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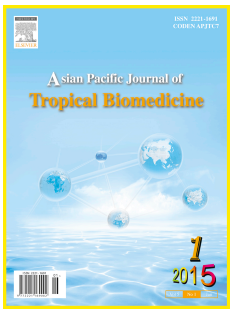

Bee pollen extract of Malaysian stingless bee enhances the effect of cisplatin on breast cancer cell lines

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ABSTRACT

Objective: To evaluate the antioxidant and antiproliferative effect of methanolic bee pollen extract (BPE) of Malaysian stingless bee [*Lepidotrigona terminata* (*L. terminata*)] and its synergistic effect with cisplatin (a chemotherapeutic drug) on MCF-7 cancer cell line.

Methods: The antioxidant activity of BPE from *L. terminata* was measured by using 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) assay. Antiproliferative activity at different concentrations of BPE and cisplatin was determined through using MTT assay on MCF-7 and L929 cell lines. An interaction effect (synergistic, additive and antagonistic) between BPE and cisplatin was determined by CompuSyn software based on MTT assay data.

Results: The EC_{50} (50% decrement of DPPH inhibition) of BPE was 0.5 mg/mL. *L. terminata* BPE exhibited antiproliferative activity on both cancer and normal cell lines. The IC_{50} (concentration of drug that was required for 50% of cell inhibition *in vitro*) of BPE on MCF-7 was 15 mg/mL whereas in normal cell line L929 was 26 mg/mL. The IC_{50} for cisplatin on MCF-7 was 20 µmol/L. The combination effect of BPE and cisplatin on MCF-7 cells showed that BPE at 15 mg/mL was able to potentiate the inhibitory effect of cisplatin at all different concentrations (2.5–20.0 mg/mL). The average of cancer cells inhibition which was potentiated by BPE was around 50%. A combination index values of less than 1 reported in the CompuSyn software further proved the synergistic effect between BPE and cisplatin, suggesting that BPE was working synergistically with cisplatin.

Conclusions: Our study therefore suggested that BPE of Malaysian stingless bee, *L. terminata* is a potential chemopreventive agent and can be used as a supplementary treatment for chemotherapy drugs. BPE might be able to be used to potentiate the effect of chemotherapy drugs with the possibility to reduce the required dose of the drugs. The molecular mechanisms of how the BPE exerts antiproliferative activity will be a much interesting area to look for in future studies.

1. Introduction

Bee pollen is considered as a functional food due to its compositions of carbohydrates, proteins, amino acids, lipids, sugars, fibres, vitamins and mineral salts[1,2]. It is a collection of pollen grains collected by the bees from various botanical sources, mixed with nectar and secretion from the hypopharyngeal glands such as β-glycosidase enzymes. Its beneficial effect on health is due to the presence of phenolic compounds with high antioxidant activities[1,3].

Oxidative damage and carcinogenesis may gradually lead to the formation of tumors through several mechanisms. Polyphenols present in bee pollen and other bee products contain antioxidant and antiproliferative activities that can regulate cell proliferation and induce apoptosis[4]. Bee pollen has the antioxidative capacity and the ability to neutralize active oxygen species, extensively due to the action of polyphenols combination. Previous studies have demonstrated that bee pollen can inhibit tumor growth and alleviate the pain of chemotherapy in cancer patients[5,6].

In China, bee pollen of *Brassica campestris* L. (*B. campestris*) has been widely used as food supplement to strengthen body resistance against cancer[7]. A steroid fraction of bee pollen chloroform extract of *B. campestris* was shown to strongly induce apoptosis in human prostate cancer PC-3 cells[7]. A study by Izuta *et al.* showed that bee pollen was able to suppress cells proliferation in *in vitro* model of vascular endothelial growth factor-induced human umbilical vein endothelial cells proliferation and migration[8]. Constituents of bee pollen such as polysaccharides were also shown by Wang *et al.* to have significant antiproliferative activity in colon cancer cell lines[5]. Thus the aim of the study is to evaluate the antiproliferative activity of bee pollen extract (BPE) from Malaysian stingless bee, *Lepidotrigona terminata* (*L. terminata*) and its effect when combined with cisplatin on MCF-7 cancer cell lines.

2. Materials and methods

2.1. Samples and reagents

Bee pollen or bee bread sample was acquired from a local stingless bee company, K.B meliponini. The source of bee pollen was from the species of *L. terminata*. The species was identified by the Entomology Section, Malaysian Agriculture and Research Development Institute, Serdang, Malaysia. Bee pollen sample was dried at 37 °C for two consecutive days and once dried, was kept at 4 °C until further use.

