of crude extracts and pure compounds on cancer cell lines: The bioactivity of four crude extracts and two pure compounds were studied on four cancer cell lines. The yield of four crude extracts from fine powder of *P. taxodiifolius* leaves and twigs are 40.2, 37.3, 90.0 and 170.1 g for hexane, ethyl acetate, acetone and methanol, respectively. The bioactivity study of four crude extracts presented the strong cytotoxic on all cancer cell lines (Table 1).

**Fig. 1:** The chemical structures of glochidone (1) and lupeol (2) and their physico-chemical properties

**Note: Compound 1:** Lupa-1,20(29)-diene-3-one (Glochidone). Crystals: white needles from CH₂Cl₂-MeOH, m.p. 167.7-168.1°C (Lit. 22 164-165°C). UV λₘ₉₈ nm(log ε): 228(1.50), 330(1.03) . FTIR νₑₑ cm⁻¹: 2939, 2873, 1670 (C=O stretching of conjugated ketone), 1454, 1380, 1215, 1099, 1014, 945, 871, 825 EIMS m/z (% relative intensity): 422[M⁺], 369(6), 300(5), 298(14), 281(20), 269(13), 256(17), 227(17), 208(23), 200(24), 178(44), 167(24), 149(100), 125(32), 119(28), 97(34), 81(46), 77(49), 67(74), 55(77). ¹H-NMR (400 MHz,CDCl₃) δ (ppm): Table 1. COSY correlations H/H : 1/2; 2/1; 3/2; 13/18; 18/21a; 19/18, 22a/22b; 29b/30; 30/18 HMBC correlations C-H : 1/3, 5, 9, 25 ; 2/3 ; 3/23, 24 ; 4/3 ; 5/6, 23, 24, 25 ; 6/5 ; 7/26, 27 ; 8/6, 7, 9 ; 9/25, 26 ; 10/5, 9, 25 ; 11/9 ; 12/18, 27, 30 ; 13/11b, 26, 27 ; 14/18, 26, 27, 28 ; 15/9, 16 ; 16/28 ; 17/18, 22b ; 18/21a, 19, 21b, 28 ; 19/13b, 29a, 29b ; 20/18, 19, 21b, 30 ; 21/19 ; 22/21b, 18, 28 ; 23/24 ; 24/23 ; 25/1, 2 ; 26/7, 9 ; 27/13 ; 28/16a, 18, 22a, 22b ; 29/19, 30 ; 30/19, 29a, 29b

**Note: Compound 2:** (20(29)-lupen-3-ol) (Lupeol). Crystals: white needles from CH₂Cl₂-MeOH, m.p. 210.3-210.8°C (Lit. 38 210-211°C). UV λₘ₉₈ nm(log ε): 211(1.31) . FTIR νₑₑ cm⁻¹: 3336 (OH), 2934, 2869, 1649, 1454, 1380, 1041, 879. EIMS m/z (% relative intensity): 427 [M+H⁺], 369(14), 368(16), 354(26), 338(23), 324(13), 306(9), 281(11), 256(12), 229(17), 218(23), 206(26), 204(31), 192(17), 178(48), 161(26), 149(100), 147(19), 121(29), 97(23), 95(29), 79(31), 69(28), 55(41). ¹H-NMR (400 MHz,CDCl₃) δ (ppm): Table 1. COSY correlations H/H : 3/2 ; 13/18 ; 18/21a ; 29b/30 ; 30/18 HMBC correlations C-H : 1/3, 5, 9, 25 ; 2/3 ; 3/23, 24 ; 4/3 ; 5/6, 23, 24, 25 ; 6/5 ; 7/26, 27 ; 8/6, 7, 9 ; 9/25, 26 ; 10/5, 9, 25 ; 11/9 ; 12/18, 27, 30 ; 13/11b, 26, 27 ; 14/18, 26, 27, 28 ; 15/9, 16 ; 16/28 ; 17/18, 22b ; 18/21a, 19, 21b, 28 ; 19/29a, 29b ; 20/18, 19, 21b, 30 ; 21/19 ; 22/21a, 28 ; 23/3 ; 24/23 ; 25/11b ; 26/9, 11b, 12a ; 27/13 ; 28/16a, 18, 22a, 22b ; 29/19, 30 ; 30/19, 29a, 29b
Table 1: The cytotoxic activity present as the %IC$_{50}$ value and resistance factor value (R value) of crude extract from hexane, ethyl acetate, acetone and methanol fraction and two pure compounds on K562, K562/Adr, GLC4 and GLC4/Adr cell lines

