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Antioxidant and Cytotoxic Activity of the Wild Edible *Pimpinella brachycarpa* (Kom.) Nakai

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ABSTRACT

Pimpinella brachycarpa (Kom. Nakai) is one of the most favored wild vegetables in Asian regions. In the present study, a series of solvent fractions of *Pimpinella brachycarpa* were evaluated for their Total Phenolic Content (TPC), Total Flavonoid (TFC) content, antioxidant capacity and cytotoxic activity against Bel-7404 cancer cell line. PBbu had the highest TPC of 29.0567±0.27014 mg of gallic acid equivalents (GAE)/g of extract and PBet had the highest TFC of 872.422±12.0185 μg of quercetin equivalents/g of extract. There was a significantly high correlation between DPPH radical scavenging activity with TPC and $ABTS^{-+}$ Radical Scavenging Activity with TFC. While a poor correlation between cytotoxicity with TPC or TFC was obtained. Among the four solvent fractions, PBpe showed the most potent cytotoxic activity. The results indicated that the solvent fractions from *Pimpinella brachycarpa* have potent health benefit effects, thus can be used as a dietary source for functional food.

Key words: *Pimpinella brachycarpa* (Kom.) Nakai, total phenolics, total flavonoid, antioxidant activities, cytotoxicity

INTRODUCTION

Pimpinella brachycarpa (Kom.) Nakai (PB) is one of the most favored and increasingly popular wild vegetable grown in Asian regions (Na *et al*., 2007). It belongs to *Pimpinella* L., which has shown many health properties (Sun *et al*., 2009). Polyphenols have been proven to exhibit many health protective effects, such as antioxidant and anticancer activities, therefore, have received most attention (Kunyanga *et al*., 2012). Heo *et al*. (2009) reported that methanol extract of PB, which contained a reasonable mount of polyphenols, had antitumor effect against lung cancer, breast cancer, liver cancer and colon cancer. This indicated that PB should be considered as a promising functional food source.

In order to fully understand the antioxidant and cytotoxic activity of PB, four solvent fractions were prepared from 90% ethanol extract and their phenolics and flavonoids content, scavenging activity of DPPH, ABTS radical and inhibitory effects on human hepatoma cell Bel-7404 were analyzed. Moreover, the correlation between the phenolics and flavonoids content, antioxidant activity and cytotoxic activity were also studied.

MATERIALS AND METHODS

Chemicals and reagents: The human hepatoma cell Bel-7404 was procured from Shanghai Institute of Cell Biology, Chinese Academy of Science. 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,20-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma-Aldrich (Shanghai, China). RPMI 1640 was obtained from Gibco Invitrogen Co.

Plant material: The plant materials used for this investigation were purchased from LCNEWF Company Ltd. (Liaoning, China) in June 2010. Collected plant material was dried in a shaded and well-ventilated place. Plant material was pulverized into a particle size of 40 mesh before further processing.

Preparation of fractions: The PB (4.395 kg) was extracted with 90% EtOH (1:10, v/v) at 75°C 3 times and each extraction period lasted 2 h. After filtration, the solution was combined and concentrated under reduced pressure to afford the residue. The residue was dissolved in distilled water (1:10, v/v), then extracted with petroleum ether (Pet et. bp, 60-90) (1:1, v/v) 3 times. The Pet.et fraction (PBpe) was separated. Then the aqueous residue was extracted with EtOAc (1:1, v/v) for 3 times and each extraction period lasted 2 h. The EtOAc fraction was filtered and evaporated under reduced pressure to afford residue (PBet). The residue was extracted with n-BuOH (1:1, v/v) 3 times and each extraction period lasted 2 h. The n-BuOH fraction (PBbu) was evaporated under reduced pressure. The remained aqueous residue (aqueous fraction, PBaq) was freeze-dried. Therefore, four solvent fractions (PBpe, PBet, PBbu and PBaq) were obtained.

Total phenolic content (TPC) assay: Total phenolic content was measured by using the Folin-Ciocalteu method with slight modifications (Feng *et al*., 2010). The samples were diluted appropriately with water prior to analysis. Gallic acid (200, 100, 50, 25 and 12.5 mg L^{-1}) was used to establish the standard curve. Folin-Ciocalteu reagent (500 μL) was diluted ten times from the original reagent and mixed with 400 μL of 30 mg mL^{-1} sodium carbonate and 100 μL of sample in each well. Absorbance was measured at 765 nm after 30 min of reaction at 25°C. Data were expressed as milligrams of gallic acid equivalents (GAE)/g of extract.