2.2. Bee pollen crude extract

The crude BPE was prepared by using modified method from LeBlanc *et al.* by extracting 10 g of bee pollen in 25 mL of 100% methanol[9]. The mixture was vortexed for 10 min and sonicated for 1 h at 41 °C before kept overnight at

4 ˚C. The mixture was then centrifuged at 6 000 r/min for 10 min and the precipitation was discarded. The supernatant was filtered by using a 0.2 μm sterile filter unit (Millipore, USA). Once filtered, it was dried in the rotary evaporator at 41 ˚C and freeze dried. The freeze dried BPE was kept at 4 ˚C until further analysis.

2.3. Antioxidant activity

The free radical-scavenging capacity of bee pollen methanolic extract was determined by its ability to bleach the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) and was expressed as the percentage of its neutralization. A modification of a method reported by Baharum *et al.* was employed[10]. The reaction mixture contained 150 μL of DPPH (600 μmol/L) with 7.5 μL of different concentrations of BPEs (3.125, 6.250, 12.500, 25.000, 50.000 and 100.000 mg/mL). The final concentrations of bee pollen were 0.15, 0.30, 0.60, 1.20, 2.40 and 4.80 mg/mL. After 30 min at room temperature, the reaction mixture was measured by the absorbance at λ = 517 nm. Trolox, a water-soluble derivative of vitamin E at concentration of 25–600 μmol/L, was used as standard. DPPH free radical scavenging activity was calculated through using the following formula:

% Scavenging = (Absorbance of control - Absorbance of test sample/Absorbance of control) × 100

2.4. Cell culture

MCF-7 and L929 cell lines used in this study were obtained from the Advanced Medical and Dental Institute, Universiti Sains Malaysia. The cell lines were cultured in a complete Dulbecco's modified Eagle's medium (DMEM) containing 4 mmol/L L-alanyl-glutamine (GlutaMAX™) and supplemented with 10% (v/v) fetal bovine serum, 100 IU/mL of penicillin and 0.1 mg/mL of streptomycin. The cells were grown in humidified atmosphere under the conditions of 37 ˚C with 5% (v/v) CO_2.

2.5. Cell counts

MCF-7 and L929 cells were harvested by removing the medium, washed with phosphate buffer saline (pH 7.45 without Ca^{2+} and Mg^{2+}) and then incubated with 1 mL of 0.25% trypsin for 2 min. The flask was gently tapped to detach them from the plastic surface. Five milliliters of medium was added to the cell suspension while the remaining cells were vigorously washed from the bottom of the culture vessel. The suspended cells were then collected in a 15 mL centrifuge tube and an aliquot of 10 μL was taken out prior centrifugation for cell count by using haemocytometer. The number of cells per milliliter and the total cell number were calculated. Subsequently after centrifugation, an appropriate volume of complete medium was added to the cell pellet.

2.6. Preparation of compound treatments

BPE stock solution (100 mg/mL) was prepared by diluting 0.1 g of crude extracts in 1 mL DMEM. The solution was then filtered by using a 0.2 µL sterile filter unit (Millipore, USA) and diluted with culture medium to the final concentration of 2.5 mg/mL, 5 mg/mL, 10 mg/mL, 20 mg/mL and 40 mg/mL for treatments.

Cisplatin stock solution (10 mol/L) was prepared by dissolving 6 mg of cisplatin (molecular weight = 300.01 g/moL) in 2 mL DMEM. The stock solution was then diluted with culture medium to the final concentration of 2.5 µmol/L, 5 µmol/L, 10 µmol/L, 20 µmol/L and 40 µmol/L for treatments. The working solutions of the drug were freshly prepared prior usage.

2.7. Cytotoxicity determination by using MTT assay

Briefly, the cells were seeded at density of 1×10^4 cells per well of flat-bottomed 96-well microplate. The cells were incubated at 37 ˚C in a humidified 5% (v/v) CO_2 for 24 h to let the cells attach to the bottom of each well. The cultured cells were then treated with different concentrations of BPE and cisplatin, alone and with combination when the cells reached 70% confluence.