| Crude extract | K562 IC$_{50}$ (µg mL$^{-1}$) n =16 | K562/Adr R-value | GLC4 IC$_{50}$ (µg mL$^{-1}$) n =16 | GLC4/Adr R-value |
|---------------|------------------------------------|-----------------|------------------------------------|-----------------|
| Hexane        | 28.0±7.5                           | 70.0±14         | 18.6±4                             | 33.8±13.7       |
| Ethyl acetate | 1.2±0.3                            | 3.90±3          | 4.5±2.3                            | 3.0±1.5         |
| Acetone       | 1.2±0.5                            | 7.70±7.2        | 4.2±2.7                            | 2.8±1.4         |
| Methanol      | 2.4±1                              | 13.30±10.2      | 6.2±2.8                            | 14.0±4.7        |
| Pure compound | Glochidone (1)                     | 2.2±0.6         | 4.20±1.5                           | 3.2±0.9         |
|               | Lupeol (2)                         | 2.3±0.6         | 4.50±1.7                           | 2.6±0.5         |

These results revealed not only toxicity of crude extracts but also the R value that illustrate the cytotoxic effect of these crude extracts on resistance cancer cell lines compare with its sensitive cell lines. From the calculation, hexane crude extract show the lowest level of R value on both resistance cell lines that is 2.5 for K562/Adr and 1.8 for GLC4/Adr. According to cytotoxic result, hexane crude extract was further purified and produced two pure compounds. These two compounds (glochidone (1) and lupeol (2)) were identified their chemical structure by spectrometric methods and demonstrated in Fig. 1. These pure compounds were then tested the cytotoxic effect on all same cell lines and they showed the stronger toxic than their crude extract of about 10 times and the R value close to 1 for GLC4/Adr and about 2 for K562/Adr.

The cellular energetic state modification by pure compounds: The effect of 2 pure compounds (glochidone and lupeol) on the mitochondrial membrane potential ($\Delta$$\Psi_m$) was investigated in four cancer cell lines. The treatment of 2 pure compounds...
result in the $\Delta\Psi_m$ modification. We found that lupeol can reduce the $\Delta\Psi_m$ of both sensitive cell line K562 and GLC4 but not their resistance cell lines K562/Adr and GLC4/Adr while glochidone can slightly reduce $\Delta\Psi_m$ of GLC4 (Fig. 2).

**DISCUSSION**

*Phyllanthus taxodiifolius* is a Thai plant used as diuretics drug in Thai traditional herbal medicine. The cytotoxic on cancer cell line not only show the anticancer activity of all extractions and pure compounds but also the anti multidrug resistance. The certain part of extractions and purification, we obtained triterpenoids compound glochidone and lupeol. Lupeol is a triterpenoids existent in foliage, flowering and fruit trees. Its bioactive was reported as an anti-leukemia and anti-inflammatory (Saleem, 2009). Our result showed that lupeol can disturb the cellular energetic state by the mitochondria membrane potential modification of two sensitive cancer cell lines which involve in the initiation of apoptotic cell death. These results are supported by several studied on triterpenoids also lupeol which are reported as the apoptotic potential by caspase activation and also effect via mitochondria (Chaturvedi et al., 2008; Li et al., 2005; Nigam et al., 2009; Reyes-Zurita et al., 2009).

**CONCLUSION**

All crude extractions and two triterpenoids (glochidone and lupeol) purified from hexane fraction of *P. taxodiifolius* leaves and twigs show the clear evidence of anticancer activity of both sensitive and resistance of erythroleukemic and small cell lung cancer cell lines. Our results also indicated that lupeol initiated cell death by mitochondria membrane potential modification.

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