Total flavonoid content (TFC) assay: The TFC was determined by a method described previously with slight modifications (Alvarez-Parrilla *et al*., 2011). A mixture was made of 20 μL of the properly diluted sample, 80 μL of water and 6 μL of 5% $NaNO_2$. After 5 min, 6 μL of 10% $AlCl_3$ were added to the mixture and then, after 3 min, 80 μL of 0.5 M NaOH were added and incubated at room temperature for 30 min. The absorbance was read at 520 nm using a microplate reader (RT6000, Shenzhen, China). The standard curve was prepared with quercetin and the absorbance was converted to the flavonoid content in terms of mg of quercetin equivalence/g of extract.

DPPH assay: The DPPH radical scavenging activity of sample was evaluated as previously described (Kim *et al*., 2010). Briefly, 50 μL of sample solution (or EtOH) was added to 200 μL of 200 μM DPPH radical solution which was freshly made. After 30 min of incubation at room temperature, the absorbance at 520 nm was measured. DPPH radical solution with EtOH served as control. All tests were carried out in triplicate. DPPH radical-scavenging activity was expressed as the inhibition percentage (SC %) and was calculated by using the following formula:

$$SC(\%) = \frac{A_{control} - A_{test}}{A_{control}} \times 100 \quad (1)$$

where, $A_{control}$ is the absorbance of the control at 30 min reaction (containing all reagents except the test compound) and A_{test} is the absorbance of the sample at 30 min. Antiradical activity was expressed as EC_{50}, defined as the concentration of the extract generating 50% inhibition.

ABTS assay: The method was determined as described previously (Yang *et al.*, 2010). ABTS radical cation was freshly prepared by mixing 14 mmol L^{-1} ABTS with an equal volume of 4.95 mmol L^{-1} potassium persulphate and kept for 24 h in the dark at room temperature. This ABTS radical cation solution was used for the assay after dilution in Phosphate Buffer Saline (PBS) appropriately. To 300 μL of various concentrations of sample, 900 μL of ABTS radical solution was added. After 1 min incubation at room temperature, the absorbance was measured at 732 nm. ABTS solution with EtOH served as a control. All determinations were performed three times. The inhibition percentage (SC %) was calculated by Eq. 1.

The antioxidant capacity was expressed as EC_{50}, the concentration necessary for 50% reduction of $ABTS^{-+}$.

Cytotoxicity assay: Bel-7404 was cultured in RPMI-1640 medium containing 10 mmol L^{-1} HEPES, supplemented with 10% fetal calf serum, 100 U mL^{-1} penicillin and 100 U mL^{-1} streptomycin. The cytotoxicity assay was assessed by the methods of MTT assay described previously with slight modifications (Zhang *et al.*, 2009). Briefly, cells seeded on 96-well culture plates at 1×10^5/well were incubated with fractions for 22 h. Then 10 μL of the MTT solution (5 mg mL^{-1}) was added into each well and made the final concentration 0.5 mg mL^{-1}, followed by further 2 h incubation. After the medium was removed, DMSO (100 μL) was added into each well before reading the microplates at 570 nm. Cell viability was expressed as Optical Density (OD).

Statistical analysis: All analyses were carried out at least in triplicate. Values were presented as the mean±SD. Statistical differences between mean values were determined by Student's t test. The significance level was set at $p<0.05$.

RESULTS AND DISCUSSION

Preparation of fractions: Five solvents (EtOH, Pet.et, EtOAc, n-BuOH, and water) were used in order to separate the bioactive chemicals from the plant (4.395 kg), according to their polarity, yielding four fractions: PBpe (Pet.et fraction, 25 g), PBet (EtOAc fraction, 125 g), PBbu (n-BuOH fraction, 60 g) and PBaq (aqueous fraction, 760 g) (Fig. 1). To our knowledge, only Heo *et al.* (2009) and Lee *et al.* (2006) have used methanol and ethanol respectively to gain crud extracts from PB. Thus, this is the first time different solvents were used to further separate the sub-extracts.