The cells were treated with 5 different concentrations of BPE (2.5, 5.0, 10.0, 20.0 and 40.0 mg/mL) and 5 different concentrations of cisplatin (2.5, 5.0, 10.0, 20.0 and 40.0 µmol/L). A total of 200 µL of medium containing compound treatments was added into each well. Treatments were carried out in triplicate. Well with only treatment solution (either BPE or cisplatin) was used as blank or background control, whereas well containing cells without treatment was used as negative control. Once treated, cells were incubated for 48 h.

The percentage of cell proliferation was determined by using MTT assay with the Vybrant® MTT cell proliferation assay kit (Thermo Fisher Scientific, USA). In brief, the fresh medium was replaced after 48 h of incubation. Ten microlitres of the 12 mmol/L MTT stock solution were added into each well. The cells with MTT solution were incubated in the CO_2 incubator for 4 h. The medium was then removed from the well after the cells were labelled with MTT. DMSO (50 µL) was added to each well and mixed thoroughly with a pipette. The plate was incubated again at 37 ˚C for 10 min. Finally the absorbance was read at 540 nm. The percentage of cell proliferation was calculated by using the formula below:

% Cell proliferation = (Absorbance sample - Absorbance blank)/ (Absorbance negative control - Absorbance blank) × 100

2.8. Analysis of synergistic effects

To determine the synergistic, additive or antagonistic effect, experiment with non-constant combination of BPE and cisplatin concentrations was done following the method by Qiao *et al.* with some modifications[11]. The cells were

treated with cisplatin at concentrations of 2.5, 5.0, 10.0, 15.0, 20.0 µmol/L in combination with fixed concentration of IC_{50} of BPE at 15 mg/mL. The data from this experiment were analysed through using CompuSyn software to determine the synergistic, additive and antagonistic effect of the drugs combination. The statistical analysis was performed and the result was presented as combination index (CI). A CI value is a mathematical and quantitative representation of the pharmacological interplay of two drugs (CI > 1: antagonism; CI = 1: additive; CI < 1: synergism)[12].

2.9. Statistical analysis

All values were expressed as the mean ± SD. The differences in the mean of treated and untreated cells were analysed by using IBM SPSS Statistics 22 (US).

3. Results

3.1. Antioxidant capacity of BPE

In this study, the antioxidant activity of methanolic crude extract of *L. terminata* BPE measured by using DPPH assay showed a concentration dependent manner in the dose response curve (Figure 1). The EC_{50} (50% decrement of DPPH inhibition) of BPE was 0.5 mg/mL.

3.2. Antiproliferative effect in vitro

The potential antiproliferative effect of BPE was assayed by using the surrogate cell viability MTT assay. Crude BPE was shown to inhibit the growth of both cancer and normal cell lines. The inhibition of cell growth on the breast cancer MCF-7 and normal L929 cell lines by BPE of *L. terminata* was in dose–dependent manner (Figure 2). The IC_{50} of BPE on MCF-7 was 15 mg/mL, whereas in normal cell line was 26 mg/mL. BPE at 20 mg/mL inhibited 70% of cell viability in the MCF-7 cells (Figure 2A) whereas in normal L929 cell lines, inhibition of 70% of cell viability was observed with much higher concentration of 40 mg/mL (Figure 2B).

3.3. Cytotoxic synergism of BPE with chemotherapy drug

To determine the combination effect of BPE and cisplatin (an anticancer chemotherapy drug), we have designed a non-constant combination treatment of cisplatin and BPE on MCF-7 cells. Serial concentrations of cisplatin were combined with the fixed concentration of BPE at 15 mg/mL. Higher MCF-7 cells inhibition was seen in BPE (15 mg/mL) and cisplatin at all concentrations when compared to cisplatin alone. BPE combination with 2.5 µmol/L cisplatin inhibited 55% of MCF-7 cells proliferation when compared with 2.5 µmol/L cisplatin alone. Whereas BPE in combination with 20 µmol/L cisplatin could further reduce cell proliferation up to 76% compared to 52% reduction

seen in treatment of 20 µmol/L cisplatin alone, although it was not statistically significant. The result showed that the BPE was able to potentiate the inhibitory effect of cisplatin in breast cancer cells when combined with cisplatin at all concentrations (Figure 3).

To further confirm the interaction between cisplatin and BPE, the data was computed in CompuSyn software. CI values of less than 1 were seen in all interactions between BPE and cisplatin. Reported results in the CompuSyn software were between 0.46 and 0.97 (Table 1). The lowest CI value was seen in combination of BPE and cisplatin at 10.0 µmol/L dose.

4. Discussion

Our study showed that the antioxidant activity of *L. terminata* BPE exhibited much higher percentage of DPPH inhibition than other Malaysian stingless bee species reported recently. BPE from species *Trigona apicalis* and *Trigona itama* at concentration of 1 mg/mL showed 39% and 19% of DPPH inhibition, respectively, compared to 70% DPPH inhibition of 1 mg/mL methanolic BPE from *L. terminata*[13]. However, the result of this study showed lower inhibition than that of Silva *et al.* which reported that crude BPE from the Brazilian stingless bee species *Melipona rufiventris* at concentration 0.1 mg/mL was shown to exhibit 50% DPPH inhibition[14].

The difference in antioxidant activity seen in our study could be due to the difference of the solvent used (methanol in this study *vs.* ethanol in the study by Silva *et al.*), which may give different compounds extraction efficacy, therefore contributed to the different chemical activities[14,15]. Furthermore specific pollen foraging activities, different pollen species and different diets of stingless bee itself may give different compounds activities found in BPE[14,16]. Previous studies have shown that bee pollen has good antioxidant properties related to the presence of phenolic compounds[2,17].

The antiproliferative activity of *L. terminata* BPE against MCF-7 and L929 cell lines was evaluated by using MTT assay. The concentration of BPE required to inhibit 50% of MCF-7 cells growth was 15 mg/mL. At 20.0 mg/mL, 70% inhibition of MCF-7 cells was seen. Whereas in normal L929 cell line, 26 mg/mL was required to exhibit 50% cells inhibition. The response different between normal and cancer cells may suggest that BPE can be relatively safe in normal cells and considered to be a useful agent for anticancer application in future. The much lower IC_{50} of BPE in cancer cells suggests that BPE may be specifically targeting the MCF-7 cancer cells.

Similar antiproliferative effect of BPE had been reported in the study by Kustiawan *et al.* by using four stingless bee species[18]. The result revealed that all four species were cytotoxic to different cell lines tested although their antiproliferative activities were rather low compared to the chemotherapeutic drugs used. Another study by Wu and Lou also showed strong antiproliferative activity of *B. campestris* bee pollen chloroform extract in inducing apoptosis in human prostate cancer[7]. The observed cytotoxic activity exhibited by bee pollen varied with the different solvent used, bee species and different cell lines.

In cytotoxic synergism assay, the average of MCF-7 cells inhibition by BPE in combination of cisplatin was around

50%. This study shows that BPE is able to potentiate and enhance the cisplatin effect on MCF-7 cells at all concentrations.

Active compounds in the BPE might either enhance (synergistic) or decrease (antagonistic) the therapeutic activity of chemotherapy drugs. There was a great concern that some compounds present in the natural product might work antagonistically instead of synergistically with the therapeutic activity of drugs[19]. In this study, CI values reported in all interactions between BPE and cisplatin were less than 1. These CI values further proved the synergistic effect between the two compounds.

This study may suggest that BPE from Malaysian stingless bee *L. terminata* can be used to supplement the chemotherapeutic agents due to its antiproliferative activitiy and its ability to enhance the effect of chemotherapy agent, even at low concentration. In a recent review by Komosinska-Vassev *et al*., bee pollen supplement can help to improve the condition of early prostate cancer when given together with chemotherapeutic drug[20]. Furthermore, bee pollen as supplement can be combined with chemotherapy treatment to treat side effects of cancer as reported in a recent study by Salles *et al*. which showed that bee pollen was able to improve muscle mass and metabolism in undernourished rats[21]. Decreasing muscle mass and poor metabolisms were commonly seen in patients with cancer[22]. The combination of chemotherapy drug and natural product such as BPE may be potentially effective in cancer treatment in future.

The data of this study suggest that bee pollen extract of Malaysian stingless bee is a potential chemopreventive agent and can be used as a supplement during chemotherapy treatment. The same data indicate that BPE works synergistically with chemotherapy drug, cisplatin and enhances the effect of cisplatin even at lower concentration. The molecular mechanisms of how the BPE exerts its antiproliferative and synergistic activities with cisplatin will be a promising area to further explore in future studies.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

We thank Dr. Hasni Arshad (Advanced Medical and Dental Institute, USM) for providing MCF-7 cell line and Dr. Rosliza Jajuli (Malaysian Agricultural Research and Development Institute) for stingless bee identification. This study was funded by Fundamental Research Grant Scheme, Ministry of Education Malaysia (Grant No: FRGS/2/2013/SKK01/USM/03/3).