Total TPC and TFC assay: Using the colorimetric determination, it was found that PBbu fraction and the highest Total Phenolics Content (TPC) of 29.0567±0.27014 mg of gallic acid equivalents (GAE)/g of extract and PBet had the highest Total Flavonoid Content (TFC) of 872.422±12.0185 μg of quercetin equivalents/g of extract (Table 1). Phenolics are a group of

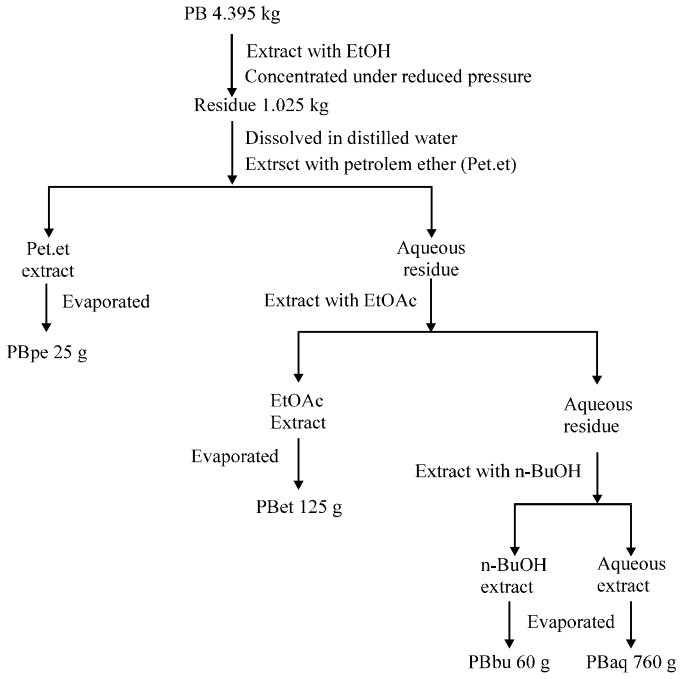

Fig. 1: Process of separation of solvent fractions from *Pimpinella brachycarpa*

Table 1: Phytochemical Properties of *Pimpinella brachycarpa* fractions

Fractions	TPC (mg of GAE g^{-1} of extract)	TFC (µg of QE g^{-1} of extract)
PBpe	7.2751±0.00664[a]	435.756±10.00[a]
PBet	10.1831±0.0673[b]	872.422±12.0185[b]
PBbu	29.0567±0.27014[c]	725.756±28.7389[c]
PBaq	11.5395±0.06897[d]	331.311±1.9245[d]

Data are per gram of dry botanical basis and are expressed as mean±SD TPC: Total phenolic content, TFC: Total flavonoid content. GAE: Gallic acid equivalents, QE: Quercetin equivalents. PBpe, PBet, PBbu and PBaq stand for different fractions from PB (Pet.et fraction, EtOAc fraction, n-BuOH fraction and aqueous fraction, respectively). Means in a row without a common superscript letter are statistically different ($p<0.05$)

bioactive compounds, such as anthocyanins, phenolic acids, flavonol glycosides and so on. Different extraction methods, genetic and environmental factors will alter the phenolic profiles and concentrations (Lee *et al*., 2012). The total phenolics and total flavonoid content were not consistent. It indicated that different type of phenols existed in different fractions besides flavonoid.

DPPH assay: DPPH (1,1-diphenyl-2-picrylhydrazyl) is a free-radical compound that has been widely used to determine the free-radical scavenging ability of various samples (Chen *et al*., 2011). Natural antioxidants which are capable of quenching radicals may prevent cellular components from oxidative damage and benefit human health. As shown in Fig. 2a, all of the four fractions showed significant DPPH radical scavenging capacity under the experimental conditions. The DPPH radical scavenging capacities of the four fractions were in the following order from highest to lowest: PBbu>PBaq>PBet>PBpe. The EC_{50} of DPPH scavenging activity was 0.6292±0.1257, 1.7179±0.1823, 4.2340±0.0575 and 4.3720±0.2416 mg mL^{-1}, respectively. The DPPH radical scavenging capacities are due to their hydrogen donating abilities. Therefore, PB had the