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Table 1
CI data for combination effect of cisplatin and BPE on MCF-7 cells.

Cisplatin dose (μmol/L)	BPE dose (mg/mL)	Effect	CI
2.5	15.0	0.454 1	0.970 6
5.0	15.0	0.411 5	0.947 2
10.0	15.0	0.289 3	0.463 2
15.0	15.0	0.265 2	0.849 5

20.0	15.0	0.236 0	0.905 7

Figure legends:

Figure 1. Scavenging effect of methanolic extract from *L. terminata* BPE at different concentrations (0.15, 0.3, 0.6, 1.2, 2.4 and 4.8 mg/mL) on the stable DPPH.

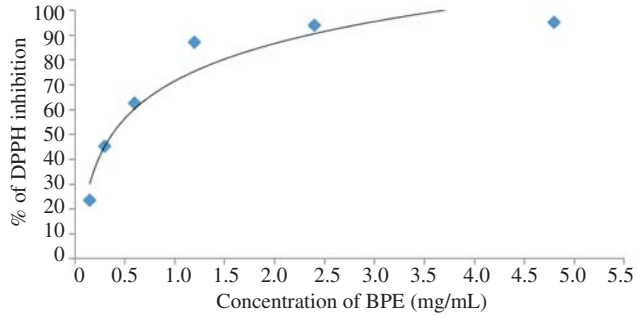
Results were expressed as percentage of DPPH inhibition with respect to control; Each value represents the mean ± SD.

Figure 2. Antiproliferative effect of BPE at different concentrations on MCF-7 cell lines and L929 cell lines.

A: MCF-7 cell lines; B: L929 cell lines; Each value represents the mean ± SD from three separate experiments.

Figure 3. The effect of cisplatin alone and combination of cisplatin and BPE on MCF-7 cells.

The percentage of cell proliferation was measured at 48 h post-treatment; Each value represents the mean ± SD from three separate experiments; Statistical analysis was determined by using Student's *t*-test with **: $P < 0.001$; *: $P < 0.05$.



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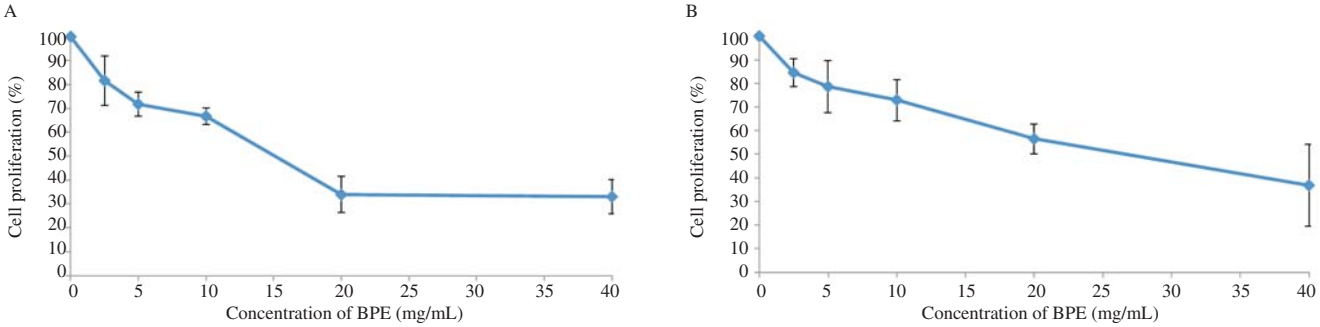

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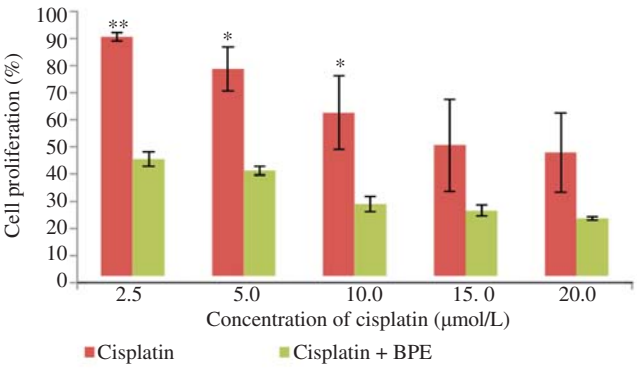

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