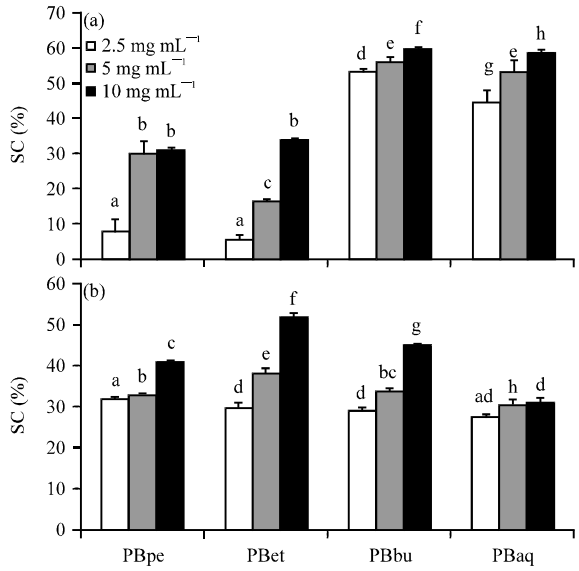

Fig. 2(a-b): Free radical scavenging capacity of PB solvent fractions. (a) DPPH radical scavenging capacity and (b) $ABTS^{-+}$ scavenging activity. PBpe, PBet, PBbu and PBaq stand for different fractions from PB (Pet.et fraction, EtOAc fraction, n-BuOH fraction, aqueous fraction, respectively). Results are mean±SD of three parallel measurements. Bars having different letters are significantly different at $p<0.05$

proton-donating ability and could serve as free radical scavengers (Serbetci *et al.*, 2012). And PBbu had the strongest ability to scavenge DPPH free radicals. Additional statistical analysis revealed that there was a significantly high correlation between DPPH radical scavenging activities with TPC. The results were in agreement with previous reports that the phenolic compounds contribute significantly to the antioxidant activity (You *et al.*, 2012).

ABTS assay: In the $ABTS^{-+}$ scavenging assay, All PB fractions inhibited radical formation in a dose-dependent manner and the $ABTS^{-+}$ Radical Scavenging Activity of the four fractions were in the following order from highest to lowest: PBet>PBbu>PBpe>PBaq (Fig. 2b). The EC_{50} of $ABTS^{-+}$ scavenging activity was 2.9387±0.0036, 3.7810±0.0581, 5.2059±0.0891 and 14.7522±1.9936 mg mL^{-1}, respectively. These were consistent with the TFC. Statistical analysis showed that there was a strong correlation between $ABTS^{-+}$ Radical Scavenging Activity with TFC. $ABTS^{-+}$ scavenging assay is always employed to test the total antioxidant capacity because it can react with all phenolic hydroxyl groups. But some antioxidants are more active in reducing $ABTS^{-+}$ than in trapping DPPH (Li *et al.*, 2010). Therefore, we could conclude that flavonoid has a higher reductive ability to reduce $ABTS^{-+}$. It also showed us that different antioxidants had different mechanisms (Prochazkova *et al.*, 2011). Thus, approaches from single antioxidant are not adequate to assess the health benefit of food mixtures.

Cytotoxicity assay: Phenolics, with high antioxidant activity have shown antiproliferative effects in different cell lines (Encalada *et al.*, 2011). However, antiproliferative effects are not always consistent with antioxidant capacities. The anticancer effect of four solvent fractions was

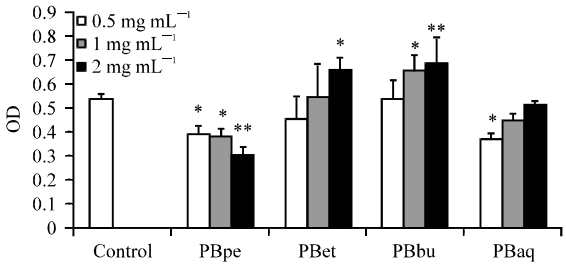

Fig. 3: Cytotoxic effect of PB fractions on Bel-7404. PBpe, PBet, PBbu and PBaq stand for different fractions from PB (Pet.et fraction, EtOAc fraction, n-BuOH fraction, aqueous fraction, respectively). *p<0.05,**p<0.01, compared to control.

assessed by cytotoxicity (Fig. 3). PBpe dose dependently reduced the Bel-7404 viability. PBaq suppressed cell viability at lower dose, while increased cell viability at higher dose. On the contrary, PBet and PBbu dose dependently elevated the cell viability. Besides, a very poor correlation of cytotoxicity with TPC or TFC was documented, suggesting that polyphenols may not be the main anticancer chemicals of PBpe, its composition should be further investigated.

CONCLUSION

The results of this study indicated that PB had a reasonable mount of polyphenols and flavonoids. Furthermore, PB has antioxidant and cytotoxic properties and could be used as potential dietary sources to exhibit beneficial effects. While the precise composition and chemical characterizations of active compounds in PB need to be explored further.

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